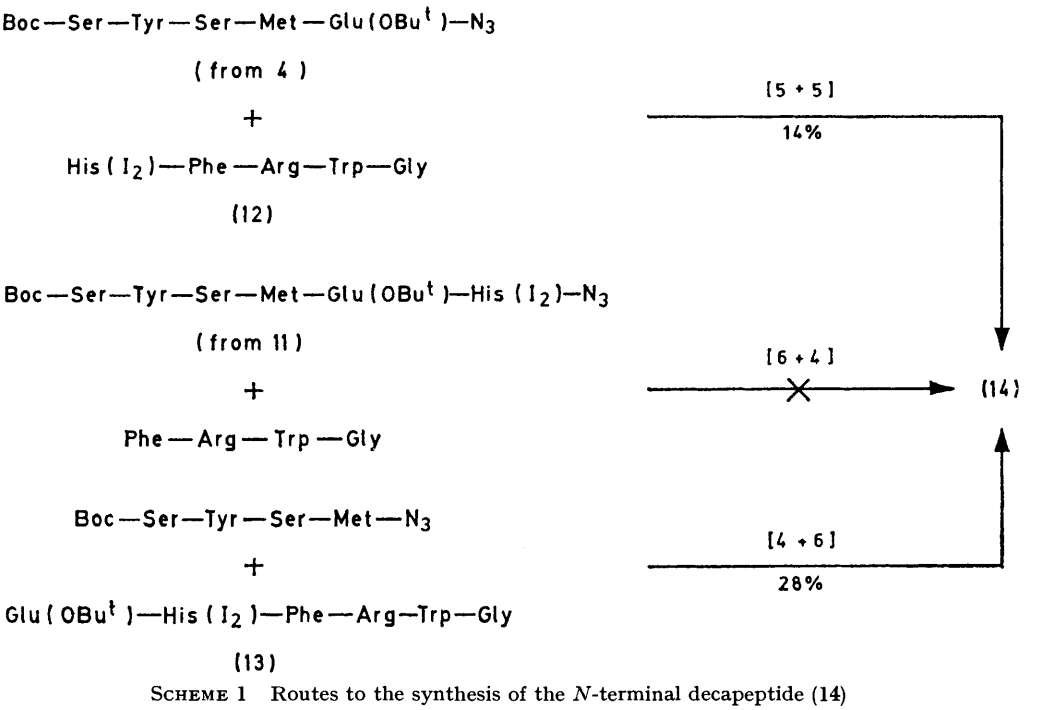
Tritiated Peptides. Part 7.[1] Synthesis of [2,5-$^3H_2$-His^6]-β-Corticotrophin-(1—24)-tetracosapeptide †

By Mark C. Allen, Derek E. Brundish, and Roy Wade,* CIBA Laboratories, Horsham, West Sussex RH12 4AB

The synthesis is described of β-corticotrophin-(1—24)-tetracosapeptide ‡ labelled with tritium in the histidine residue at position 6 at a specific radioactivity of 30 Ci mmol^{-1} by reductive dehalogenation of a protected precursor. Evidence for the integrity of the final product is provided by amino-acid analysis, column chromatography, and bioassay, supported by chemical and enzymic analytical data on the protected precursor and the derived free peptide containing di-iodohistidine.

FOLLOWING the syntheses of β-corticotrophin-(1—24)-tetracosapeptide labelled with tritium in the tyrosine [2,3] and phenylalanine [4] residues, we wished to extend our investigations to ascertain whether the labelling method, catalytic dehalogenation, could be used to label specifically the aromatic side-chain of the amino-acid histidine, which is also present in Synacthen.



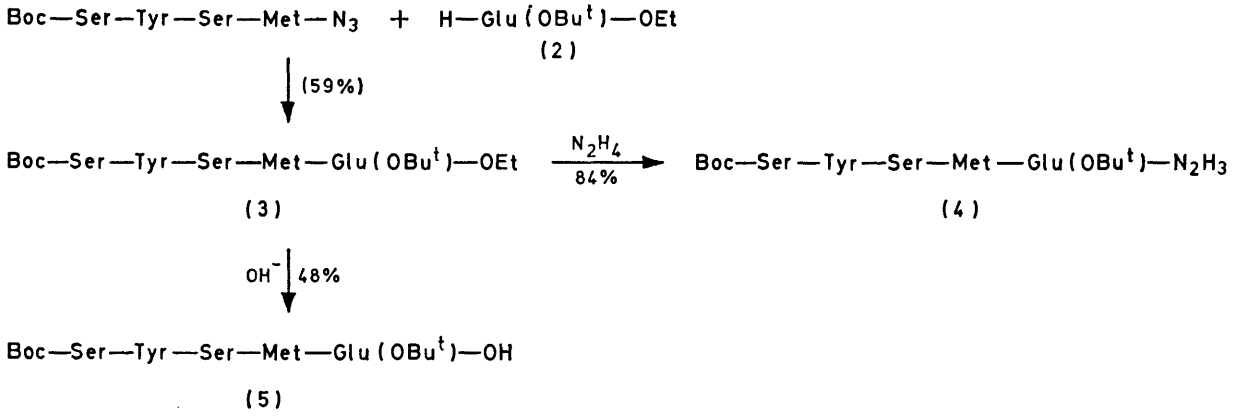
SCHEME 1 Routes to the synthesis of the *N*-terminal decapeptide (14)

No undue difficulties were foreseen in the synthesis of protected (6-di-iodohistidine)-tetracosapeptide although this cannot be achieved by direct iodination due to the presence of other more susceptible amino-acid residues (two tyrosines, methionine). The approach would therefore be totally synthetic in order to ensure subsequent entry of the label into the specific site required. Labelling of a free peptide by iodination at histidine followed by catalytic tritiation has been demonstrated for the tripeptide thyrotropin-releasing hormone (TRH) [5] and implicated for the 61-residue α-neurotoxin of *Naja nigricollis*,[6] and we were confident that protected synthetic peptides containing di-iodohistidine could be similarly labelled by reductive de-iodination.

