Tritiated Peptides. Part 9.[1] Synthesis of [3,5-$^3H_2$-Tyr^{12}]- and [2,5-$^3H_2$-His^{20}]-Human Calcitonin †

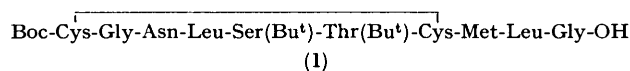
By **Derek E. Brundish** and **Roy Wade**,* Ciba-Geigy Pharmaceuticals Division, Horsham, West Sussex RH12 4AB

The syntheses are described of human calcitonin dotriacontapeptides separately labelled with tritium in the tyrosine and histidine residues at positions 12 and 20 to specific radioactivities of 50.5 and 8.2 Ci mmol^{-1}, respectively, by reductive de-iodination of suitable 11—32 fragments followed by rapid coupling to the 1—10 fragment and column-chromatographic purification of the reaction mixtures, using gel-filtration, and ion-exchange and high-pressure liquid chromatographic techniques. It was demonstrated that the peptide containing a disulphide bridge poisons the catalysts used for tritiation.

WE have reported the syntheses of various corticotrophin analogues labelled with tritium in aromatic amino-acid residues (ref. 1 and preceding papers in the series) and we wished to see whether the labelling method would be applicable to synthetic peptides which contain disulphide bridges.

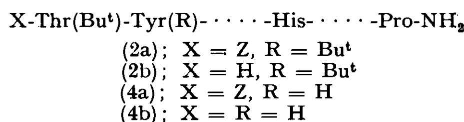
Fromageot has observed that iodinated free peptides containing disulphide bridges could be cleanly dehalogenated in aqueous buffers without adverse effects,[2] and this encouraged us to hope that de-iodination of protected peptides might be similarly possible in aprotic solvents. groups are present that require hydrogenation for removal) was a mixture of three peptides containing histidine, monoiodohistidine, and di-iodohistidine [compound (3), $x = 0$, 1, and 2]. As expected, *O*-butyltyrosine had not been affected.[5] Compound (3) contained 0.73 atoms of iodine per molecule, and yielded on reduction pure compound (2b), confirming that ring-cleavage of histidine had not occurred.

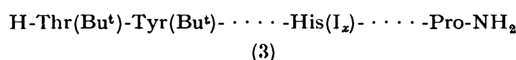
We also had available compound (4a), with a free side-chain hydroxy-group on the tyrosine residue at position 12. Iodination of this peptide after removal of the

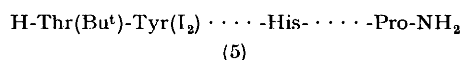


X-Thr(Bu^t)-Tyr(R)-Thr(Bu^t)-Gln-Asp(OBu^t)-Phe-Asn-Lys(Boc)-Phe-His-Thr(Bu^t)-Phe-Pro-Gln-Thr(Bu^t)-Ala-Ile-Gly-Val-Gly-Ala-Pro-NH_2

represented by







We carried out experiments using Fromageot's approach to see whether tritiated calcitonin could be prepared by this relatively simple method. Synthetic calcitonin was iodinated with methanolic iodine chloride in phosphate buffer. However, we were unable to de-iodinate the product using a variety of catalysts, including those reported by Fromageot.

Rapid assembly of two iodinated precursors for labelled calcitonin was possible from (1) and (2).[3] The docosapeptide (2b) contains tyrosine and histidine as the only residues susceptible to oxidative iodination, and is sulphur-free. We decided to iodinate (2b) to the level of only one iodine atom per molecule, as at higher levels cleavage of the imidazole nucleus can be expected.[4] The product of iodination (in which no protecting amino-terminal benzyloxycarbonyl group (4b) gave material in which monoiodotyrosine, di-iodotyrosine, and monoiodohistidine were present. The peptide containing di-iodotyrosine and histidine, compound (5), was isolated by counter-current distribution in 28% yield.

From compounds (3) and (5) we prepared the protected calcitonin precursors (6) and (7). These compounds could not be reduced in DMF using the usual palladium–rhodium catalyst mixture [1] due to catalyst poisoning.

