Synthesis of 5- and 7-Bromotryptophan and of [5-Bromotryptophan[9]]-β-corticotrophin-(1—24)-tetracosapeptide, a Highly Potent Corticotrophin Analogue †

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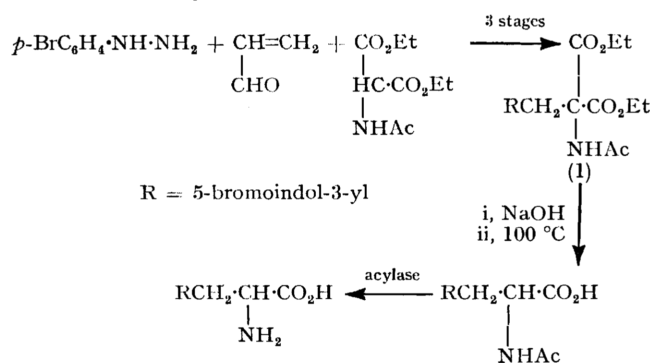
The synthesis of 5- and 7-bromo-L-tryptophans *via* Fischer cyclisation of the appropriate bromophenylhydrazone of 4-acetamido-4,4-bis(ethoxycarbonyl)butanal is described. The title tetracosapeptide was synthesised using standard methods of stepwise and fragment condensation. The product was pure as judged by amino-acid analysis after acidic or enzymic hydrolysis and by high pressure liquid chromatography. In an isolated adrenal cell bioassay, the peptide had a steroidogenic potency 2.4 times that of Synacthen.

WE have previously described the synthesis of a number of analogues of corticotrophin containing halogenated aromatic amino-acids.[1] The protected precursors of these peptides have been used by us as substrates for the production of peptides containing high levels of tritium located at specific positions by catalytic dehalogenation. These labelled peptides are used to trace their distribution and metabolic fate *in vivo*.[2]

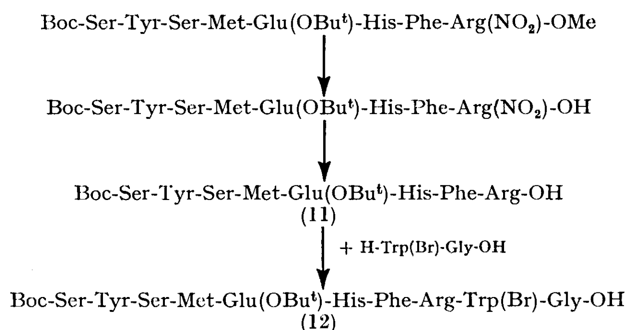
It was decided to investigate whether peptides containing tryptophan residues could be tritium-labelled by this reductive technique. Attempts to synthesise iodotryptophans by routes involving a Fischer indolisation were not successful due to instability of the intermediates and we therefore decided to undertake the present synthesis using the readily prepared 5-bromo-derivative. It had previously been observed that, while 4-iodophenylalanine-containing derivatives of corticotrophin could be readily and completely dehalogenated,[1,3,4] a 4-bromophenylalanine-containing tetracosapeptide was dehalogenated too slowly to be of practical use.[3] This experience is now extended by our inability, to date, to find suitable experimental conditions for the clean and



SCHEME 1

rapid replacement of bromine by hydrogen in the protected tetracosapeptide whose synthesis we detail here.

N-Acetyl-5-bromo-L-tryptophan was synthesised by a route (Scheme 1) based on that used by Rydon and Tweddle [5] for the synthesis of 5-chloro-DL-tryptophan. The Fischer cyclisation product (1) was saponified and decarboxylated by heating to give acetyl-5-bromo-DL-tryptophan (2). The L-isomer of this racemate was hydrolysed using the acylase present as an impurity in crude fungal α-amylase. Acetyl-5-bromo-D-tryptophan



SCHEME 2

was recovered, racemised, and recycled to increase the yield of required isomer. It was found possible to carry out a similar series of reactions using 2-bromophenylhydrazine to give 7-bromo-L-tryptophan.