Of the various possibilities (Scheme 1), the most attractive synthetic approach to the key intermediate, the *N*-terminal decapeptide, seemed to be by a 5 + 5 coupling at the glutamic acid residue as this allowed fewest synthetic steps from the scarce unnatural amino-acid di-iodohistidine to the final product. We prepared the hydrazide (4) and carboxylic acid (5) of the *N*-terminal pentapeptide by conventional procedures (Scheme 2). Coupling of the free acid to the *C*-terminal pentapeptide (12) by hydroxybenzotriazole-assisted carbodi-imide condensation (DCC–HOBt) was completely unsuccessful and is not detailed here. This was surprising since successful couplings in high yield at this bond have been reported. Biossonnas and his co-workers synthesised α-MSH [7] and the *N*-terminal eicosapeptide sequence of ACTH [8] by DCC condensation at the Glu–His bond. The γ-carboxy-function of the glutamic

† 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.

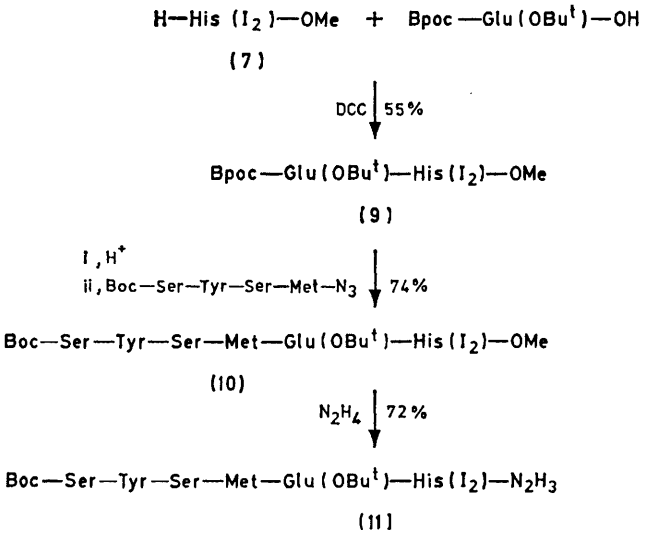
‡ In this paper, all amino-acid residues are L.



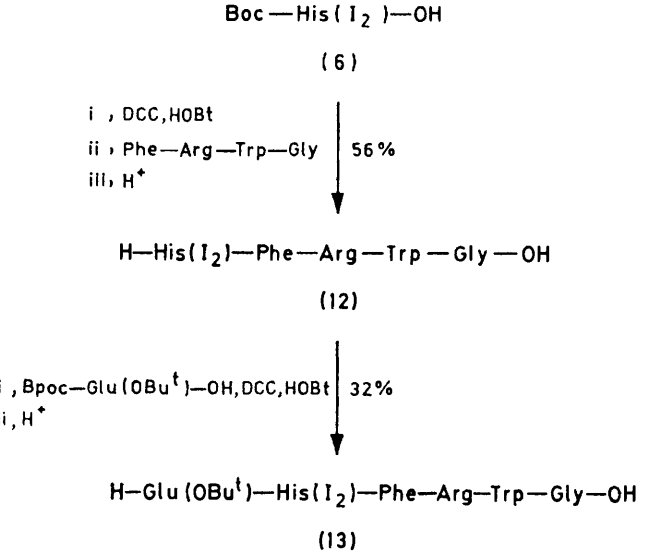
SCHEME 2 Synthesis of *N*-terminal pentapeptide precursors (4) and (5)

acid residue was protected as the benzyl ester in these instances whereas we have used the t-butyl ester. To check whether failure was due to steric hindrance by the di-iodohistidine residue, biphenylylisopropoxycarbonyl-glutamic acid γ-t-butyl ester was coupled to di-iodohistidine methyl ester. The reaction proceeded with excellent yield. Accordingly, the *N*-terminal pentapeptide free acid was coupled to histidine methyl ester by the DCC–HOBt procedure. The coupling was inefficient and yielded grossly impure product. The reason for the failure of these couplings is not obvious. Recently, it has been reported that side-reactions can occur in the presence of hydroxybenzotriazole between active esters and unprotected serine hydroxy-groups in the presence of histidine-containing peptides.[9]

We next attempted the 5 + 5 coupling using the azide procedure. Decapeptide was obtained with difficulty from the reaction mixture in low yield (14%). Recovery of additional decapeptide by counter-current distribution was not possible owing to severe problems of emulsification. Since the yield was disappointing, we carried out a model azide coupling using histidine methyl ester and the azide generated from benzyloxycarbonyl γ-t-butyl glutamic acid hydrazide. A negligible yield of dipeptide resulted.



SCHEME 3 Synthesis of the *N*-terminal hexapeptide precursor (11)



SCHEME 4 Synthesis of the C-terminal penta(12)- and hexa-(13)-peptide

A 6 + 4 approach was next attempted using the azide method as this had been successful for the protected [7-(iodophenylalanine)]-decapeptide.[4] The *N*-terminal hexapeptide hydrazide (11) was prepared without difficulty (Scheme 3) but the derived azide completely failed to couple with the *C*-terminal tetrapeptide.

Finally a 4 + 6 azide coupling was investigated as a route to the decapeptide. This involved the formation of a peptide bond between residues of methionine and glutamic acid which is well documented [3,4,10] and was successful in this instance also. The *C*-terminal hexapeptide (13) was rigorously purified at each stage of synthesis (Scheme 4) from the tetrapeptide through to the hexapeptide by counter-current distribution to ensure the elimination of all traces of by-products. Although the overall yield of decapeptide by this 4 + 6 route is less than by the 5 + 5 approach in terms of the yield from a given starting quantity of di-iodohistidine, it

became the route of choice as a result of the ease of crystallisation of the product in a high state of purity from the reaction mixture.

