Tritiated Peptides. Part 8.[1] Synthesis of [3,5-$^3H_2$-Tyr^2]-, [4-^{3}H-Phe^7]-, and [3,5-$^3H_2$-Tyr^{23}]-Human Corticotrophin

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Syntheses are described of human corticotrophin nonatriacontapeptides separately labelled with tritium in the tyrosine residues at positions 2 and 23 and in the phenylalanine residue at position 7 at specific radioactivities of 49, 25, and 31 Ci mmol^{-1} respectively. Labelling was by reductive deiodination of protected precursors. Evidence for the integrity of the final products is provided by amino-acid analysis, column chromatography, and bioassay, supported by chemical and enzymic analytical data.

SEVERAL accounts exist of the preparation of tritium-labelled corticotrophins, either of the full nonatriacontapeptide or of shorter sequences. Nishizawa *et al.*[2] prepared porcine ^{3}H-corticotrophin with a specific activity of 10 mCi mmol^{-1} by Wilzbach exchange labelling and von Werder *et al.*[3] obtained a tricosapeptide analogue by the same procedure labelled to the level of 1 Ci mmol^{-1}. Neither product was examined for possible damage by racemisation which is a known hazard of Wilzbach labelling.[4] We have reported [1] the syntheses of several corticotrophin tetracosa- and octadeca-peptides labelled at various aromatic amino-acid residues. The specific activities achieved have varied from 17 to 46 Ci mmol^{-1}. It was shown that our materials are indistinguishable from the corresponding non-radioactive peptides by a combination of analytical methods.

Corticotrophin nonatriacontapeptide has been labelled to an activity of 15 Ci mmol^{-1} by microwave discharge activation.[5] The material recovered was claimed to be fully biologically active and the scale of preparation was claimed to be suitable for 10 mg or less of material. However, at the 10 mg level the specific activities achieved were low, *ca.* 0.5 Ci mmol^{-1}, and when high activities were achieved the quantity obtained, 2 μg, was too small to allow analysis by any other means than bioassay, which is not a critical approach when unsupported by other data. The method does, nonetheless, have the attraction of being directly applicable to natural (rather than synthetic) material. Our procedure has the limitation of being totally synthetic, thus requiring a large commitment to produce the precursor compounds. Once these are available though, it is a simple matter to produce routinely specifically-labelled peptides to order in any quantity and full analytical data can be obtained for each batch prepared.

Recently, two accounts [6,7] have appeared of the preparation by synthesis of human corticotrophin nonatriacontapeptide labelled with tritium at a high specific radioactivity in the tyrosine residue at position 23. One approach was by hydrogenation of the di-iodotyrosine [23] (Dit^{23})-containing free peptide, synthesised by the solid phase procedure.[8] Hydrogenation was carried out in a mixture (1 : 10 : 90, v/v) of 0.1M-acetic acid, hexamethylphosphoramide, and dimethylformamide in which the exchangeable hydrogen atom content was kept to a minimum, and by this means a specific activity of 46.6 Ci mmol^{-1} was achieved. The hormone was judged pure on the basis of its bioactivity in two systems and its chromatographic and electrophoretic properties.

The synthesis which we have briefly reported [7] was by the hydrogenation of the fully-protected Dit^{23}-containing synthetic nonatriacontapeptide in the aprotic solvent dimethylformamide. This afforded, after work-up, a product with a specific activity of 25 Ci mmol^{-1} which was shown to be identical to human corticotrophin by amino-acid analysis, enzymic digestion, bioassay, and in its chromatographic properties. Our precursor peptide was synthesised by classical procedures, and we consider that classical synthesis gives more certainty of obtaining a final product that can be demonstrated to be pure than does solid phase synthesis. This is because the intermediates in a classical synthesis can be subjected to analytical investigation. In solid phase synthesis, only the end product is available for analysis.

This paper describes the synthesis of three tritium-labelled corticotrophin nonatriacontrapeptides with full details of synthesis and analysis. The syntheses have been achieved by analogous routes to those which furnished the corresponding tetracosapeptides.

The Dit^{23}-nonatriacontapeptide was prepared from the iodinated 11—39 fragment. This was prepared from the normal (*i.e.* Tyr^{23}-containing) 11—39 fragment by direct iodination with iodine chloride. The fragment was unprotected only at the α-amino terminus and was suitable for direct iodination in that the tyrosine residue is the only residue susceptible to substitution by iodine chloride and in that no protecting groups remain on the fragment that require catalytic hydrogenation for removal. Iodination was not complete although the same conditions applied to the corresponding (11—24) sequence were adequate.[9] Amino-acid analysis following enzymic digestion of the derived nonatriacontapeptide showed the presence of an appreciable content of monoiodotyrosine (Table 2). Paucity of material prevented improvement of this step.