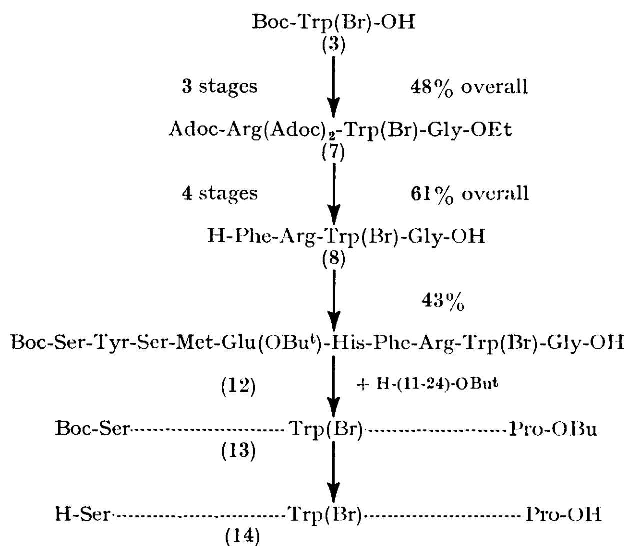
We synthesised the tetracosapeptide by the usual (1—10) + (11—24) fragment condensation.[6] To avoid lengthy synthetic approaches to the required bromine-containing *N*-terminal decapeptide (12) (Scheme 2), we wished to prepare the octapeptide (11) for coupling with the dipeptide bromotryptophyl-glycine. Our first approach which is not detailed here, was *via* the fully protected octapeptide containing nitroarginine methyl ester and was unsuitable for the following reasons: (i) the fully protected octapeptide could not be purified due to its low solubility; (ii) saponification of the methyl ester proved difficult and entailed a lengthy separation of the acid from the ester by counter-current distribution. The acid was obtained in low yield; (iii) reduction of the nitro-group (not reported here) proceeded very slowly due to the poisoning of the catalyst by methionine and gave an impure product which needed another lengthy purification by counter-current distribution.

To avoid the difficult saponification and reduction steps, we synthesised the octapeptide by a 4 + 4 azide

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

coupling using the appropriately deprotected *C*-terminal tetrapeptide. The octapeptide was recovered as the tosylate salt of the arginine guanidine group so that it was in a suitable form for coupling. In a trial coupling with tryptophyl-glycine, inefficient reaction was observed and this approach was abandoned.

We then aimed to synthesise (12) by a 6 + 4 approach as had been successfully adopted in the synthesis of the substituted phenylalanine decapeptides.[3,4] This approach required protection of the guanidine function of arginine by an acid-labile group. L-Bromotryptophan was converted into the dipeptide (4) in high yield. Bis-(t-butoxycarbonyl)arginine gave the tripeptide in poor yield whereas tris-(1-adamantyloxycarbonyl)arginine allowed an approach to the required tetrapeptide (8) in good yield. The subsequent couplings to the tetracosapeptide product (1) proceeded smoothly. The successful synthesis is shown in Scheme 3.



SCHEME 3

The free peptide (14) was prepared by deprotection using aqueous trifluoroacetic acid followed by ion-exchange chromatography. The recovered peptide was shown to be pure by amino-acid analysis following acidic or enzymic hydrolysis and behaved as a single compound when investigated by high pressure liquid chromatography (h.p.l.c.). The steroidogenic potency as measured in an isolated adrenal cell bioassay[7] was 2.4 times that of Synacthen. To the best of our knowledge, this is the first report of enhanced steroidogenic activity due to a single substitution with an L-amino-acid in corticotrophin analogues. Ramachandran has described enhanced melanocyte-stimulating activity in the case of a corticotrophin analogue containing 2-nitrophenylsulphenyl (NPS) in the 2 position of the tryptophan indole nucleus. This compound was three times more potent as a melanophore-stimulating agent but possessed only 0.01 times of the steroidogenic potency of corticotrophin.[8] It has recently been reported that analogues of somatostatin containing 5-fluoro- and 5-bromo-D-tryptophan have greatly enhanced activities in suppressing growth-hormone release from cultured rat pituitary cells compared with somatostatin containing D-tryptophan.[9] It was postulated that the increased availability of electrons in the indole nucleus might be responsible for the enhanced bioactivity. This type of consideration could also apply to our observation as the tryptophan residue is located in the active centre of corticotrophin.[10] This implies that the region close to the indole nitrogen plays a key role in the initiation of steroidogenesis.