The protected tetracosapeptide was obtained without difficulty and from it the free tetracosapeptide containing di-iodohistidine was prepared which had a potency of 17% compared to Synacthen in an isolated adrenal cell bioassay.[11] This value is high compared with that of 0.4% for the (7-iodophenylalanine)-containing analogue.[4]

Peptides containing di-iodohistidine were found to yield monoiodohistidine and a smaller amount of histidine on acidic hydrolysis. This has been reported previously [12] though no information on the position of substitution with iodine in the generated monoiodohistidine was available at that time. We isolated monoiodohistidine generated by hydrolysis of di-iodohistidine with hot aqueous hydrochloric acid and showed by n.m.r. spectroscopy that the iodine had been retained in the 2-position.[13] By contrast, we observed that treatment of di-iodohistidine with hot thionyl chloride in methanol resulted in the loss of the iodine atom at position 2 and the production of 5-iodohistidine methyl ester.

The precursor yielded the tritiated peptide by the method used previously although the product was purified by a different ion-exchange technique. The peptide was fully active in the adrenal cell bioassay. By scintillation counting of the amino-acids produced by complete enzymic digestion of the peptide [3] it was found that label had been introduced exclusively into the histidine residue. No evidence was found for non-specific exchange labelling into the tyrosine residue for example. Further, examination of the amino-acid mixture produced by hot, constant-boiling hydrochloric acid (115 °C, 18 h) revealed that the total radioactivity eluted in the histidine position on the amino-acid analyser was unaffected (94.5 ± 12%) by the acid treatment. We also applied this test to the non-specifically labelled histidine residue present in [D-Ser[1], 4-^{3}H-Phe[7], Lys [17,18]]-β-corticotrophin-(1—18)-octadecapeptide amide [1] with the same result (103 ± 12%). The non-specific exchange labelling of the histidine residue observed by us previously [1,3,4] may involve the 2- and 5-positions of the imidazole nucleus.

Menez and his co-workers [6] observed with their preparation of tritium-labelled α-neurotoxin that, after acidic hydrolysis, only 10% of the total initial radioactivity was recoverable and that this was bound to histidine. From this they concluded that there had been exchange of label ' between aromatic hydrogen and protons of the solvent promoted by the acid hydrolysis of the peptide '. If our own conclusion concerning the stability of histidine ring protons and tritons under hot strongly acidic conditions is correct then we would infer that the 10% was, in fact, all the radioactivity associated with histidine initially in the α-neurotoxin. This would imply that the remaining 90% was associated with some other residue. Tyrosine is an obvious possibility as it is known that radioactivity associated with this amino-acid is exchanged out under these conditions.[14]

EXPERIMENTAL

Analytical methods have been given in Part 1.[2] 2,5-Diiodohistidine was found to be decomposed under the conditions of acidic hydrolysis to a mixture of histidine and 2-iodohistidine. These two products were estimated using the colour yield for histidine and added together and are quoted as ' His species ' in analyses—the recovery was, therefore, not quantitative. Enzymic digestion of peptides containing di-iodohistidine was not complete under the conditions used.

N-Benzyloxycarbonyl-γ-t-butyl Glutamic Acid α-Hydrazide (1).—This compound was prepared from the α-ethyl ester [10] (5.5 g) as described for the 4-methoxybenzyloxycarbonyl analogue.[15] Crystallisation from ethanol–diethyl ether allowed removal of bis-hydrazide by-product and the *product* was obtained by trituration of the recovered residues under diethyl ether (3.54 g, 67%). It had m.p. 69—71 °C, $[\alpha]_D^{28}$ +2.3 ± 0.1° (*c* 1.6, EtOAc) (Found: C, 58.1; H, 7.1; N, 11.95. $C_{17}H_{25}N_3O_5$ requires C, 58.08; H, 7.17; N, 11.96%).

γ-t-Butyl Glutamic Acid α-Ethyl Ester Hydrochloride (2).—This compound was prepared from the benzyloxycarbonyl derivative [10] (10.0 g) as described for the analogous α-methyl ester hydrochloride.[16] The *product* was recrystallised twice from ethyl acetate–light petroleum (b.p. 60—80 °C), yield 5.0 g (79%). It had m.p. 94—96 °C, $[\alpha]_D^{28}$ +26.3 ± 0.2° (*c* 0.9, EtOH) (Found: C, 49.45; H, 8.3; Cl, 13.15; N, 5.35. $C_{11}H_{21}NO_4$·HCl requires C, 49.35; H, 8.22; Cl, 13.46; N, 5.23%).

N-t-Butoxycarbonyl-seryl-tyrosyl-seryl-methionyl-γ-t-butyl Glutamic Acid Ethyl Ester (3).—Boc-Ser-Tyr-Ser-Met-N_2H_3 [17] (3.08 g) was dissolved in *NN*-dimethylformamide (DMF) (22 ml), cooled to −5 °C and 5M-HCl (1.48 ml) was added while the temperature of the mixture was kept below 0 °C. Isopentyl nitrite (0.75 ml) was added and the mixture was stirred at −7 °C for 7 min.[18] Triethylamine (Et_3N) (1.03 ml) was added and the mixture was added to a solution of compound (2) (1.54 g) and Et_3N (0.73 ml) in DMF (15.0 ml) at −10 °C. The mixture was stirred at −10 °C for 1 h and then at 4 °C for 72 h. Solids were filtered off and the filtrate evaporated below 40 °C. The residue was dissolved in a mixture of n-butanol (40 ml) and ethyl acetate (20 ml) and washed at 0 °C with saturated citric acid solution (3 × 50 ml), water (2 × 50 ml), and saturated $NaHCO_3$ solution (2 × 50 ml); it was then dried (Na_2SO_4) and evaporated to dryness below 40 °C. The residue was dissolved in chloroform and applied to a column (25 × 2 cm) of silica gel. The product was recovered by elution with 3% (v/v) methanol in chloroform. The recovered material (2.4 g, 59%) was sufficiently pure for further use. A sample for analysis was crystallised from ethyl acetate–light petroleum (b.p. 60—80 °C). The *ester* had m.p. 123—124 °C, $[\alpha]_D^{28}$ −8.2 ± 0.3° (*c* 0.7, EtOAc) (Found: C, 53.95; H, 7.2; N, 8.95; S, 4.0. $C_{36}H_{57}N_5O_{13}S$ requires C, 54.08; H, 7.14; N, 8.76; S, 4.00%).

