Synthesis of [3,5-$^{3}H_2$-Tyr^{23}]-β-Corticotrophin-(1—24)-tetracosapeptide

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

The synthesis is described of β-corticotrophin-(1—24)-tetracosapeptide † labelled with tritium in the tyrosine residue at position 23 to a specific radioactivity of 46 Ci mmol^{-1} by reductive deiodination of a protected precursor. Evidence for the integrity of the final product is provided by amino-acid analysis, column and thin-layer chromatography, and bioassay, supported by chemical and enzymic analytical data on the protected precursor and the derived tetracosapeptide containing di-iodotyrosine.

TETRACOSACTRIN labelled with radioisotope to a high specific activity was required for metabolic studies. Owing to the low physiological level of corticotrophin, only ^{3}H, ^{125}I, and ^{131}I are sufficiently radioactive for consideration.

The iodine isotopes are attractive on account of their high specific radioactivity but a fundamental objection can be raised against the use of iodinated peptides for investigations such as binding studies and metabolism. Owing to the large atomic radius of iodine and the lipophilic character of iodinated aromatic amino-acids, iodinated peptides are unlikely to have the same physical properties as the unsubstituted peptides from which they are prepared. At pH values near physiological, the change in lipophilicity of a peptide brought about by iodination causes alteration of physical properties in solution. This has been put to use in column chromatographic procedures for separating iodine-labelled from unlabelled corticotrophin.[1] We report here the synthesis of an analogue of tetracosactrin in which the tyrosine residue at position 23 has been replaced by di-iodotyrosine and have confirmed a change in behaviour on ion-exchange chromatography and altered potency in a bioassay system. It is to be expected that residue modification in peptides

† 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.

[1] R. J. Lefkowitz, J. Roth, W. Pricer, and I. Pastan, *Proc. Nat. Acad. Sci., U.S.A.*, 1970, **65**, 745.

could cause changes of conformation in solution and would alter binding to carrier or receptor proteins.

With these considerations in mind, we chose to investigate the possibility of preparing tetracosactrin labelled with tritium to a level of about 50 Ci mmol^{-1}. Theoretically obtainable activity with one [3,5-$^3H_2$]-tyrosine residue in the peptide would be 58 Ci mmol^{-1}.

Several reports exist of tritium labelling of peptide hormones. Wilzbach exchange-labelling of corticotrophins has afforded randomly labelled materials with specific activities of 10 mCi mmol^{-1} (ref. 2) and about 1 Ci mmol^{-1} (ref. 3). Neither product was investigated for possible damage by racemisation which is known to take place during Wilzbach labelling.[4] In addition, these materials would be unsuitable for metabolic studies if the tritium proved exchangeable under physiological conditions. Carlsson and Sjöholm[5] synthesised tritium-labelled oxytocin and lysine-vasopressin from amino-acid of high specific activity. Such an approach is likely to be wasteful and hazardous in an extended synthesis.

We decided to examine the possibility of specifically labelling tetracosactrin by catalytic tritiation of a protected synthetic iodinated analogue[6] followed by removal of protecting groups. Difficulties envisaged were that the sulphur of methionine might poison the catalyst and that it might be oxidised by the halogen released during work-up.

There are many reports of the preparation of peptide hormones labelled, presumably in the ring of tyrosine, with isotopes of iodine. Although many workers obtained chromatographically homogeneous materials by suitable work-up procedures, we know of no report of the chemical investigation of a radioiodinated corticotrophin sample which would establish whether iodination is accompanied by other changes in the structure of the peptide. While iodination would be expected to be applicable safely to small peptides containing few susceptible residues, such as angiotensin II,[7] more complex molecules such as tetracosactrin might be expected to be subject to other reactions during the procedures commonly employed for iodination. Oxidative conditions can convert methionine into its *S*-oxide. An excess of iodine causes degradation of the imidazole nucleus of histidine[8] and modification of serine and tryptophan.[9]