EXPERIMENTAL

Routine analytical procedures were as described by Brundish and Wade.[11]

5-Bromo-3-[2-acetamido-2,2-bis(ethoxycarbonyl)ethyl]indole (1).—Sodium (0.32 g) in dry methanol (16 ml) was added to a solution of diethyl acetamidomalonate (68 g) in dry benzene (110 ml) contained in a 1-l 3-necked flask fitted with stirrer, dropping funnel, and condenser closed with a drying tube ($CaCl_2$). Acrolein (22 ml) in dry benzene (24 ml) was added at a rate such that the temperature of the reaction mixture was maintained below 35 °C. Stirring was continued for 2 h and then glacial acetic acid (7.5 ml) was added followed by 4-bromophenylhydrazone (65 g). The mixture was warmed to 50 °C for 1 h and the resulting clear solution left overnight at room temperature. After removal of solvent by evaporation the residual brown oil was dissolved in glacial acetic acid (140 ml), water (1 200 ml) and concentrated H_2SO_4 (50 ml) were added, and the mixture was stirred at gentle reflux for 4 h and then cooled. The aqueous layer was decanted, the brown solid was dissolved in ethyl acetate–benzene (1 : 1), and the organic layer was washed with 1M-sodium hydrogen carbonate solution and with water; it was then dried (Na_2SO_4) and evaporated. Trituration of the solid residue with ether was followed by several recrystallisations from methanol (charcoal) to give pale yellow needles, m.p. 160—163 °C; yield was 53 g (39%). The *product* was recrystallised for analysis from methanol and had m.p. 165—167 °C (Found: C, 50.9; H, 5.05; Br, 19.0; N, 6.7. $C_{18}H_{21}BrN_2O_5$ requires C, 50.83; H, 4.97; Br, 18.79; N, 6.58%).

*Acetyl-5-bromo-*DL*-tryptophan* (2).—The substituted malonate (4 g) was heated under reflux with 10% NaOH solution (20 ml) for 1 h, cooled, and acidified with concentrated HCl below 25 °C. The crystals of diacid were filtered off, washed with water, and sucked dry; they had m.p. 167—170 °C (decomp) (yield 3.2 g). The diacid was heated with water (100 ml) under reflux for 2½ h. On cooling fine crystalline plates separated. These were filtered off and recrystallised from water containing a little methanol. *Acetyl-5-bromo-*DL*-tryptophan* (2.35 g, 77%) was obtained having m.p. 212—214 °C (Found: C, 47.85; H, 3.8; Br, 24.45; N, 8.45. $C_{13}H_{13}BrN_2O_3$ requires C, 48.01; H, 4.02; Br, 24.57; N, 8.61%).

*5-Bromo-*L*-tryptophan.*—Crude fungal α-amylase (Calbiochem B grade, 3 g) was stirred vigorously (Whirlimixer) with water (30 ml), the mixture centrifuged, and the supernatant liquid added to a solution of acetyl-5-bromo-DL-tryptophan (3.5 g) in water (20 ml) containing lithium hydroxide (0.45 g) and of pH 7.0 (adjusted with acetic acid if necessary). After incubation at 37 °C for 3 days the mixture was stored in the refrigerator overnight and the cream solid was filtered off. The filtrate was retained for

the recovery of acetylbromo-D-tryptophan (see below). The solid was suspended in warm water (7 ml), dissolved by adding the minimum of concentrated HCl dropwise and the solution was filtered hot to remove a trace of coagulated protein. Ammonia (*d* 0.88) was added to pH 7.0, and the solid was filtered off, washed with water, and sucked dry. Yield of 5-*bromo*-L-*tryptophan* was 1.0 g (66%), m.p. 288—291 °C (decomp) $[\alpha]_D^{20}$ +31.8° (*c* 1, M-HCl), −28.5° (*c* 1, AcOH) (Found: C, 46.7; H, 3.95; Br, 27.9; N, 9.75. $C_{11}H_{11}BrN_2O_2$ requires C, 46.65; H, 3.89; Br, 28.27; N, 9.39%).