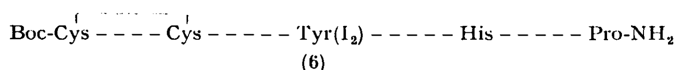
We next prepared the amino-terminal decapeptide with the thiol functions substituted by trityl groups (8).[6] Hydrogenation of mixtures of (3) or (5) with (8) resulted in dehalogenation to regenerate (2b) and (4b), confirming that the bulky blocking group stopped sulphur poisoning of the catalyst. We then prepared the precursors (9) and (10) by condensation of (3) or (5) with (8). The

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

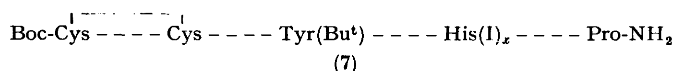
products were extremely insoluble and were isolated by gel-filtration chromatography using 5% aqueous hexamethylphosphoramide (HMPA) on G50-Sephadex.[7] They gave satisfactory amino-acid analyses but could not be reduced with the palladium-rhodium catalyst mixture, even after gel-filtration to remove traces of HMPA. Failure was possibly due to either steric factors or to partial detritylation.

Further purification was not possible as the compounds were not soluble in solvents suitable for counter-current distribution, the only purification technique of sufficient power to tackle this sort of problem, and this illustrates a disadvantage of the ' maximal protection ' approach. The ' minimal protection ' synthesis of large peptides is accompanied by side-reactions but the tonins recovered after ion-exchange chromatography were still impure and h.p.l.c. was necessary to obtain pure preparations. The ion-exchange step could not be omitted as it removed impurities which were difficult to resolve from calcitonin by h.p.l.c.

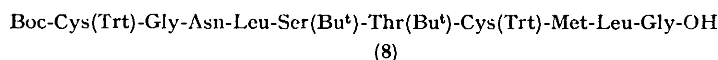
When the reaction sequences were repeated with radioactive (11—32)-amides, no additional problems were encountered. The radiochemical efficiency of replacement of iodine by tritium was 86% for iodinated histidine and 93% for iodinated tyrosine. These values are higher than those observed in the Synacthen series, probably because the calcitonin precursors are ' maximally protected '. The amide protons in the peptide backbone appear not to exchange under the experimental conditions.



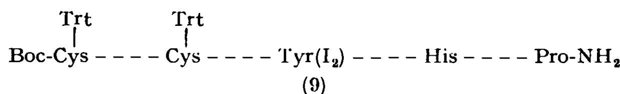
(6)



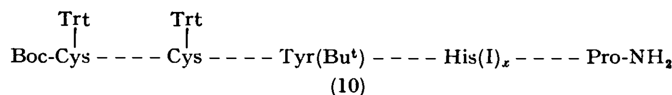
(7)



(8)



(9)



(10)

products are frequently sufficiently soluble for attempted purification by counter-current distribution. We have not detailed the experimental work concerned with these unsuccessful approaches.

We then decided to investigate the possibility of catalytically tritiating the docosapeptides (3) and (5), followed by rapid coupling of the products (2b) and (4b) to the decapeptide (1). A ten-fold excess of the hydroxybenzotriazole ester of (1) reacted completely within 5 min with the reduced (11—32) amide fragments. We included four molar proportions of triethylamine in the reaction mixtures as a precaution against the inhibition of coupling by anions, and also to prevent generation of iodine from iodide ion. The protected calcitonin dotriacontapeptides were separated from excess of decapeptide by gel-filtration chromatography.[7]

In early experiments, examination of the products obtained after ion-exchange chromatography by h.p.l.c. showed that the products were contaminated with significant amounts (10—20%) of a more lipophilic component. This was isolated by preparative high-pressure liquid chromatography (h.p.l.c.) and analysed following modification of cysteine [8] and enzymic digestion.[9] The peptide contained no serine but had an apparently elevated level of alanine. Alanine and *O*-t-butylserine elute together in the system we were using. When we treated the crude products with trifluoroacetic acid for 1 h, serine was completely deprotected. The calci-

The labelled peptides were enzymically hydrolysed [9] after reduction with 2-mercaptoethanol and reaction with ethyleneimine,[8] and the mixtures were separated using the amino-acid analyser. The histidine-labelled peptide was labelled in the histidine residue exclusively, while the tyrosine-labelled calcitonin had 4.8% of the label in the histidine residue.[10] A small amount (1.5%) of this is due to iodohistidine in the starting material (5), while the remainder arises from non-specific exchange.[1]

EXPERIMENTAL

The general remarks given in Part I [11] apply. Thin-layer chromatograms were developed in the following solvent systems: (A), upper phase of ethyl acetate–pyridine–water (2 : 1 : 2 v/v); (B), n-butanol–glacial acetic acid–water (67 : 10 : 23 v/v). The behaviour of di-iodohistidine on acidic hydrolysis is detailed in Part 7.[12] Analytical values for proline in the presence of cystine may be high due to overlap of these two amino-acids. Such values are marked with an asterisk (*) in the text.

