The Synthesis of Tritium-Labelled Human Corticotropin of High Specific Radioactivity

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Human $[[3,5\text{-}^3\text{H}_2]\text{Tyr}^{23}]$ corticotropin-(1–39)-nonatriacontapeptide of specific radioactivity 25.2 Ci/mmol, identical with unlabelled human corticotropin by several criteria, was prepared via the fully protected di-iodotyrosine compound. The latter was synthesized by classical procedures.

The recent report of the synthesis of the 2- and 23-di-iodotyrosine and 2,23-bis(di-iodotyrosine) analogues of corticotropin by the solid-phase method (Lemaire *et al.*, 1977) and the suggestion that one of these would be suitable for the preparation of ^3H -labelled human corticotropin prompted us to describe our experiences during the synthesis of the labelled hormone. The method used by us follows directly that applied in the synthesis of the ^3H -labelled (1–24)-corticotropin analogue described previously (Brundish & Wade, 1972, 1973).

We report the synthesis of the protected [23-di-iodotyrosine]nonatriacontapeptide by classical procedures and the use of this to prepare pure ^3H -labelled human corticotropin in good yield. Improved handling procedures for the labelled hormone are presented, which minimize treatment of, and hence damage to, the compound.

Methods and Materials

Protected corticotropin-(11–39)-nonacosapeptide (Sieber *et al.*, 1972) was kindly provided by Dr. W. Rittel, CIBA-GEIGY A.G., Basle, Switzerland. Enzymes were attached to Sepharose 4B as described by Bennett *et al.* (1972) and were from the following sources: aminopeptidase M from Rohm and Haas (U.K.) Ltd., Croydon, Surrey, U.K.; trypsin and chymotrypsin from Worthington Biochemical Corp., Freehold, NJ, U.S.A.; prolidase was prepared from pig kidney by using the first two steps of the method of Davis & Smith (1957). All solvents were A.R. grade and were redistilled before use. Thin-layer chromatograms on silica-gel plates (BDH Chemicals Ltd., Poole, Dorset, U.K.) were developed in the solvent system butan-1-ol/acetic acid/water (10:1:3, by vol.). Tritium gas ($^3\text{H}_2$) was from The Radiochemical Centre, Amersham, Bucks., U.K.

Experimental and Results

H-Lys(Boc)-Pro-Val-Gly-Lys(Boc)-Lys(Boc)-Arg-Arg-Pro-Val-Lys(Boc)-Val-Tyr(I₂)-Pro-Asn-Gly-Ala-Glu(OBu^t)-Asp(OBu^t)-Glu(OBu^t)-Ser-Ala-Glu(OBu^t)-Ala-Phe-Pro-Leu-Glu(OBu^t)-Phe-OBu^t-1½H₂SO₄ (II)*

(a) *Hydrogenation.* Protected (11–39)-peptide (0.78 g) was dissolved in methanol (150 ml) and hydrogenated for 16 h at 20°C in the presence of 10% palladium/charcoal (0.6 g; Engelhardt, Cinderford, Glos., U.K.). Examination of a portion of solution by t.l.c. showed that deprotection was complete. Catalyst was removed by filtration and the combined filtrate and washings were rotary-evaporated to yield compound (I) [partially protected corticotropin-(11–39)-nonacosapeptide; see Scheme 1] (693 mg, 92%).

(b) *Iodination.* Compound (I) (229 mg) was dissolved in methanol (6.0 ml), and ICl (36 mg) in carbon tetrachloride (0.6 ml) was added in portions (0.1 ml) over 1 min. The mixture was stirred for 15 min at room temperature (20°C) and water (1.5 ml) was added. SO₂ was blown over the surface of the solution with shaking to decompose the excess of ICl, and the solution was passed through a column (1.1 cm × 1.1 cm) of Dowex 1-X8 (acetate form) resin. The column was washed with aq. 75% (v/v) methanol (7.5 ml). Methanol was removed by rotary evaporation, and the aqueous suspension was transferred to a 15 ml centrifuge tube and freeze-dried over conc. H₂SO₄ and solid KOH to yield 236 mg (96%) of the triacetate salt of the iodinated peptide. A sample (2 mg) was deprotected with trifluoroacetic acid and divided into two por-