The protected 23-di-iodotyrosine analogue was readily accessible by modification at a late stage in the synthetic scheme of preparation of protected tetracosactrin.[10] The 11—24 fragment, deprotected only at the α-amino-terminus by removal of the benzyloxycarbonyl function by catalytic reduction, afforded a material particularly suitable for modification in that it contains no amino-acid residues other than tyrosine susceptible to iodination and that no protecting groups remain on the fragment that require catalytic hydrogenation for removal. Iodination was effected by the action of iodine monochloride in acetic acid. The product, isolated as the tritosylate salt, was coupled in the normal manner with the protected 1—10 fragment using dicyclohexylcarbodi-imide and hydroxysuccinimide, and the protected tetracosapeptide was purified by counter-current distribution. Treatment of the product with trifluoroacetic acid followed by ion-exchange chromatography afforded the free iodinated peptide in good yield. It differed from tetracosactrin in its behaviour on ion-exchange chromatography and in its potency in an isolated adrenal cell bioassay ($57 \pm 5\%$ compared to tetracosactrin).[11] Enzymic digestion followed by amino-acid analysis showed that the first 22 amino-acids are released quantitatively and that the di-iodotyrosylproline link was completely resistant.

Tritiation of the protected tetracosapeptide was effected in *NN*-dimethylformamide (DMF) by using a mixture of 5% palladium–charcoal (Engelhard) and 5% rhodium–calcium carbonate (Engelhard) as catalyst. Uptake was complete within 45 min at room temperature. Experiments not reported here with other catalysts confirmed that base was an essential part of the catalyst system. In its absence iodine was liberated into solution during work-up. Organic bases were not satisfactory in use and the combined catalyst–base rhodium–calcium carbonate was adopted. The catalyst mixture used gave the most rapid deiodination of all the combinations tested. Neither catalyst was effective in the absence of the other. Prolonged reduction was avoided on account of possible radiation damage to the peptide and also of the tendency of catalysts to break down mechanically under prolonged agitation in DMF causing difficulties during work-up. For determining the rate of deiodination, we used the ^{125}I-labelled β-corticotrophin-(11—24)-tetradecapeptide derivative mixed with an equimolar amount of methionine. Dehalogenation was assessed by t.l.c. followed by examination of the plate for radioactivity, and the extent of methionine oxidation was assessed by visualisation with ninhydrin reagent on the same chromatogram. It was found necessary to develop the chromatogram in an atmosphere of sulphur dioxide to ensure complete reduction of any ^{125}iodine formed back to iodide ion, as iodine ran concurrently with the tetradecapeptide derivative.

After deprotection of tritiated material with tri-

[2] E. E. Nishizawa, R. B. Billiar, J. Karr, and K. B. Eik-Nes, *Canad. J. Biochem.*, 1965, **43**, 1489.

[3] K. von Werder, K. Schwarz, and P. C. Scriba, *Klin. Woch.*, 1968, **17**, 940.

[4] E. A. Evans, in 'Tritium and Its Compounds,' Butterworths, London, 1966, p. 136.

[5] L. Carlsson and I. Sjöholm, *Acta Chem. Scand.*, 1966, **20**, 259.

[6] K. J. M. Andrews, F. Bultitude, E. A. Evans, M. Gronow, R. W. Lambert, and D. H. Marrian, *J. Chem. Soc.*, 1962, 3440: C. R. Ball and R. Wade, *J. Chem. Soc.* (*C*), 1968, 1338.

[7] J. L. Morgat, L. T. Hung, and P. Fromageot, *Biochim. Biophys. Acta*, 1970, **207**, 374.

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

[9] L. K. Ramachandran, *Chem. Rev.*, 1956, **56**, 199.

[10] R. Schwyzer and H. Kappeler, *Helv. Chim. Acta*, 1963, **46**, 1550.