N-t-Butoxycarbonyl-seryl-tyrosyl-seryl-methionyl-γ-t-butyl-glutamic Acid Hydrazide (4).—The ester (3) (7.8 g) was dissolved in DMF (40 ml), $N_2H_4·H_2O$ (5.0 ml) was added and the mixture was stirred under N_2 at room temperature for 3 h. Water (50 ml) was added and the mixture was kept at 4 °C for 16 h. The solid was filtered off, washed with 50% aqueous methanol, and dried *in vacuo* over conc. H_2SO_4. The *product* (6.43 g, 84%) had m.p. 198—201 °C, $[\alpha]_D^{28}$ −21.9 ± 0.3° (*c* 0.6, CH_3CO_2H) (Found: C, 51.8; H, 7.15; N, 12.65; S, 4.15. $C_{34}H_{55}N_7O_{12}S$ requires C, 51.98; H, 7.01; N, 12.48; S, 4.08%).

N-t-Butoxycarbonyl-seryl-tyrosyl-seryl-methionyl-γ-t-butyl-glutamic Acid Monohydrate (5).—The ester (3) (8.2 g) was dissolved in methanol (90 ml) and 1M-NaOH (15 ml) was added. The mixture was stirred under N_2 at room temperature for 30 min, and 1M-HCl (15 ml) was added. The mixture was evaporated to dryness, dissolved in equal volumes (30 ml each) of the phases of the system $CHCl_3$–CH_3OH–CCl_4–H_2O (3 : 3 : 1 : 1; v/v) and loaded into tubes 1—3 of a 123-tube counter-current distribution machine. After 110 upper-phase transfers, unchanged ester (3) (2.21 g, 27%) was recovered from tubes 28—40 and product was recovered from tubes 41—82. The residue obtained by evaporation was triturated under light petroleum (b.p. 60—80 °C) to give the amorphous *product* (3.9 g, 48%). It had $[\alpha]_D^{27}$ +23.8 ± 0.3° (*c* 1.4, MeOH) (Found: C, 51.95; H, 7.05; N, 8.85; S, 4.05. $C_{34}H_{55}N_5O_{14}S$ requires C, 51.68; H, 7.02; N, 8.87; S, 4.05%).

N$^{\alpha}$-t-Butoxycarbonyl-2,5-di-iodohistidine (6).—This compound was prepared in 89% yield from di-iodohistidine [19] [His(I_2)] in the usual manner [20] at pH 9.8 and 20 °C during 72 h. It was crystallised from ethyl acetate–light petroleum (b.p. 60—80 °C). The *product* had m.p. 181 °C, $[\alpha]_D^{20}$ −4.2 ± 0.4° (*c* 0.6, EtOH) (Found: C, 26.1; H, 3.05; N, 8.1; I, 49.85. $C_{11}H_{15}I_2N_3O_4$ requires C, 26.04; H, 2.96; N, 8.28; I, 50.09%).

2,5-Di-iodohistidine Methyl Ester Dihydrochloride Dihydrate (7).—His(I_2) (15.2 g) was suspended in dry methanol (60 ml) and cooled in ice; dry HCl gas was then passed through the stirred suspension for 5 h. The resulting solution was stored overnight at 4 °C with exclusion of moisture and the resulting crystals were recrystallised from dry methanol to yield 13.5 g (73%) of product, m.p. 186 °C, $[\alpha]_D^{28}$ +9.8 ± 1.0° (*c* 0.5, H_2O). The compound failed to give a satisfactory analysis after repeated recrystallisations from methanol but appeared pure as judged by t.l.c. and high-voltage paper electrophoresis. An amorphous sample (m.p. 61 °C, softens) obtained from methanol–diethyl ether had an analysis consistent with its formulation as the *dihydrate* (Found: C, 16.2; H, 2.55; I, 47.85; N, 8.1. $C_7H_{11}Cl_2I_2N_3O_2 \cdot 2H_2O$ requires C, 15.85; H, 2.45; N, 7.92; I, 47.92%).

5-Iodohistidine Methyl Ester Dihydrochloride (8).—(a) *From 2,5-di-iodohistidine.* His(I_2) (23 g) was added to a solution of $SOCl_2$ (4.75 ml) in methanol (50 ml) at −10 °C and the mixture was stirred with exclusion of moisture at −7 °C for 45 min and then at 45 °C for 3 h.

Examination of the mixture by low-voltage paper electrophoresis (25 V cm^{-1}, 30 min) in pyridinium acetate buffer (pH 6.0) showed that esterification was not complete. The reaction was completed by stirring the mixture for a further 7 h at 55 °C. The mixture was evaporated to dryness and several portions of fresh methanol were evaporated from the residue. The gum obtained was kept *in vacuo* over solid KOH for 16 h and then crystallised from methanol–diethyl ether to give 7.0 g (27%) of crude ester. A sample recrystallised from the same solvent mixture gave the *ester dihydrochloride* which had m.p. 202—204 °C, $[\alpha]_D^{20}$ +18.1 ± 0.4° (*c* 0.5, MeOH) (Found: C, 22.65; H, 3.35; I, 34.4; N, 11.55. $C_7H_{12}Cl_2IN_3O_2$ requires C, 22.82; H, 3.29; I, 34.50; N, 11.41%).

(b) *From 5-iodohistidine.*[19] His(I) (0.77 g) was suspended in dry methanol (10 ml) and dry HCl gas was passed through the stirred mixture at 0 °C for 9 h. The mixture was stored at 4 °C for 5 weeks and the solid filtered off. It was recrystallised (charcoal) from methanol–diethyl ether to afford the ester dihydrochloride (0.50 g, 47%) which had m.p. 202—204 °C, $[\alpha]_D^{26}$ +18.6 ± 0.2° (*c* 2.0, MeOH).