Racemisation of Acetyl-5-*bromo*-D-*trytophan*.—The mother-liquor after filtration of 5-bromo-L-trytophan, combined from several resolution experiments, was acidified to pH 2.0 with concentrated hydrochloric acid and extracted three times with ethyl acetate; the extract was dried (Na_2SO_4) and evaporated. The residue crystallised on trituration with light petroleum. The solid (8 g) was heated with acetic anhydride (20 ml) to reflux temperature, poured into cold water (80 ml), and the mixture evaporated. Water (100 ml) was evaporated from the residue which was then dissolved in methanol (80 ml). The solution was boiled (charcoal), filtered, concentrated to 20 ml, water (20 ml) was added, and the solution set aside to crystallise. Further crops of racemate were obtained by concentrating the mother-liquor. After a further recrystallisation from methanol the product, m.p. 208—211 °C, was resolved with enzyme as described above.

3-[2-*Acetamido*-2,2-*bis*(*ethoxycarbonyl*)*ethyl*]-7-*bromo-indole*.—This compound was prepared by the method described above starting from 2-bromophenylhydrazine. The *product* which was recrystallised from aqueous methanol or from toluene had m.p. 138—141 °C and was obtained in similar yield (Found: C, 50.7; H, 4.95; Br, 18.9; N, 6.55. $C_{18}H_{21}BrN_2O_5$ requires C, 50.83; H, 4.97; Br, 18.79; N, 6.58%).

Acetyl-7-*bromo*-DL-*tryptophan*.—Saponification and decarboxylation of the above malonate was carried out as described for the corresponding 5-bromo-isomer. The *product* was crystallised from aqueous methanol and had m.p. 216—218 °C (Found: C, 47.95; H, 4.1; Br, 24.7; N, 8.7. $C_{13}H_{13}BrN_2O_3$ requires C, 48.01; H, 4.02; Br, 24.57; N, 8.61%).

7-*Bromo*-L-*tryptophan*.—The acetyl derivative of the racemate was resolved as described above for the 5-bromo-isomer. Yield of pure L-*amino-acid* was 63%, m.p. 276—280 °C (decomp), $[\alpha]_D^{20}$ −7.6° (*c* 1, AcOH) (Found: C, 46.35; H, 3.95; Br, 27.75; N, 9.7. $C_{11}H_{11}BrN_2O_2$ requires C, 46.65; H, 3.89; Br, 28.27; N, 9.89%).

N-*t*-*Butoxycarbonyl*-5-*bromotryptophan* (3).—This compound was prepared from 5-bromotryptophan in 94% yield by the method of Schnabel at pH 9.8.[12] The *product* was crystallised from ethyl acetate–hexane and had m.p. 164—165 °C, $[\alpha]_D^{28}$ = +1.5 ± 0.7° (*c* 0.8, MeOH) (Found: C, 50.2; H, 5.05; Br, 20.9; N, 7.3. $C_{16}H_{19}BrN_2O_4$ requires C, 50.11; H, 5.00; Br, 20.88; N, 7.31%).

N-*t*-*Butoxycarbonyl*-5-*bromotryptophylglycine Ethyl Ester* (4).—Glycine ethyl ester hydrochloride (5.28 g) was dissolved in dimethylformamide (DMF) (25 ml), cooled to −10 °C, and Et_3N (5.25 ml) was added. A solution of (3) (14.5 g) in DMF (25 ml) at −10 °C was added followed by a solution of dicylohexylcarbodi-imide (DCC) (8.5 g) in DMF (8 ml). The mixture was stirred at 4 °C for 16 h. Dicyclohexylurea (DCU) was filtered off and the filtrate was dried by rotary evaporation. The residue was dissolved in ethyl acetate (50 ml) and the solution was washed with cold citric acid and hydrogen carbonate solutions, dried (Na_2SO_4), and evaporated to dryness. Crystallisation from benzene–cyclohexane gave the *product* (13.9 g, 79%) which had m.p. 130—132 °C, $[\alpha]_D^{28}$ = −15.1 ± 0.7° (*c* 1.0, MeOH) (Found: C, 51.3; H, 5.55; Br, 17.15; N, 9.0. $C_{20}H_{26}BrN_3O_5$ requires C, 51.27; H, 5.60; Br, 17.07; N, 8.97%).