O-*t-Butyl-threonyl-*O-*t-butyl-tyrosyl-*O-*t-butyl-threonyl-glutaminyl-β-t-butyl-aspartyl-phenylalanyl-asparaginyl-*N$^\epsilon$-*t-butoxycarbonyl-lysyl-phenylalanyl-iodohistidyl-*O-*t-butyl-threonyl-phenylalanyl-prolyl-glutaminyl-*O-*t-butyl-threonyl-alanyl-isoleucyl-glycyl-valyl-glycyl-alanyl-proline Amide* (3).—Compound (2a) (1 g) was dissolved in glacial acetic acid (50 ml), diluted with methanol (200 ml), and hydrogenated for 16 h in the presence of 10% palladium–charcoal (0.4 g). Removal of the *N*-terminal benzyloxycarbonyl group was then complete (t.l.c. in system A). The catalyst was

removed by filtration through a pad of Celite and the filtrate was evaporated to dryness below 35 °C. The residue of (2b) was dissolved in methanol (20 ml) and a solution of ICl (54 mg) in methanol (2.5 ml) was added with stirring in five equal portions at intervals of 1 min. The mixture was stirred for a further 5 min, water (5 ml) was added, and SO_2 was blown on the surface of the solution to destroy unreacted ICl. The solution was added dropwise to vigorously-stirred saturated $NaHCO_3$ solution (250 ml) and stored at 4 °C for 1 h. Compound (3) was collected by filtration, washed with water and ether, and dried over concentrated H_2SO_4 *in vacuo* (yield 853 mg, 87%).

A portion (2 mg) of the product was treated with 90% aqueous trifluoroacetic acid for 30 min, and the recovered peptide was converted to the acetate salt using Dowex 1 resin. Digestion with a mixture of carrier-bound enzymes [9] followed by amino-acid analysis gave: Ala, 2.04; Asn + Gln + Thr, 6.49; Asp, 0.98; Gly, 2.05; His, 0.38; Ile, 1.06; Lys, 0.99; Phe, 3.00; Pro, 1.97; Tyr, 0.97; Val, 1.01; His(I), 0.39; His(I_2), 0.17; Tyr(I), 0; Tyr(I_2), 0.

O-*t-Butyl-threonyl*-3,5-*di-iodotyrosyl*-O-*t-butyl-threonyl-glutaminyl-*β*-t-butyl-aspartyl-phenylalanyl-asparaginyl*-N$^{\epsilon}$-*t-butoxycarbonyl-lysyl-phenylalanyl-histidyl*-O-*t-butyl-threonyl-phenylalanyl-prolyl-glutaminyl*-O-*t-butyl-threonyl-alanyl-isoleucyl-glycyl-valyl-glycyl-alanyl-proline Amide* (5).—Compound (4a) (1 g) was hydrogenated as described for compound (2a), and compound (4b) was recovered and iodinated as described above for compound (2b), using 110 mg of ICl added over a period of 30 min. Crude compound (5) was recovered and dried as described for compound (3). Deprotection of a sample (2 mg) of the product, and analysis after enzymic hydrolysis, gave: Ala, 1.89; Asn + Gln + Thr, 4.96; Asp, 0.70; Gly, 1.90; His, 0.84; Ile, 1.02; Lys, 0.88; Phe, 2.75; Pro, 2.01; Tyr, 0.12; Val, 1.03; His(I), 0.07; Tyr(I), 0.02; Tyr(I_2), 0.49.

The crude product (0.79 g) was dissolved in the upper phase (30 ml) of the solvent system toluene–chloroform–methanol–water (5 : 5 : 8 : 2 v/v), put in tubes 1—3 of a 123-tube counter-current machine and 120 transfers of upper phase were performed. Examination of the contents of the tubes by t.l.c. (systems A and B) showed that the major product was present in tubes 90—117. All other tubes were re-filled with fresh solvents and a further 500 transfers were performed in the recycling mode. Pure *product* (295 mg, 28%) was recovered from tubes 112—120 by collection of the upper phases, partial evaporation, and azeotropic distillation with benzene–ethanol. Deprotection of a sample (7.2 mg) of the product and analysis after enzymic digestion gave: Ala, 2.05; Asn + Gln + Thr, 6.38; Asp, 0.94; Gly, 2.04; His, 0.97; Ile, 1.05; Lys, 0.98; Phe, 3.00; Pro, 2.00; Tyr, 0.03; Val, 1.02; His(I), 0.03; Tyr(I), 0; Tyr(I_2), 0.93.