* Abbreviations: Boc, t-butoxycarbonyl; OBu^t, t-butoxy; Tyr(I₂), 3,5-di-iodo-L-tyrosine; Z, benzyloxy-carbonyl.

tions after the addition of an internal standard. One half was hydrolysed with acid and examined by amino acid analysis (Brundish & Wade, 1973). Found: Ala, 3.02; Arg, 2.01; Asp, 2.02; Glu, 4.01; Gly, 2.05; Leu, 0.98; Lys, 3.99; Phe, 1.98; Pro, 4.00; Ser, 0.88; Tyr, 0.89; Val, 3.02. The other half gave, after enzymic hydrolysis (Bennett *et al.*, 1972), Tyr, 0.01.

The remainder (234 mg) was dissolved in 90% (v/v) methanol (3 ml) and six 1 ml portions of 1 M-(NH₄)₂SO₄ were added with stirring, followed by water (8 ml). The sesquisulphate was collected by centrifugation and washed with water (4 × 8 ml) to remove excess of salt. The damp solid was dried at reduced pressure over conc. H₂SO₄ and KOH pellets to yield 208 mg (89.6%; 84% overall) of product.

Boc-Ser-Tyr-Ser-Met-Glu(OBu^t)-His-Phe-Arg-Trp-Gly-Lys(Boc)-Pro-Val-Gly-Lys(Boc)-Lys(Boc)-Arg-Arg-Pro-Val-Lys(Boc)-Val-Tyr(I₂)-Pro-Asn-Gly-Ala-Glu(OBu^t)-Asp(OBu^t)-Glu(OBu^t)-Ser-Ala-Glu(OBu^t)-Ala-Phe-Pro-Leu-Glu(OBu^t)-Phe-OBu^t, 1½ H₂SO₄ (III)

Compound (II) (208 mg, 47 μmol), protected corticotropin-(1–10)-peptide (89.1 mg, 61 μmol) (Schwyzer & Kappeler, 1961) and hydroxybenzotriazole (9.5 mg, 62 μmol) were dissolved in dimethylformamide (1 ml) at 50°C with stirring under N₂. Then aqueous 0.5 M-H₂SO₄ (14 μl) was added, followed by dicyclohexylcarbodi-imide (41 mg, 200 μmol) in dimethylformamide (0.5 ml), and the mixture was stirred for 2 h at 50°C under N₂. The mixture was evaporated to dryness at 20°C and put in tube 1 of a 123-tube countercurrent machine (10 ml each phase) and subjected to 122 upper-phase transfers in the system methanol/buffer/chloroform/carbon tetrachloride (20:7:12:8, by vol.) (the buffer contained 29 ml of acetic acid, 19 g of ammonium acetate and 960 ml of water) (Sieber *et al.*, 1972). Samples of the phases were examined by t.l.c. and fresh solvent was put in tubes 25–42 and 92–111, which were found to contain impurities. A further 512 transfers were performed in the recycling mode, after which product (95 mg, 34%) was isolated from tubes 76–117. It had the following amino acid analysis after acid hydrolysis: Ala, 3.01; Arg, 3.06; Asp, 2.06; Glu, 5.13; Gly, 3.09; His, 1.06; Leu, 0.99; Lys, 3.89; Met, 1.06; Phe, 3.08; Pro, 4.00; Ser, 2.86; Trp, 0.44; Tyr, 2.00; Val, 2.98.

[3,5-³H₂][Tyr²³]-β-Corticotropin-(1–39)-nonatriacontapeptide (IV)

A solution of compound (III) (11.0 mg) in dimethylformamide (0.6 ml) was catalytically ³H-labelled over 5% palladium/charcoal (9.6 mg) and 5% rhodium/CaCO₃ (9.2 mg) with 98%