The Dit^2- and Phe(I)^7-nonatriacontapeptides were prepared using the appropriate halogenated *N*-terminal decapeptides which have been described previously.[10,11]

All three nonatriacontapeptides were obtained from the 1—10 and 11—39 fragments by HOBt-assisted DCC-coupling followed by counter-current distribution purification as described in the synthesis of the natural human hormone.[12] Labelling, de-protection, and preliminary work-up were exactly as described for the tetracosapeptide analogues but we encountered difficulty with the ion-exchange chromatography. When trimethylammonium acetate buffer was used as eluant, the peptide became only partially soluble in water after repeated drying to remove the volatile buffer. We therefore purified labelled materials using short columns of carboxymethylcellulose (Na^+ form) and a gradient of sodium chloride buffered to pH 7 with sodium phosphate. This procedure is attractive in that the recovered material has merely to be diluted for storage and any damage that might occur during repeated evaporations to dryness is avoided. We now use similar column chromatographic procedures routinely for preparations of labelled corticotrophin tetracosapeptides and octadecapeptide amides. Respectively, these involve the use of linear gradients of sodium chloride, 0—0.40 and 0—0.55M, each buffered in 0.05M-sodium phosphate (pH 7).

One inconvenience of the preparation of end-products in salt buffers is that the phosphate present inhibits the enzymic digestion used as one of the analytical tests. We found it necessary to exchange phosphate for chloride ions in the sample before digestion.

The products were pure as judged by amino-acid analysis after both acidic and enzymic hydrolysis and were fully active in an isolated adrenal cell bioassay.[13] In the case of [Tyr(I_2)^{23}]-ACTH difficulty was experienced in obtaining complete hydrolysis by the enzyme mixture.[14] The final columns (a and b) of Table 2 show the results of amino-acid analysis of the products of enzymic digestion using two different batches of enzyme. From column a it would appear that there is a deficiency of Gly, His, Lys, and Met, and possibly of Pro, indicating there may have been incomplete digestion of the regions -Met-Glu-His- and Gly-Lys-Pro. On the other hand, the full residue of Asp liberated would imply that the region -Glu-Asp-Glu- which often proves resistant (as for example in Table 3) has been completely hydrolysed. However, the figures shown in column b where Asp and Glu are deficient indicate that the -Glu-Asp-Glu sequence has not been fully broken down by this particular batch of enzymes. In this case, figures for Gly and Lys are satisfactory and the deficiency of Pro is therefore probably not due to resistance of -Gly-Lys-Pro but to -Val-Tyr(X)-Pro, where X represents the iodine substitution. The low Val value would also support this suggestion. The values given for monoiodotyrosine are not regarded as accurate, since Trp and Tyr(I) have similar elution times. Overall the two analyses differ considerably but together allow a confident statement about the product.

The specific activities of the Phe^7- and Tyr^2-labelled products were satisfactory. That of the Tyr^{23}-labelled product was much lower than that of the Tyr^2-labelled product as a result of the incomplete iodination of the tyrosine residue in the 11—39 fragment.

EXPERIMENTAL

General procedures are given in Part 1. Iodophenylalanine was estimated as described in Part 3.[11] Thin layer chromatograms were developed in the following solvent systems: A, butan-1-ol–acetic acid (glacial)–water, 10 : 1 : 3 v/v; B, ethyl acetate–pyridine–formic acid–water, 63 : 21 : 10 : 6 v/v.

H-Lys(Boc)-Pro-Val-Gly-Lys(Boc)-Lys(Boc)-Arg-Arg-Pro-Val-Lys(Boc)-Val-Tyr-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. 3/2 H_2SO_4 (1).—Protected (11—39)-peptide[12] (0.78 g) was dissolved in methanol (150 ml) and hydrogenated at 20 °C in the presence of 10% palladium–charcoal (0.6 g) for 16 h when t.l.c. (solvent system A) showed that deprotection was complete. The catalyst was filtered off and the combined filtrate and washings were evaporated to yield 693 mg (92%) of product.

A portion (455 mg) of this was converted into the sesquisulphate (1) as described by Sieber, Rittel, and Riniker.[12] The yield was 410 mg (90%).