The i.r. spectra of the materials from experiments (a) and (b) were identical. A 220 MHz n.m.r. spectrum of a mixture of the two materials in D_2O showed a single ring proton signal at τ −3.92 relative to HOD. Iodohistidine recovered after acidic hydrolysis of di-iodohistidine showed a single ring proton signal at τ −2.70 relative to HOD.

N-(2-Biphenyl-4-ylisopropoxycarbonyl)-γ-t-butylglutamyl-2,5-di-iodohistidine Methyl Ester (9).—Compound (7) (7.0 g) was suspended in methanol (36 ml) cooled to 10 °C, and a solution of sodium (655 mg) in methanol (17.5 ml) was added. The suspended ester salt immediately went into solution. Dry diethyl ether (24 ml) was added and the mixture was stirred in ice for 20 min and filtered through a pad of Celite to remove solid NaCl. The combined filtrate and washings were dried by rotary evaporation below 30 °C and the solid ester was dissolved in a mixture of acetonitrile (44 ml) and DMF (18 ml) together with 6.0 g of *N*-2-biphenyl-4-ylisopropoxycarbonyl-γ-t-butyl-glutamic acid.[21] The mixture was cooled to −5 °C and a solution of dicyclohexylcarbodi-imide (DCC) (3.0 g) in DMF (6 ml) was added. The mixture was stirred at 4 °C for 72 h, dicyclohexylurea (DCU) was filtered off, and the filtrate was evaporated to dryness. The residue was dissolved in ethyl acetate (30 ml) and the solution was extracted with cold citric acid solution (3 × 20 ml), water (20 ml), saturated $NaHCO_3$ solution (2 × 20 ml), and water (20 ml), and then dried (Na_2SO_4) and evaporated to dryness. The residue was applied as a solution in benzene to a column (50 × 3 cm) of silica and the product was recovered by elution with 7.5% diethyl ether in benzene. It was crystallised from benzene–cyclohexane to yield 6.26 g (55%) of *product*, m.p. 109—116 °C, $[\alpha]_D^{27}$ +51.0 ± 2.5° (*c* 0.15, EtOAc) (Found: C, 45.55; H, 4.6; I, 29.75; N, 6.55. $C_{32}H_{38}I_2N_4O_7$ requires C, 45.48; H, 4.54; I, 30.08; N, 6.63%).

N-t-Butoxycarbonyl-seryl-tyrosyl-seryl-methionyl-γ-t-butyl-glutamyl-2,5-di-iodohistidine Methyl Ester (10).—Compound (9) (4.17 g) was stirred for 5 h at 20 °C with 50 ml of a mixture of acetic acid–formic acid (98%)–H_2O (7 : 1 : 2 v/v). The solution was diluted with water (200 ml) at 0 °C and extracted with diethyl ether (2 × 250 ml). The aqueous phase was evaporated to dryness below 20 °C, and the residue was dissolved in water (50 ml) and adjusted to pH 8 by addition of saturated $NaHCO_3$ solution. The solution was extracted with ethyl acetate (3 × 100 ml) and the combined extracts were washed with brine (100 ml), dried (Na_2SO_4), and evaporated to dryness to yield 2.6 g (87%) of base as a gum.

Boc-Ser-Tyr-Ser-Met-N_2H_3 [17] (3.7 g) was dissolved in DMF (18 ml) cooled to −10 °C and 4.55M-HCl (2.18 ml) in ethyl acetate was added. t-Butyl nitrite (0.51 ml) was added and the mixture was stirred at −7 °C for 10 min.[18] Et_3N (1.38 ml) was added and the base prepared above was added at −5 °C in solution in DMF (15 ml). The mixture was stirred at 4 °C for 120 h, filtered, and the filtrate was evaporated to dryness below 30 °C. The residue was dissolved in n-butanol–ethyl acetate (1 : 1, v/v; 200 ml) and the solution was extracted at 0 °C with citric acid solution (3 × 100 ml), water (100 ml), saturated $NaHCO_3$ solution (2 × 100 ml), and water (100 ml), and then dried (Na_2SO_4) and evaporated to dryness. The residue was dissolved in 3% (v/v) methanol in chloroform (200 ml) and applied to a column of silica (45 × 2 cm). The product was recovered by elution with 5% (v/v) methanol in chloroform

and was obtained as a solid (4.3 g, 74%) by trituration with diethyl ether. The *ester* had m.p. 117 °C, $[\alpha]_D^{27}$ −11.4 ± 0.5 °C (*c* 1.0, MeOH) (Found: C, 41.95; H, 5.25; N, 9.3. $C_{41}H_{60}I_2N_8O_{14}S$ requires C, 41.89; H, 5.15; N, 9.54%).

N-*t-Butoxycarbonyl-seryl-tyrosyl-seryl-methionyl-γ-t-butyl-glutamyl-2,5-di-iodohistidine Hydrazide Monohydrate* (11).—Compound (10) (3.3 g) was dissolved in DMF (20 ml), $N_2H_4 \cdot H_2O$ (1.4 ml) was added, and the mixture was stirred under N_2 for 16 h in the dark. The solvent was removed by rotary evaporation below 20 °C, the residue was kept *in vacuo* over conc. H_2SO_4 for 16 h and then triturated under H_2O. The solid obtained was crystallised from aqueous methanol to give 2.28 g (72%) of *product* which had m.p. 172 °C (decomp.), $[\alpha]_D^{23}$ −11.4 ± 1.4° (*c* 0.35, DMF) (Found: C, 40.3; H, 5.3; I, 21.15; N, 11.8. $C_{40}H_{60}I_2N_{10}O_{13}S \cdot H_2O$ requires C, 40.25; H, 5.24; I, 21.30; N, 11.74%).

2,5-*Di-iodohistidyl-phenylalanyl-arginyl-tryptophyl-glycine Acetate* (12).—Phe-Arg-Trp-Gly·HOAc was prepared from *Z*-Phe-Arg(NO_2)-Trp-Gly-OMe [22] by saponification followed by reduction, and was purified by counter-current distribution in the solvent system n-butanol–10% aqueous acetic acid (1 : 1, v/v). It was obtained as a solid by freeze-drying from aqueous solution.