N^{α},N^{ω}-*Bis*-(*t*-*Butoxycarbonyl*)*arginyl*-5-*bromotryptophylglycine Ethyl Ester* (6).—The dipeptide (4) (14.8 g) was dissolved in M-HCl in acetic acid (150 ml) and kept at room temperature for 45 min. The solution was evaporated to dryness below 30 °C, a portion (100 ml) of fresh methanol was evaporated from the residue and this residue was stored *in vacuo* over KOH pellets for 16 h to give 12.9 g (100%) of H-Trp(Br)-Gly-OEt·HCl (5).

N^{α},N^{ω}-Bis(t-butoxycarbonyl)arginine (11.9 g) and *N*-hydroxybenzotriazole (HOBt) (4.85 g) were dissolved in DMF (30 ml) and the solution was cooled to 0 °C. A solution of DCC (7.2 g) in DMF (12 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 (5) (12. 9 g) and Et_3N (4.42 ml) in DMF (40 ml) at 0 °C was added the mixture was stirred at 4 °C for 16 h. DCU was filtered off and the filtrate was evaporated to dryness by rotary evaporation. The residue was dissolved in ethyl acetate (250 ml) and purified by extraction in the normal manner. The recovered material was purified by silica gel chromatography. The product was eluted by chloroform–methanol (97 : 3, v/v). The recovered *product* (2.65 g, 11%) could not be crystallised. It had $[\alpha]_D^{30}$ = −18.7 ± 0.6° (*c* 1.0, MeOH) and amino-acid analysis after acidic hydrolysis: Arg, 0.97; Gly, 1.00; Trp, 0.31.

N^{α},N^{δ},N^{ω}-*Tris*-(1-*Adamantyloxycarbonyl*)*arginyl*-5-*bromotryptophylglycine Ethyl Ester* (7).—Tris-(1-Adamantyloxycarbonyl)arginine (15.38 g) and HOBt (3.34 g) were dissolved in DMF (50 ml) and the solution was cooled to 0 °C. A solution of DCC (4.95 g) in DMF (15 ml) was added and the mixture was stirred at 0 °C for 90 min and then at 10 °C for 90 min.

The hydrochloride (3) (8.8 g) was dissolved in DMF (50 ml), the solution was cooled to 0 °C and adjusted to pH 9 by addition of Et_3N (3.05 ml). The solution was added to the active ester solution and the mixture was stirred at 4 °C for 17 h. The solution was filtered to remove a little insoluble material which had separated and the filtrate was evaporated to dryness. The residue was dissolved in ethyl acetate (300 ml) and purified by extraction in the usual manner. The recovered residue afforded impure tripeptide (20.1 g) on trituration under light petroleum. Pure material was obtained by silica gel chromatography. The product was eluted by benzene–ether (7 : 3, v/v). Crystallisation from cyclohexane afforded the *tripeptide* (14.1 g, 61%) which contained solvent of crystallisation. It had m.p. 146—148 °C, $[\alpha]_D^{30}$ = −10.6 ± 0.5° (*c* 1.0, MeOH) (Found: C, 62.1; H, 7.05; Br, 7.25; N, 8.85. $C_{54}H_{72}BrN_7O_{10}\cdot\frac{1}{2}C_6H_{12}$ requires C, 62.16; H, 7.13; Br, 7.25; N, 8.90%).

Phenylalanylarginyl-5-*bromotryptophylglycine Acetate* (8).—Compound (7) (7.0 g) was dissolved in a mixture of trifluoroacetic acid (225 ml) and water (25 ml) and kept at room temperature for 45 min. The solvent was removed by rotary evaporation below 10 °C and the residue was triturated with ether and dried. The residue was dissolved in water (50 ml) and passed through a column (46 × 1.5 cm) of Dowex 1 (acetate form) resin which was eluted with water (200 ml). The solution was freeze-dried and gave 3.64 g (96%) of solid.

t-Butoxycarbonylphenylalanine (2.48 g) was dissolved in a mixture of pure $CHCl_3$ (10 ml) and sodium-dried dioxan (10 ml) and Et_3N (1.31 ml) was added; the solution was cooled to −10 °C. Isobutyl chloroformate (1.16 ml) was added to the mixture which was stirred at −10 °C for 30 min with exclusion of moisture.