³H₂ (4 ml, 10 Ci); the peptide was recovered, deprotected and worked-up as described for the corresponding tetracosapeptide analogue (Brundish & Wade, 1973). Final purification was achieved on a column (3 cm × 0.7 cm) of CM-cellulose (Na⁺ form); the peptide was applied in water (0.4 ml) and eluted with a linear gradient (40 ml total) of NaCl (0–0.125 M) in a buffer of 25 mM-sodium phosphate, pH 7. Fractions were monitored for radioactivity, and those containing the peptide (tubes 9–12) were combined. The product (568 nmol, 30%) had the following amino acid analysis: Ala, 2.97; Arg, 2.98; Asp, 2.07; Glu, 5.00; Gly, 3.07; His, 1.00; Leu, 1.00; Lys, 3.96; Met, 0.99; Phe, 3.00; Pro, 4.02; Ser, 2.70; Trp, 0.75; Tyr, 1.93; Val, 2.97. The specific radioactivity was 25.2 Ci/mmol. A sample (1 ml) of the solution was passed through a column (0.6 cm × 3.5 cm) of Dowex 1-X8 (acetate form) resin contained in a Pasteur pipette and the column was eluted with water. Fractions (0.5 ml) were collected and their A₂₈₀ was measured. Fractions 2 and 3 contained peptide and were combined.

Hydrolysis of a sample of the peptide solution with a mixture of carrier-bound enzymes followed by amino acid analysis showed: Ala, 2.46; Arg, 2.97; Asn+Ser, 3.24; Asp, 0.34; Glu, 3.93; Gly, 3.04; His, 0.99; Leu, 0.92; Lys, 3.90; Met, 1.00; Phe, 2.96; Pro, 3.91; Trp, 0.95; Tyr, 1.89; Val, 3.03; and that the radioactivity was distributed among the residues in the ratio Tyr/His = 98.7:1.3 (Brundish *et al.*, 1976). For storage, samples of the peptide were diluted to 100 μg/ml with 0.9% NaCl and kept at –19.5°C.

Discussion

We prepared human corticotropin labelled with ³H at the tyrosine-23 residue by essentially the same route (see Scheme 1) as used for the corresponding tetracosapeptide (Brundish & Wade, 1973), but starting from the partially protected 11–39 sequence of the human hormone (I). The fact that again the tyrosine-23 residue was the only residue susceptible to iodination by ICl in nonacosapeptide (I) allows this rapid approach. Compound (III) was obtained in a high state of purity after countercurrent distribution. We consider that the classical approach using the condensation of fragments each capable of purification to a high degree is a more certain route to pure peptides in this molecular-weight range than the solid-phase procedure.

The precursor (III) was ³H-labelled by catalytic iodination, deprotected with 90% (v/v) trifluoroacetic acid and purified by ion-exchange chromatography on CM-cellulose (Na⁺ form). Attempts to purify the hormone on the same ion-exchange resin by using the volatile trimethylammonium acetate buffer system (Brundish & Wade, 1973) used for

Scheme 1. Sequence of reactions used in the synthesis of ³H-labelled human corticotropin nonatriacontapeptide
Reaction conditions are described in the text.

the tetracosapeptides is not recommended, as in this case the nonatriacontapeptide is difficult to redissolve in water after evaporation to remove the buffer. Additional dangers are that concentration of solutions of such highly radioactive peptides leads to enhanced radiolysis and that adsorption losses arise as a consequence of the evaporations which have to be carried out in glass apparatus. These drawbacks are avoided by the present procedure. The hormone as obtained in NaCl/sodium phosphate buffer, pH7, is suitable for direct use in animal systems to study distribution and metabolism (Baker *et al.*, 1976; Bennett *et al.*, 1974).

We observed that phosphate ions inhibited the enzymes used for breakdown of the peptide in our system. It was therefore necessary to exchange phosphate ions for Cl⁻ before digestion. The highly acidic region of the peptide, residues 27–30, was partially resistant to enzymic hydrolysis. The labelled hormone was fully active (potency 108%, 95% confidence limits 59–202%) compared with synthetic human corticotropin in the isolated adrenal-cell bioassay (Lowry *et al.*, 1973).

Our route to the labelled hormone, using ³H-labelling of the protected peptides and thus minimizing absorptive losses on the catalyst, allows considerably higher overall yields than can be obtained by similar exchange with free peptides in aqueous media. In this instance, our yield was 30%, whereas ³H-labelled oxytocin could be recovered in only 2.5% yield from di-iodo-oxytocin (Morgat *et al.*, 1970).

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