A solution of (6) (18.3 g) and hydroxybenzotriazole hydrate (HOBt, 5.5 g) was prepared in DMF (60 ml) and cooled to 0 °C. A solution of DCC (7.5 g) in DMF (15 ml) was added and the mixture was stirred at 0 °C for 1 h and then at 10 °C for 1 h. A solution of Phe-Arg-Trp-Gly·HOAc (18.0 g) and Et_3N (4.0 ml) in DMF (60 ml) at 0 °C was added and the mixture was stirred for 16 h at 4 °C. Insoluble matter was filtered off and the filtrate was evaporated to dryness below 40 °C to give a gum which, on trituration with ethyl acetate, afforded a yellow powder (33.9 g). This was dissolved in acetic acid (108 ml) and treated for 30 min at room temperature with trifluoroacetic acid (900 ml). The solution was evaporated to dryness and the residue was kept *in vacuo* over NaOH pellets for 16 h. It was dissolved in 50% aqueous ethanol (200 ml) and passed through a column (50 × 4 cm) of Dowex 1 (acetate form) resin. The eluate and washings were dried by evaporation to a reduced volume and freeze-dried to give a yellow powder (25.6 g).

A portion (3.25 g) of this was dissolved in 80 ml of each phase of the solvent system n-butanol–10% aqueous acetic acid (1 : 1, v/v) and put in tubes 1—8 of a 123-tube counter-current distribution machine, then 115 transfers of upper phase were performed after which the product was located by t.l.c. in solvent No. 7 [23] in tubes 28—70. All other tubes were refilled with fresh solvent and an additional 215 transfers were performed in the recycling mode. The product was recovered as a white powder from tubes 94—16 by evaporation followed by freeze-drying of an aqueous suspension in a yield of 2.12 g (56% overall). The *product* had $[\alpha]_D^{28}$ −13.7 ± 0.3° (*c* 0.6, MeOH) (Found: C, 42.7; H, 4.55; I, 24.9; N, 15.0. $C_{34}H_{41}I_2N_{11}O_6 \cdot CH_3CO_2H$ requires C, 42.65; H, 4.44; I, 25.07; N, 15.20%).

Trial Azide Couplings at a Glutamic Acid Residue with (1) *and* (4).—(*a*) A solution of (4) (1.0 g) in DMF (11.7 ml) was cooled to −10 °C and 5M-HCl (0.77 ml) was added. Isopentyl nitrite (0.17 ml) was added and the mixture was stirred at −7 °C for 7 min.[18] Triethylamine (0.55 ml) was added and the mixture was poured into a solution of (12) (0.97 g) and triethylamine (0.135 ml) in DMF (11.7 ml). The mixture was stirred at −5 °C for 30 min and then at 4 °C for 72 h. Triethylamine hydrochloride was filtered off, and the filtrate was evaporated to dryness below 40 °C. The residue was triturated with cold water (80 ml) and yielded 1.05 g of solid. Crystallisation from 90% aqueous MeOH gave 226 mg (14%) of *decapeptide* which had m.p. 210 °C (decomp.) (Found: C, 47.7; H, 5.5; I, 15.1; N, 12.95. $C_{68}H_{92}I_2N_{16}O_{18}S$ requires C, 47.84; H, 5.39; I, 14.89; N, 13.13%).

(*b*) A solution of (1) (1.0 g) in DMF (12.5 ml) was cooled to −10 °C and 5M-HCl (0.81 ml) was added. Isopentyl nitrite (0.40 ml) was added and the mixture was stirred at −10 °C for 10 min.[18] Triethylamine (0.56 ml) was added and the azide solution was poured into a mixture of histidine methyl ester dihydrochloride (0.69 g) and Et_3N (0.80 ml) in DMF (12.5 ml) at −10 °C. The mixture was stirred at −5 °C for 30 min and then at 4 °C for 16 h. The neutral fraction (0.2 g, 14%) was recovered in the usual manner and examined by t.l.c. in chloroform–methanol (9 : 1, v/v). The desired product, *Z*-Glu(OBu^t)-His-OMe, was one of four compounds present in approximately equal amounts in the recovered material.

Trial Coupling with Compound (5) *at a Glutamic Acid Residue.*—Compound (5) (233 mg) and HOBt (46 mg) were dissolved in DMF (1 ml) and cooled to −10 °C. DCC (63 mg) in DMF (0.5 ml) was added and the mixture was stirred at −10 °C for 1 h and then at 0 °C for 1 h. A solution of His-OMe·2HCl (72 mg) and Et_3N (85 μl) in DMF (1 ml) was added and the mixture was stirred for 30 min at 0 °C and then allowed to come to room temperature overnight. The hexapeptide derivative was worked up in the usual manner.[4] The crude material obtained (100 mg, 36%) was examined by t.l.c. in chloroform–methanol (85 : 15, v/v). The desired product, Boc-Ser-Tyr-Ser-Met-Glu(OBu^t)-His-OMe, was one of three compounds present in approximately equal amounts.