[11] P. J. Lowry, C. McMartin, and J. M. Peters, *J. Endocrinol.*, 1973, **59**, 43.

fluoroacetic acid, iodine in various oxidation states was removed by reduction with sulphur dioxide and use of ion-exchange resin, which also served to replace trifluoroacetate and tosylate ions by acetate. The preparation then contained finely-divided material probably derived from the catalyst. Treatment with 0·75% thioglycolic acid solution for a minimum of 3 h at 50° under nitrogen, originally intended to reduce any methionine *S*-oxide, converted the impurity into a soluble form which was removed by the use of ion-exchange resin. The impurity remained at the top of the column as a purple band, indicating that it was probably a noble metal compound. Final purification of the peptide was effected by ion-exchange chromatography on carboxymethylcellulose, affording material which was pure as judged by t.l.c. in several solvent systems, amino-acid analysis, and enzymic digestion.[12] Bioassay in an isolated adrenal cell system[11] showed the product to be equipotent (99 ± 27%) with tetracosactrin. When stored at −20° in solution containing suitably selected peptidase-free albumin the product had a half-life of 2—3 months.[13] The specific activity of the peptide over a series of several preparations was commonly about 45 Ci $mmol^{-1}$, indicating only partial exchange of tritium with the potentially exchangeable protons of the protected peptide during catalytic deiodination. The specific activity of our preparation makes it acceptable for use as a tracer in metabolic studies at physiological concentration.

After the completion of our work, a report appeared of the preparation of angiotensin II labelled with tritium to a specific activity of 56·3 Ci $mmol^{-1}$.[7] The method used (iodination of free peptide followed by catalytic tritiation) would not be applicable to preparing labelled tetracosactrin because of the damage that would be caused by the iodination procedure. Over 90% of the material remained adsorbed on the catalyst. The same authors[14] have reported the preparation of tritium-labelled oxytocin in 2·5% yield. Our method, employing protected peptides and thus minimising absorptive losses on the catalyst, allows considerably higher overall yields. A more recent publication from the same laboratory[15] reports the application of the iodination–reduction method to the α-neurotoxin of *Naja nigricollis*. This peptide contains 61 amino-acid residues and is iodinated at a histidine rather than a tyrosine residue. The tritiated product contained 10% of labelled impurities and had a biological potency of 60—70% of that of native toxin; this demonstrates the difficulties which arise when the technique is applied to complex polypeptides.

A report has appeared of the synthesis of [2-phenylalanine, 4-$^3H_2$-norvaline]-β-corticotrophin-(1—24)-tetracosapeptide by the catalytic reduction of the 4-allylglycine analogue in the absence of the sulphur of the usual 4-methionine peptide.[16] This afforded material with two unnatural residues in the tetracosapeptide.

EXPERIMENTAL

Thin-layer chromatograms were run on layers (0·25 mm) of silica gel G (Merck) in the following solvent systems: (A), butan-1-ol–acetic acid–water (10 : 1 : 3 v/v); (B), 2-methylpropan-1-ol–acetic acid–water (67 : 10 : 23 v/v); (C), butan-1-ol–pyridine–acetic acid–water (30 : 20 : 6 : 24 v/v). Compounds were detected by ninhydrin, by chlorine and starch–iodide, and by use of a Panax Thin-Layer Radiochromatogram Scanner System E.0111/XPD-05. Radioactive compounds were assayed with a Packard model 3003 Liquid Scintillation Spectrometer in 10 ml of scintillator [containing 2,5-bis-(5-t-butylbenzoxazol-2-yl)-thiophen (30 g) and naphthalene (340 g) in a mixture of toluene (2·5 l) and methylCellosolve (1·7 l)], with [αβ-$^3H_2$]-hexadecane as internal standard. Samples for amino-acid analysis were hydrolysed in 6N-hydrochloric acid for 16 h at 115°. Phenol (2—4 mg) was added to samples containing di-iodotyrosine prior to acidic hydrolysis. Under these conditions di-iodotyrosine was converted quantitatively into tyrosine.