[3,5-$^3H_2$-Tyr^{12}]-*Calcitonin*.—Compound (5) (11.4 mg) was dissolved in DMF (0.5 ml) and catalytically tritiated in the presence of 10% palladium–charcoal (11.1 mg) and 5% rhodium–calcium carbonate (9.7 mg) using 98% tritium gas (3.2 ml, 8 Ci). After 45 min, the catalysts were removed by filtration through a cellulose powder pad and the filtrate and washings were evaporated to dryness. The residue was dissolved in a solution of compound (1) (60 mg), HOBt (13 mg) and DCC (18 mg) in DMF (0.75 ml), which had been stirred under N_2 for 2 h at 50 °C; the solution and one DMF washing (0.25 ml) of the vessel were then transferred to a tapered centrifuge tube and kept under N_2 at 50 °C for 10 min and then at −20 °C for 40 min. The crystalline DCU was removed by centrifugation and the supernatant solution was applied to a column (50 × 2 cm) of G50 Sephadex swollen in 5% aqueous DMF.[7] This was eluted by upward flow at a rate of 5 ml h^{-1} and fractions (2 ml) were collected automatically. Those fractions containing radioactivity (24—31) were combined and evaporated to dryness. The residue was treated with 90% trifluoroacetic acid (3 ml) for 60 min. The mixture was evaporated to dryness, dissolved in water (1 ml), and passed through a column (1 ml) of Dowex 1 (acetate form) resin, which was eluted with water (3 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 (12 × 0.7 cm) of carboxymethylcellulose (trimethylammonium form) which was eluted with water (5 ml) and then with a linear gradient (0—02M) of trimethylammonium acetate, pH 5.0. Fractions (1 ml) were collected and assayed for radioactivity. Fractions 25—29 were combined and evaporated, and portions (3 ml) of water containing a little ethanol (to minimise frothing) were evaporated from the residue until all the buffer had been removed. The *product* (1.38 μmol, 37%) was dissolved in water (0.53 ml) and the bulk (0.50 ml) of the solution was applied to a column (50 × 0.7 cm) of Nucleosil $10C_{18}$ using a Rheodyne six-port injection valve. The column was eluted at 7 ml min^{-1} with a constant volume (100 ml) gradient starting with methanol–water–phosphoric acid (400 : 600 : 1 v/v) and ending with a composition of 800 : 200 : 1 v/v. The eluate was monitored at 210 nm and fractions (0.5 min) were collected automatically. Fractions 34—39 were combined and evaporated to a volume of approx. 3 ml. The solution was passed through a column (1 ml) of Dowex 1 (acetate form) resin and the eluate was evaporated to dryness. The residue was dissolved in water (1.0 ml) and stored in liquid N_2. On amino-acid analysis the material gave: Ala, 2.01; Asp, 3.03; Cys, 0.70; Glu, 2.01; Gly, 4.05; His, 1.01; Ile, 1.00; Leu, 2.07; Lys, 0.99; Met, 0.95; Phe, 3.00; Pro, 2.53*; Ser + Thr, 5.63; Tyr, 1.00; Val, 1.01, and had a specific radioactivity of 50.5 Ci mmol^{-1}. After reduction and aminoethylation,[8] enzymic hydrolysis gave: Ala, 2.07; Asp, 0.92; Cys(AE), 1.67; Glu, 1.89; Gly, 3.80; His, 1.01; Ile, 0.97; Leu, 2.04; Lys, 0.99; Met, 0.96; Phe, 2.93; Pro, 1.95; Ser, 0.99; Thr, 4.93; Tyr, 1.00; Val, 1.09. The radioactivity was distributed amongst the residues [10] in the ratio Tyr : His = 95.2 : 4.8.