γ-t-Butyl-glutamyl-2,5-di-iodohistidyl-phenylalanyl-arginyl-tryptophyl-glycine Tosylate (13).—*N*-2-Biphenyl-4-yl-isopropoxycarbonyl-γ-t-butyl-glutamic acid (3.55 g) and HOBt (1.27 g) were dissolved in DMF (35 ml) and cooled to 0 °C. DCC (1.66 g) in DMF (5.5 ml) was added and the mixture was stirred for 1 h at 0 °C and then for 1 h at 10 °C. A solution of (12) (6.54 g) and Et_3N (0.89 ml) in DMF (35 ml) at 0 °C was added and the mixture was stirred for 17 h at 4 °C. A small amount of insoluble material was filtered off and the filtrate was evaporated to dryness below 35 °C. The residue obtained was triturated with diethyl ether and yielded 8.49 g (96%) of light yellow solid. This was dissolved in a mixture of acetic acid–formic acid–water (7 : 1 : 2 v/v) (186 ml) and kept at room temperature for 5 h. The solution was evaporated to approximately one-third volume below 25 °C, water (50 ml) was added, and the solution was freeze-dried to yield 8.26 g of material. A portion (3.1 g) was dissolved in a mixture of both phases (150 ml each) of the solvent system 1.25% (w/v) aqueous toluene-4-sulphonic acid–t-butyl alcohol–toluene (10 : 10 : 3, v/v), loaded into tubes 1—15 of a 123-tube counter-current machine and subjected to 108 upper-phase transfers. Samples of the upper phases were examined in the t.l.c. system No. 7; [23] the contents of tubes 1—82 and 111—123 were replaced with fresh solvent and a further 1 000 transfers were performed in the recycling mode with examination of the distribution at intervals and removal of impurities. The contents of the tubes finally containing purified product were combined, the upper phase was separated, and the lower phase was extracted with fresh upper phase (2 × 100 ml). The combined upper phases were diluted with water

(600 ml) and reduced by rotary evaporation below 25 °C until a single phase was obtained. The solution was adjusted to pH 6 by addition of saturated $NaHCO_3$ solution and freeze-dried. The solid obtained was triturated with ice-cold water (50 ml), and the solid was filtered off and washed with a further portion of ice-cold water (50 ml). The solid was dried *in vacuo* over conc. H_2SO_4 to yield 1.01 g of *product* (overall yield, 32%). It had amino-acid analysis after acidic hydrolysis: Arg, 0.98; Glu, 0.97; Gly, 1.00; His species, 1.02; Phe, 1.00; Trp, 0.37. Different preparations gave widely variable analyses for S indicating non-stoicheiometric recoveries of combined toluenesulphonic acid.

N-t-Butoxycarbonyl-seryl-tyrosyl-seryl-methionyl-γ-t-butyl-glutamyl-2,5-di-iodohistidyl-phenylalanyl-arginyl-tryptophyl-glycine (14).—Boc-Ser-Tyr-Ser-Met-N_2H_3 [17] (1.43 g) dissolved in DMF (5 ml) was cooled to −10 °C and 2.1M-HCl in EtOAc (2.84 ml) was added with stirring, the temperature being kept below −5 °C. t-Butyl nitrite (0.18 ml) was added and the mixture was stirred at −10 °C for 10 min.[18] Triethylamine (0.83 ml) was added and the mixture was added to a solution of (13) (2.5 g) containing sufficient triethylamine to establish pH 9 in DMF (5 ml) at −10 °C. The mixture was stirred at −10 °C for 1 h and at 4 °C for 72 h. Triethylamine hydrochloride was filtered off and the filtrate was evaporated to dryness below 35 °C. The residue was triturated with water and afforded a light yellow solid (3.24 g) which was crystallised twice from DMF–MeOH–H_2O (10 : 30 : 3, v/v) to give the *product* (0.90 g, 28%), which had m.p. 210 °C (decomp.), $[\alpha]_D^{25}$ −7.0 ± 1.0° (*c* 0.5, DMF) and amino-acid analysis after acidic hydrolysis: Arg, 1.00; Glu, 1.01; Gly, 1.00; His species, 0.96; Met, 1.00; Phe, 1.00; Ser, 1.84; Trp, 0.43; Tyr, 1.00

t-Butoxycarbonyl-seryl-tyrosyl-seryl-methionyl-γ-t-butyl-glutamyl-2,5-di-iodohistidyl-phenylalanyl-arginyl-tryptophyl-glycyl-Nε-t-butoxycarbonyl-lysyl-prolyl-valyl-glycyl-Nε-t-butoxycarbonyl-lysyl-Nε-t-butoxycarbonyl-lysyl-arginyl-arginyl-prolyl-valyl-Nε-t-butoxycarbonyl-lysyl-valyl-tyrosyl-proline t-Butyl Ester Trihydrochloride (15).—*C*-Terminal tetradecapeptide base dihydrochloride [24] (315 mg) was dissolved with (14) (300 mg) and HOBt (27 mg) in DMF (3 ml) and warmed to 50 °C with stirring until a solution was obtained. 1M-HCl (0.176 ml) was added followed by DCC (119 mg) in DMF (0.5 ml) and the solution was stirred at 50 °C for 2 h under N_2. After storage at 4 °C, DCU was filtered off and the filtrate was evaporated to dryness below 30 °C. The residue was dissolved in the lower phase (50 ml) of methanol–ammonium acetate buffer (pH 4.5)–chloroform–carbon tetrachloride (8 : 4 : 5 : 2, v/v) and loaded into tubes 1—5 of a 123-tube counter-current distribution machine (10-ml each phase) and subjected to 577 upper-phase transfers.[24] Samples of the lower phases were examined by t.l.c. in solvent system No. 7.[23] *Product* (379 mg, peptide content 77%, yield 52%) was recovered from tubes 52—74. It contained DCU and had amino-acid analysis after acidic hydrolysis: Arg, 2.98; Glu, 1.01; Gly, 2.03; His species, 0.79; Lys, 3.96; Met, 0.99; Phe, 0.99; Pro, 3.02; Ser, 1.79; Trp, 0.48; Tyr, 1.99; Val, 3.00.