N^{ϵ}-*Butoxycarbonyl-*L*-lysyl-*L*-prolyl-*L*-valylglycyl-*N^{ϵ}*-butoxycarbonyl-*L*-lysyl-*N^{ϵ}*-butoxycarbonyl-*L*-lysyl-*L*-arginyl-*L*-arginyl-*L*-prolyl-*L*-valyl-*N^{ϵ}*-butoxycarbonyl-*L*-lysyl-*L*-valyl-*L*-3,5-di-iodotyrosyl-*L*-proline t-Butyl Ester Tritosylate Salt.*—Protected tetradecapeptide dihydrochloride[10] (1 g) was hydrogenated overnight at room temperature in 90% acetic acid (10 ml) over 5% palladium–charcoal (100 mg). The catalyst was removed by filtration and washed. The combined filtrate and washings were evaporated to dryness. The residue was dried *in vacuo* (KOH and conc. H_2SO_4) and dissolved in glacial acetic acid (10 ml), and a solution of iodine monochloride (280 mg, 4 equiv.) in glacial acetic acid (8 ml) was added. The mixture was kept at room temperature for 20 min, water (18 ml) was added, and sulphur dioxide was passed through the solution until the excess of reagent was destroyed. The resultant solution was passed through a column (10 × 1 cm) of Dowex 1 (acetate form) resin and material was eluted with 50% aqueous acetic acid. The eluate was evaporated to dryness at 20° and several portions of chloroform–methanol (1 : 1; 10 ml) were evaporated from the residue until all traces of water had been removed. Trituration of the residue with ether gave the triacetate salt (1 g, 91%) which was substantially pure as judged by t.l.c. in system (A). The solid was dissolved in methanol (10 ml) and toluene-*p*-sulphonic acid hydrate (225·5 mg, 3 equiv.) dissolved in freshly-distilled pyridine (3 ml) was added. The mixture was evaporated below 20° several times with addition of portions (10 ml) of methanol until solidification occurred. The solid was triturated with ether and the recovered solid dried at 50° and 1·2 mmHg over conc. H_2SO_4 for 6 h to give the *tritosylate salt* (1·09 g, 94%), which was substantially pure as judged by t.l.c. in system (A) (Found: C, 50·95; H, 6·9; I, 8·65; N, 11·55. $C_{122}H_{196}I_2N_{24}O_{33}S_3$ requires C, 50·9; H, 6·8; I, 8·85; N, 11·7%).

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

[13] C. McMartin, personal communication.

[14] J. L. Morgat, L. T. Hung, R. Cardinaud, P. Fromageot, J. Backaert, M. Imbert, and F. Morel, *J. Labelled Compounds*, 1970, **6**, 276.

[15] A. Menez, J. L. Morgat, P. Fromageot, A. M. Ronseray, P. Boquet, and J. P. Changeux, *FEBS Letters*, 1971, **16**, 332.

[16] R. Schwyzer, P. Schiller, J. L. Fanchère, G. Karlaganis, and G. M. Pelican in ' Protein and Polypeptide Hormones,' Proceedings of the 2nd International Symposium Liège, vol. 1, 1971, p. 167.