The tripeptide acetate (3.64 g) described above was dissolved in DMF (15 ml) cooled to 0 °C and Et_3N (0.87 ml) was added. The solution was added to the mixed anhydride solution and the mixture was stirred for 18 h at room temperature with exclusion of moisture. The solution was evaporated to dryness and the residue was dissolved in a mixture of n-butanol and ethyl acetate (1 : 1, v/v; 300 ml) which was purified by extraction in the usual manner. After evaporation of the solvent, the residue was solidified by trituration with light petroleum.

The crude ester (5.3 g) was dissolved in methanol (156 ml) and M-NaOH (27.5 ml) was added. The mixture was stirred at room temperature for 1½ h, then cooled to 0 °C and M-HCl (27.5 ml) was added with vigorous stirring. The solution was evaporated to dryness and the residue was dissolved in a mixture of n-butanol and ethyl acetate (2 : 1, v/v, 300 ml); the latter was washed with brine (5 × 100 ml), dried (Na_2SO_4), and the solution evaporated to dryness. The residue was dissolved in M-HCl in acetic acid (120 ml) and kept for 1 h at room temperature. The solution was evaporated to dryness and the residue was dissolved in water (25 ml). The solution was passed through a column (30 × 1 cm) of Dowex 1 (acetate form) resin and eluted with water (100 ml). The eluate was freeze-dried to give 4.39 g of solid. This was dissolved in the upper phase (60 ml) of the solvent system n-butanol–10% acetic acid (1 : 1, v/v), loaded into tubes 1—6 of a 123-tube counter-current machine and subjected to 115 upper-phase transfers. The upper phases of each tube were analysed by t.l.c. in the solvent system No. 7[13] and all tubes other than 28—55, which contained product, were refilled with fresh solvents. After a further 340 transfers of upper phase in the recycling mode, product contaminated only with phenylalanine was recovered from tubes 115—123 and 1—39 by rotary evaporation followed by freeze-drying. The material was separated from the phenylalanine contaminant by counter-current distribution using the solvent system n-butanol–pyridine–acetic acid–water (8 : 2 : 1 : 9, v/v). The material was put in tubes 1—8 and after 114 upper-phase transfers, pure product was recovered from tubes 58—88. After rotary evaporation followed by freeze-drying, the *product* (2.90 g, 61%) was obtained as a white solid which had $[\alpha]_D^{27} = -26.9 \pm 0.6°$ (*c* 0.65, DMF) (Found: C, 48.9; H, 5.55; Br, 10.8; N, 14.75. $C_{28}H_{35}BrN_8O_5 \cdot CH_3CO_2OH \cdot 2H_2O$ requires C, 48.71; H, 5.86; Br, 10.80; N, 15.15%).

N-*Benzyloxycarbonyl-γ-t-butyl-glutamylhistidylphenylalanyl-*N^{ω}*-nitroarginine Methyl Ester* (9).—*Z*-Glu(OBu^t)-His-N_2H_3 (4.58 g) was dissolved in DMF (85 ml) and cooled to −10 °C; 5M-HCl (5.65 ml) was added in portions and then isopentyl nitrite (1.30 ml) the temperature of the solution being kept below −5 °C. The mixture was stirred at −7 °C for 7 min and Et_3N (3.93 ml) was added. A solution of H-Phe-Arg(NO_2)-OMe·HCl (prepared from 4.4 g of the N-t-butoxycarbonyl dipeptide) and Et_3N (1.38 ml) in DMF (85 ml) was added and the mixture was stirred for 60 h at 4 °C. Insoluble material was filtered off and the filtrate evaporated under reduced pressure. The residue was dissolved in a mixture of n-butanol (100 ml) and ethyl acetate (50 ml) and purified by extraction in the normal manner. Two crystallisations from ethyl acetate–light petroleum (b.p. 60—80 °C) gave the *product* (4.4 g, 56%) which had m.p. 128—130 °C, $[\alpha]_D^{28} = -27.7 \pm 0.7°$ (*c* 0.5, MeOH) (Found: C, 55.85; H, 6.1; N, 16.55. $C_{39}H_{52}N_{10}O_{11}$ requires C, 55.97; H, 6.26; N, 16.74%).