(6—2,5-Di-iodohistidine)-β-corticotrophin-(1—24)-tetracosapeptide (16).—A sample (8.4 mg) of (15) was stirred for 30 min at room temperature with 90% TFA (5 ml). The solution was evaporated to dryness and the residue was dissolved in water (1 ml) and converted into the acetate salt by passage through a column (1 ml) of Dowex 1 (acetate form) resin. The solution was dried and the residue dissolved in water (0.5 ml). The solution was applied to a column (3 × 0.8 cm) of carboxymethyl cellulose (Na^+ form) and eluted with a linear gradient (40 ml, 0—0.55M) of sodium chloride in 0.05M-phosphate buffer, pH 7. The recovered *peptide* (675 nmol, 32%) had amino-acid analysis after acidic hydrolysis: Arg, 3.01; Glu, 1.00; Gly, 2.03; His species, 0.76; Lys, 3.98; Met, 0.98; Phe, 0.99; Pro, 3.00; Ser, 1.75; Trp, 0.53; Tyr, 2.00; Val, 3.00. A portion of solution was passed through a short column of Dowex 1 (chloride form) resin to remove phosphate ions [1] and subjected to enzymic hydrolysis [25] followed by amino-acid analysis: Arg, 2.90; Glu, 0.69; Gly, 1.77; His, 0.01; His(I_2), 0.37; Lys, 4.04; Met, 0.90; Phe, 0.91; Pro, 3.04; Ser, 2.04; Trp, 0.96; Tyr, 1.81; Val, 3.00.

[2,5-$^3H_2$-His[6]]-β-Corticotrophin-(1—24)-tetracosapeptide.—This compound was prepared from (15) in 87% yield as described for the tyrosine-labelled analogue [2] with the difference that the final ion-exchange chromatographic purification was as detailed above for compound (16). The *product* had amino-acid analysis after acidic hydrolysis: Arg, 3.03; Glu, 1.00; Gly, 2.00; His, 0.99; Lys, 3.94; Met, 0.98; Phe, 0.98; Pro, 2.97; Ser, 1.76; Trp, 0.79; Tyr, 1.99; Val, 3.00. Following removal of phosphate ions as described above, amino-acid analysis after enzymic hydrolysis gave: Arg, 2.93; Glu, 0.98; Gly, 1.96; His, 0.99; Lys, 3.89; Met, 0.97; Phe, 0.99; Pro, 2.80; Ser, 1.99; Trp, 0.92; Tyr, 2.01; Val, 2.82. The specific radioactivity of the peptide was 30.0 Ci mmol^{-1} and radioactivity was located [3] exclusively in the histidine residue. Its potency in the isolated adrenal cell bioassay [11] was 98% (95% confidence limits, 71—134%) compared to Synacthen.

A portion of the acid hydrolysate was resolved on the amino-acid analyser and the distribution of radioactivity amongst the amino-acid residues was determined.[3] By comparison with the enzymic digest examined, the histidine residue contained 94.5 ± 12% of the radioactivity originally present.

We thank Miss M. Chen for bioassay measurements, Mr. B. E. Evans for enzymic digests and amino-acid analyses, Mrs. S. M. Garman for the assessment of the distribution of radioactivity amongst the amino-acid residues, and Dr. W. Rittel, CIBA-GEIGY Ltd. (Basle) for the supply of protected tetradecapeptide salt.

[8/1843 *Received*, 23*rd October*, 1978]

REFERENCES

[1] Part 6, D. E. Brundish, J. R. Martin, and R. Wade, *J. Chem. Research*, 1979, (*S*) 11.
[2] D. E. Brundish and R. Wade, *J.C.S. Perkin I*, 1973, 2875.
[3] D. E. Brundish, J. R. Martin, and R. Wade, *J.C.S. Perkin I*, 1976, 2182.
[4] D. E. Brundish and R. Wade, *J.C.S. Perkin I*, 1976, 2186.
[5] J. Pradelles, J.-L. Morgat, P. Fromageot, C. Oliver, P. Jacquet, D. Gourdji, and A. Tixier-Vidal, *FEBS Letters*, 1972, **22**, 19.
[6] A. Menez, J.-L. Morgat, P. Fromageot, A.-M. Ronseray, P. Boquet, and J.-P. Changeux, *FEBS Letters*, 1971, **17**, 333.
[7] R. H. Boissonnas, S. Guttmann, J.-P. Waller, and P.-A. Jacquenoud, *Experientia*, 1956, **12**, 446.
[8] S. Guttmann and R. H. Boissonnas, *Helv. Chim. Acta*, 1959, **42**, 1257.
[9] M. Bodanszky, M. L. Fink, Y. S. Klausner, S. Natarajan, K. Tatemoto, A. E. Yiotakis, and A. Bodanszky, *J. Org. Chem.*, 1977, **42**, 149.
[10] R. Schwyzer and H. Kappeler, *Helv. Chim. Acta*, 1961, **44**, 1991.

[11] P. J. Lowry, C. McMartin, and J. Peters, *J. Endocrinol.*, 1973, **59**, 43.
[12] H. Fraenkel-Conrat, *Arch. Biochem. Biophys.*, 1950, **27**, 109.
[13] C. T. Holloway, R. P. M. Bond, I. G. Knight, and R. B. Beechey, *Biochemistry*, 1967, **6**, 19.
[14] K. Hempel in 'Proceedings of Conference on Methods of Preparing and Storing Marked Molecules,' Euratom, 1964, p. 1009.
[15] E. Schröder and E. Klieger, *Annalen*, 1964, **673**, 196.
[16] E. Klieger and H. Gibian, *Annalen*, 1962, **655**, 195.
[17] B. Iselin and R. Schwyzer, *Helv. Chim. Acta*, 1961, **44**, 169.
[18] J. Honzl and J. Rudinger, *Coll. Czech. Chem. Comm.*, 1961, **26**, 2333.
[19] K. J. Brunnings, *J. Amer. Chem. Soc.*, 1947, **69**, 205.
[20] E. Schnabel, *Annalen*, 1967, **702**, 188.
[21] P. Sieber and B. Iselin, *Helv. Chim. Acta*, 1968, **51**, 622.
[22] H. Kappeler, *Helv. Chim. Acta*, 1961, **44**, 476.
[23] E. von Arx and R. Neher, *J. Chromatog.*, 1963, **12**, 329.
[24] R. Schwyzer and H. Kappeler, *Helv. Chim. Acta*, 1963, **46**, 1550.
[25] H. P. J. Bennett, D. F. Elliott, B. E. Evans, P. J. Lowry, and C. McMartin, *Biochem. J.*, 1972, **129**, 695.