N-*Butoxycarbonyl*-L-*seryl*-L-*tyrosyl*-L-*seryl*-L-*methionyl*-γ-*t*-*butyl*-L-*glutamyl*-L-*histidyl*-L-*phenylalanyl*-L-*arginyl*-L-*tryptophylglycyl*-N^ε^-*butoxycarbonyl*-L-*lysyl*-L-*prolyl*-L-*valylglycyl*-N^ε^-*butoxycarbonyl*-L-*lysyl*-N^ε^-*butoxycarbonyl*-L-*lysyl*-L-*arginyl*-L-*arginyl*-L-*prolyl*-L-*valyl*-N^ε^-*butoxycarbonyl*-L-*lysyl*-L-*valyl*-L-3,5-*di-iodotyrosyl*-L-*proline t-Butyl Ester Tritosylate Salt*.—The tetradecapeptide tritosylate salt (1·04 g), the protected *N*-terminal decapeptide acid [17] (610 mg), *N*-hydroxysuccinimide (80 mg), and dicyclohexylcarbodi-imide (108 mg) were stirred in freshly-distilled DMF (9 ml) for 45 h at 50° under nitrogen. The mixture was added dropwise to vigorously stirred ethyl acetate (75 ml) and the precipitated solids (1·6 g) were filtered off, washed with ethyl acetate and dried *in vacuo*. The solid was dissolved in the lower phase (100 ml) of methanol–ammonium acetate buffer (pH 4·5)–chloroform–carbon tetrachloride (8 : 4 : 5 : 2 v/v), loaded into tubes 17—26 of a 125-tube counter-current machine (10 ml each phase) and subjected to 275 upper-phase transfers. Samples of the lower phase of the fractions were examined by t.l.c. in system (B). Fractions 60—75 were combined and the lower phase was separated and evaporated to dryness below 20°. Several portions (10 ml) of chloroform–methanol (1 : 1) were evaporated from the residue, which was then solidified by trituration with petroleum (b.p. 60—80°). The *tetracosapeptide* derivative (463 mg, 30%) was chromatographically pure by t.l.c. in system (B); $\lambda_{max.}$ [0·5N-NaOH–MeOH (1 : 1 v/v)] 283 (ε 7400) and 290 nm (7610); amino-acid analysis: Arg, 3·00; Glu, 1·04; Gly, 2·00; His, 1·00; Lys, 4·02; Met, 0·98; Phe, 1·00; Pro, 2·94; Ser, 1·78; Tyr, 1·96; Val, 2·91 (Found: C, 52·8; H, 6·8; I, 6·05; N, 12·9; S, 2·8. $C_{190}H_{288}I_2N_{40}O_{50}S_4$ requires C, 52·9; H, 6·7; I, 5·9; N, 13·0; S, 2·9%).

[23-3,5-*Di-iodotyrosine*]-β-*corticotrophin*-(1—24)-*tetracosapeptide*.—A sample (12 mg) of the protected tetracosapeptide was treated with 90% trifluoroacetic acid (1 ml) for 30 min at room temperature. The solution was evaporated to dryness below 20°, the residue was dissolved in water (1 ml), and the solution was passed through a column (1 ml) of Dowex 1 (acetate form) resin which was eluted with water (8 ml). The combined eluate and washings were evaporated to dryness and the residue dissolved in 0·05M-Tris buffer (0·25 ml) containing Ca^{2+} and Mn^{2+} (5×10^{-3}M each). Trypsin (250 μg) in buffer (0·25 ml) was added and the mixture was kept at 37° for 5 h. Leucine aminopeptidase (500 μg) in buffer (0·2 ml), aminopeptidase M (1 mg) in buffer (0·5 ml), and butan-1-ol (0·1 ml) were added and the mixture was kept at 37° for 36 h; conc. HCl (0·1 ml) was then added. Amino-acid analysis showed Arg, 3·01; Glu, 1·07; Gly, 2·00; His, 0·94; Lys, 3·89; Met, 0·89; Met (O), 0·15; Phe, 1·01; Pro, 1·97; Ser, 2·08; Trp, 0·94; Tyr, 1·02; Val, 2·99.

A further sample (10·7 mg) of protected tetracosapeptide was deprotected and worked up as before. The hexa-acetate residue was dissolved in water (0·5 ml) and applied to a column (7·5 × 0·7 cm) of carboxymethylcellulose (trimethylammonium form) which was eluted with 0·42M-trimethylammonium acetate (pH 5·0). The eluted fractions were monitored by u.v. spectroscopy at 260 nm and the fractions containing the peptide were combined and evaporated to dryness several times with addition of portions (2 ml) of water until all buffer had been removed. The peptide (1·73 μmol) was stored in water (8 ml) at −20°.