γ-t-Butyl Glutamylhistidylphenylalanylarginine Diacetate Monohydrate (10).—Compound (9) (1.71 g) was dissolved in methanol (145 ml), M-NaOH (25.6 ml) was added, and the mixture was stirred at room temperature for 1 h. Water (700 ml) and M-HCl (25.6 ml) was added and the mixture was evaporated to dryness. The residue was shaken with saturated $NaHCO_3$ soln. (1 450 ml) and ethyl acetate (700 ml) and the aqueous phase was collected. It was cooled to 0 °C acidified to pH 3 with solid citric acid, and extracted with a mixture (1 : 1 v/v) of n-butanol and ethyl acetate (3 × 500 ml). The combined extracts were washed to neutrality with water, dried (Na_2SO_4), and evaporated to dryness. The white solid obtained (1.1 g) was dissolved in methanol (20 ml) containing acetic acid (4 ml) and hydrogenated at room temperature for 65 h in the presence of 10% palladium on charcoal (300 mg) and 5% rhodium on charcoal (300 mg). Catalysts were filtered off and the filtrate was evaporated to dryness. The oil obtained was dissolved in DMF (10 ml) and the solution was added dropwise with vigorous stirring to acetonitrile (120 ml) at 4 °C. A white solid (715 mg) was filtered off. This was purified by ion-exchange chromatography using carboxymethyl-cellulose (hydrogen form) and a linear gradient (0—0.5M) of acetic acid. The *product* (318 mg, 24%) had m.p. 131—133 °C, $[\alpha]_D^{30} = -11.8 \pm 0.7°$ (*c* 1.0, DMF) (Found: C, 52.2; H, 7.15; N, 16.35. $C_{30}H_{45}N_9O_7 \cdot 2CH_3CO_2H \cdot H_2O$ requires C, 52.21; H, 7.09; N, 16.13%).

N-*t-Butoxycarbonylseryltyrosylserylmethionyl-γ-t-butylglutamylhistidylphenylalanylarginine Tosylate Trihydrate* (11).—Boc-Ser-Tyr-Ser-Met-N_2H_3 (1.11 g) was dissolved in DMF (17 ml) and the solution was cooled to −10 °C, 5M-HCl (1.11 ml) was added and then isopentyl nitrite (0.26 ml), the temperature of the solution being kept below −5 °C. The mixture was stirred at −10 °C for 10 min and Et_3N (0.91 ml) was added. A solution of (10) (1.15 g) and Et_3N (0.41 ml) in DMF (17 ml) at −10 °C was added and the mixture was stirred at −10 °C for 1 h and then at 4 °C for 65 h. Insoluble material was filtered off and the filtrate was evaporated to dryness. The residue was dissolved in a mixture of n-butanol (75 ml) and ethyl acetate (50 ml) and the solution was extracted with water (2 × 100 ml), dried (Na_2SO_4), and evaporated to dryness. The residue was dissolved in 30 ml of each of the phases of the solvent system n-butanol–10% acetic acid (1 : 1 v/v) and loaded into tubes 2—4 of a 123-tube counter-current machine. After 119 upper-phase transfers and examination by t.l.c. of samples in the solvent system No.7, the contents of all tubes other than 59—94 were refilled with fresh solvents and the system was subjected to a further 410 transfers in the recycling mode. The contents of tubes 50—57 were combined and evaporated to dryness. The residue (761 mg) was dissolved in DMF (25 ml), cooled to 0 °C, and a solution of toluene-4-sulphonic acid (97 mg) in DMF (4.74 ml) was added. The solution was evaporated to dryness and the residue was triturated with ether to give the *tosylate salt* (731 mg, 35%); it had m.p. 157—160 °C (Found: C, 51.75; H, 6.75; N, 12.8; S, 4.5. $C_{55}H_{81}N_{13}O_{16}S \cdot CH_3C_6H_4SO_3H \cdot 3H_2O$ requires C, 51.75; H, 6.66; N, 12.65; S, 4.45%).