[2,5-$^3H_2$-His^{20}]-*Calcitonin*.—This was prepared in 38% yield from compound (3) as described for the tyrosine-labelled analogue. The *product* had amino-acid analysis after acidic hydrolysis: Ala, 2.02; Asp, 3.03; Cys, 0.91; Glu, 2.02; Gly, 3.99; His, 1.02; Ile, 1.00; Leu, 2.05; Lys, 1.01; Met, 0.93; Phe, 3.00; Pro, 1.90; Ser + Thr, 5.47; Tyr, 0.99; Val, 1.01, and had a specific radioactivity of 18.2 Ci mmol^{-1}. A sample was treated as described above and then hydrolysed enzymically. The analysis gave Ala, 2.01; Asp, 1.04; Cys(AE), 1.71; Glu, 2.01; Gly, 3.84; His, 0.99; Ile, 1.00; Leu, 2.02; Lys, 1.01; Met, 0.94; Phe, 2.92; Pro, 1.92; Ser, 1.01; Thr, 4.86; Tyr, 1.02; Val, 1.03. Radioactivity in the peptide was exclusively at the histidine residue.[10] Examination of a portion of the acid hydrolysate showed that all (105 ± 12%) of the radioactivity was stable to 6.7N-HCl at 110 °C for 16 h.[12]

N-*t-Butoxycarbonyl*-S-*trityl-cysteinyl-glycyl-asparaginyl-leucyl*-O-*t-butyl-seryl*-O-*t-butyl-threonyl*-S-*trityl-cysteinyl-methionyl-leucyl-glycine* (8).—Compound (1) (2.5 g) was dissolved in DMF (75 ml) and 2-mercaptoethanol (10 ml) was

added. The mixture was kept under N_2 for 90 min at 70 °C with stirring. The solution was evaporated to dryness and two portions (50 ml each) of DMF were evaporated from the residue. The resulting solid was triturated under N_2 with ether and light petroleum (b.p. 60—80 °C), collected by filtration in a stream of N_2, and dried *in vacuo* over concentrated H_2SO_4. The solid (2.4 g) was suspended in DMF (50 ml), trityl chloride (10.2 g) was added and the mixture was stirred under N_2 for 3 days. Methanol (100 ml) was added and the mixture was kept at 4 °C for 1 h. The precipitated solid was filtered off, re-dissolved in hot DMF (50 ml) containing acetic acid (0.1 ml), and methanol (200 ml) was added. After storage at 4 °C for 2 h, the precipitate was collected by filtration, re-suspended twice in water and filtered, and finally dried *in vacuo* over concentrated H_2SO_4. The product (2.16 g, 66%) had m.p. 230 °C (decomp.), $[\alpha]_D^{18}$ $-7.9 \pm 0.4°$ (*c* 0.45, DMF) {lit.,[6] m.p. 230 °C (decomp.), $[\alpha]_D^{20}$ $-8°$}, and was pure as judged by t.l.c. in chloroform–methanol (7 : 3 v/v).

We thank Dr. W. Rittel, CIBA-GEIGY AG., Basle for the supply of compound (1), Mr. K. Eisler and Dr. W. Rittel for compound (4a), Mr. J. R. Martin and Mr. P. Moritz of our laboratory for compound (2a), Mr. B. E. Evans for carrying out enzymic digests and amino-acid analyses, and Mrs. S. M. Garman for the assessment of the distribution of radioactivity amongst the amino-acid residues.

[0/461 *Received, 26th March,* 1980]

REFERENCES

[1] Part 8, D. E. Brundish and R. Wade, *J.C.S. Perkin I*, 1980, 462.

[2] (*a*) J. L. Morgat, L. T. Hung, R. Cardinaud, P. Fromageot, J. Bockaert, M. Imbert, and F. Morel, *J. Labelled Compounds*, 1970, **6**, 276; (*b*) A. Menez, J. L. Morgat, P. Fromageot, A. M. Ronseray, P. Boquet, and J. P. Changeux, *FEBS Letters*, 1971, **17**, 333.

[3] P. Sieber, B. Riniker, M. Brugger, B. Kamber, and W. Rittel, *Helv. Chim. Acta*, 1970, **53**, 2135.

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

[5] W. E. Mayberry and D. A. Bertoli, *J. Org. Chem.*, 1965, **30**, 2029.

[6] B. Kamber, H. Bruckner, B. Riniker, P. Sieber, and W Rittel, *Helv. Chim. Acta*, 1970, **53**, 556.

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

[8] R. D. Cole, 'Methods in Enzymology,' Academic Press, New York, 1967, p. 315.

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

[10] D. E. Brundish, J. R. Martin, and R. Wade, *J.C.S. Perkin I*, 1976, 2182.

[11] D. E. Brundish and R. Wade, *J.C.S. Perkin I*, 1973, 2875.

[12] M. C. Allen, D. E. Brundish, and R. Wade, *J.C.S. Perkin I*, 1979, 2087.