Model System for Investigation of the Deiodination Reaction.—The partially protected tetradecapeptide derivative was also obtained (as the triacetate salt) labelled with ^{125}I as follows. The reduced tetradecapeptide derivative (228 mg) was dissolved in methanol (5 ml) and iodine monochloride (32·4 mg; 10 mCi ^{125}I) in carbon tetrachloride (5 ml) was added. The mixture was kept at room temperature for 30 min, water (5 ml) was added, and sulphur dioxide was passed through the solution to destroy the excess of reagent. The mixture was evaporated to dryness; the residue was dissolved in 50% aqueous acetic acid (5 ml) and passed through a column (5 × 0·5 cm) of Dowex 1 (acetate form) resin which was washed with 50% aqueous acetic acid (10 ml). The eluate and washings were combined and evaporated to dryness and the residue dried briefly (KOH) *in vacuo*. The residue was dissolved in methanol (15 ml) and stored at −20°. A sample (1 μl) was examined after t.l.c. in system (A) with the radiochromatogram scanner. Eighty-two per cent of the radioactivity in the sample was associated with the tetradecapeptide derivative and the remainder was incorporated into slower-running degradation products which had probably been partially deblocked at the side-chain functions of lysine residues.

A sample (1 ml) of solution was evaporated to dryness and the residue (15 mg) was dissolved in DMF (1 ml). L-Methionine (0·86 mg), 5% palladium–charcoal (20 mg), and 5% rhodium–calcium carbonate (20 mg) were added and the mixture was stirred under hydrogen at room temperature. Samples (1 μl) were withdrawn at intervals and applied to the base-line of a thin-layer plate. The plate was developed with solvent (A) in an atmosphere containing sulphur dioxide and examined with the radiochromatogram scanner. Reduction was estimated in a sample by relating the number of counts associated with the tetradecapeptide spot to the initial count at time zero, and was 90% complete in 20 min and complete within 45 min. The plate was then developed with ninhydrin reagent. Some methionine had been converted into the *S*-oxide but the tetradecapeptide base had not been further degraded.

[3,5-$^3H_2$-Tyr[23]]-β-*Corticotrophin*-(1—24)-*tetracosapeptide*.—Protected tetracosapeptide derivative (10·4 mg) was dissolved in DMF (0·4 ml) and catalytically tritiated over 5% palladium–charcoal (10 mg) and 5% rhodium–calcium carbonate (10 mg) with 98% tritium gas (4 ml; 10 Ci). After 45 min, the mixture was added to ethanol (5 ml) and the catalyst was filtered off. The filtrate and washings from the catalyst were combined and evaporated to dryness. The residue was dissolved in 90% trifluoroacetic acid (2 ml) and kept at room temperature for 30 min. The mixture was evaporated to dryness and dissolved in water (1 ml) through which sulphur dioxide had been passed. The solution was passed through a column (1 ml) of Dowex 1 (acetate form) resin which was eluted with water (5 ml). Freshly-distilled thioglycolic acid (45 μl) was added to the solution, which was vigorously stirred at 50° for 3 h under nitrogen. The solution was passed through a column (1 ml) of Dowex 1 (acetate form) resin which was eluted with water (8 ml). The combined eluate and washings were evaporated to dryness and the residue was dissolved in water (0·4 ml). The solution was applied to a column (7·5 × 0·7 cm) of carboxymethylcellulose (trimethylammonium form) and eluted with 0·42M-trimethylammonium acetate. Fractions were monitored for radioactivity and those containing the peptide were combined and evaporated.

[17] R. Schwyzer and H. Kappeler, *Helv. Chim. Acta*, 1963, **44**, 1991.

Portions (1 ml) of water were evaporated from the residue until all the buffer had been removed. The residue (480 nmol, 20%) was dissolved in water (35 ml) and stored in a polythene vessel at 4°. Amino-acid analysis showed Arg, 3·00; Glu, 0·99; Gly, 2·03; His, 0·97; Lys, 3·95; Met, 0·93; Phe, 1·00; Pro, 2·99; Ser, 1·70; Tyr, 1·94; Val, 3·03; specific activity 46·4 Ci mmol^{-1}. Hydrolysis of a sample of the peptide with a mixture of carrier-bound enzymes [12] followed by amino-acid analysis showed Arg, 3·00; Glu, 1·01; Gly, 1·88; His, 1·04; Lys, 3·93; Met, 0·98; Phe, 1·03; Pro, 2·92; Ser, 1·96; Trp, 1·00; Tyr, 2·01; Val, 2·99.

The material was chromatographically pure as judged by t.l.c. in system (C) and examination of the plate with the Panax scanning system.

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