N-*t-Butoxycarbonylseryltyrosylserylmethionyl-γ-t-butylglutamylhistidylphenylalanylarginyl-5-bromotryptophyl-*

glycine Dihydrate (12).—Box-Ser-Tyr-Ser-Met-Glu-His-N_2H_3[3] (2.38 g) was dissolved in DMF (10 ml) and the solution was cooled to -10 °C; 4M-HCl in EtOAc (1.61 ml) was added followed by t-butyl nitrite (0.30 ml). The mixture was stirred at -10 °C for 10 min and Et_3N (0.90 ml) was added. A solution of (6) (1.45 g) and Et_3N (0.29 ml) in DMF (20 ml) at -10 °C was added and the mixture was stirred at -5 °C for 30 min and then at 4 °C for 65 h. Insoluble material was filtered off and the filtrate was evaporated to dryness. The residue was triturated with ice–water to yield a white solid (2.85 g). Crystallisation from aqueous methanol gave the *dihydrate* (1.36 g, 43%), m.p. 208 °C, $[\alpha]_D^{25} = -21.0 \pm 0.9°$ (*c* 0.5 DMF) (Found: C, 51.8; H, 6.15; Br, 5.0; N, 14.2; S, 2.1. $C_{68}H_{93}BrN_{16}O_{18}S \cdot 2H_2O$ requires C, 52.00; H, 6.22; Br, 5.08; N, 14.26; S, 2.04%).

N-*t-Butoxycarbonylseryltyrosylserylmethionyl-γ-t-butylglutamylhistidylphenylalanylarginyl-5-bromotryptophylglycyl-*N$^{\epsilon}$*-t-butoxycarbonyl-lysylprolylvalylglycyl-*N$^{\epsilon}$*-t-butoxycarbonyl-lysyl-*N$^{\epsilon}$*-butoxycarbonyl-lysylarginylarginylprolylvalyl-*N$^{\epsilon}$*-t-butoxycarbonyl-lysylvalyltyrosylproline t-Butyl Ester Trihydrochloride* (13).—*C*-Terminal tetradecapeptide base dihydrochloride[6] (336 mg) was dissolved with (12) (278 mg) and HOBt (27 mg) in DMF (3.5 ml) and warmed to 50 °C with stirring until a solution was obtained. M-HCl (0.18 ml) was added followed by DCC (126 mg) and the solution was stirred under N_2 for 2 h at 50 °C. After storage at 4 °C, DCU was filtered off and the filtrate was evaporated to dryness below 30 °C. The material was purified by counter-current distribution as previously described.[3] Product (412 mg, peptide content 68%, yield 48%) was recovered which had amino-acid analysis after acidic hydrolysis: Arg, 3.02; Glu, 1.05; Gly, 2.07; His, 1.02; Lys, 4.00; Met, 1.02; Phe, 1.04; Pro, 3.01; Ser, 1.89; Try, 0.25; Tyr, 2.00; Val, 3.00.

[9-(5-*Bromotryptophan*)]-β-*corticotrophin*-(1—24)-*tetracosapeptide* (14).—This compound was prepared from (13) (9.1 mg) as previously described for the analogous di-iodo-histidine-containing tetracosapeptide.[14] The recovered *peptide* (540 nmol, 33%) had amino-acid analysis after acidic hydrolysis: Arg, 2.99; Glu, 1.01; Gly, 2.02; His, 1.00; Lys, 3.99; Met, 0,98; Phe, 1.00; Pro, 2.93; Ser, 1.74; Trp, 0.43; Tyr, 2.01; Val, 2.97; and after enzymic hydrolysis[15]: Arg, 2.89; Glu, 1.08; Gly, 2.04; His, 0.98; Lys, 3.97; Met, 0.97; Phe, 0.98; Pro, 2.82; Ser, 2.02; Trp, 0; Trp(Br), 1.03; Tyr, 1.97; Val, 2.84.

The peptide behaved as a single component on examination by h.p.l.c. on a column (25 × 0.46 cm) of Nucleosil $10C_{18}$ ODS-silica using a constant mixing volume gradient of methanol (20—50%) in water containing phosphoric acid (0.1% by vol.). Its potency in the isolated adrenal cell bioassay[7] was 2.4 times (95% confidence limits, 1.6—3.7) compared to Synacthen.

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