Boc-Ser-Tyr-Ser-Met-Glu(OBu^t)-His-Phe(I)-Arg-Trp-Gly-Lys(Boc)-Pro-Val-Gly-Lys(Boc)-Lys(Boc)-Arg-Arg-Pro-Val-Lys(Boc)-Val-Tyr-Pro-Asn-Gly-Ala-Glu(OBu^t)-Asp(OBu^t)-Glu(OBu^t)-Leu-Ala-Glu(OBu^t)-Ala-Phe-Pro-Leu-Glu(OBu^t)-Phe-OBu^t. 3/2 H_2SO_4 (2).—Compound (1) (199 mg, 48 μmol) was dissolved with Boc-Ser-Tyr-Ser-Met-Glu(OBu^t)-His-Phe(I)-Arg-Trp-Gly-OH[11] (90 mg, 57 μmol) and HOBt (8.7 mg, 57 μmole) in dimethylformamide (DMF) (1.0 ml) at 50 °C with stirring under N_2. N-H_2SO_4 (9 μl) was added followed by DCC (37 mg, 180 μmol) in DMF (0.5 ml) and the mixture was stirred for 2 h at 50 °C under N_2 and then evaporated to dryness at 20 °C. The residue was dissolved in the lower phase (10 ml) of the solvent system methanol–buffer–chloroform–carbon tetrachloride (20 : 7 : 12 : 8, v/v),[12] put in tube 1 of a 123-tube counter-current distribution machine (10 ml each phase), and 167 upper phase transfers were performed. Samples of the lower phases were examined by t.l.c. in the solvent system B and fresh solvents were put in tubes 1—8 and 35—123. After a further 433 upper phase transfers in the recycling mode, the

TABLE 1

Amino-acid analyses after acidic hydrolysis of the protected nonatriacontapeptide precursors containing iodoamino acids

	Substituent		
Amino acid	Dit^2-	Phe(I)^7-	Dit^{23}-
Ala	3.07	3.00	3.01
Arg	3.00	3.00	3.06
Asp	2.08	2.03	2.06
Glu	5.13	5.02	5.13
Gly	3.09	3.06	3.09
His	1.02	0.96	1.06
Leu	1.00	0.99	0.99
Lys	3.92	3.94	3.89
Met	1.01	0.99	1.06
Phe	3.00	2.00	3.08
Pro	3.97	3.92	4.00
Ser	2.71	2.71	2.86
Trp	0.29	0.65	0.44
Tyr	1.97	2.00	2.00
Val	3.00	3.00	2.98
Phe(I)		1.01	

TABLE 2

Amino-acid analyses after acidic and enzymic hydrolysis and potency in the isolated adrenal cell bioassay of the free nonatriacontapeptides containing iodoamino acids

Amino acid	Tyr $(I_2)^2$-ACTH		Phe $(I)^7$-ACTH		Tyr $(I_2)^{23}$-ACTH [a]		
	Acidic digest	Enzymic digest	Acidic digest	Enzymic digest	Acidic digest	Enzymic [b] digest	
						a	b
Ala	3.03	3.03	3.00	2.83	3.02	3.01	2.92
Arg	3.00	2.87	2.98	2.82	3.01	3.00	2.93
Asn		1.00		0.94		0.99	3.31 [c]
Asp	1.98	1.05	1.96	1.00	2.00	1.09	0.66
Glu	4.95	5.07	4.93	4.85	4.91	5.13	4.39
Gly	3.04	2.89	3.04	2.59	3.10	2.88	2.97
His	0.98	0.93	0.93	0.85	0.89	0.82	0.90
Leu	1.00	0.99	1.02	0.95	0.99	0.97	1.00
Lys	4.04	3.92	4.23	3.14	4.05	3.71	3.97
Met	0.97	0.92	0.99	0.89	0.86	0.55	0.90
Phe	3.00	3.00	2.00	1.99	3.00	3.00	3.00
Pro	3.92	3.96	3.94	3.76	3.92	3.93	3.62
Ser	2.70	3.02	2.80	2.94	2.78	3.13	3.31 [c]
Trp	0.30	0.93	0.70	0.89	0.20	0.95	0.95
Tyr	1.99	1.01	2.03	1.98	1.97	1.08	1.02
Val	3.01	2.94	3.03	2.58	3.09	3.06	2.62
Tyr (I_2)		0.91				trace	0.43
Tyr (I)							0.21
Phe (I)			1.08	0.86			
Potency [d]	1.0 (0.67—1.71)		1.8 (1.22—2.73)		32 (19.7—50.4)		

[a] Mixture of [Tyr$(I_2)^{23}$]- and [Tyr $(I)^{23}$]-ACTH. [b] a and b are results obtained using two different batches of enzymes, see text. [c] Combined value for Asn and Ser. [d] As defined in ref. 13.

product (163 mg, peptide content 70%, yield 41%) was recovered from tubes 61—99. The amino-acid analysis is given in Table 1.

Boc-Ser-Tyr(I_2)*-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-Pro-Asn-Gly-Ala-Glu(OBu^t)-Asp(OBu^t)-Glu(OBu^t)-Leu-Ala-Glu(OBu^t)-Ala-Phe-Pro-Leu-Glu(OBu^t)-Phe-OBu^t*. 3/2 H_2SO_4 (3).—This compound was synthesised as described for compound (2) starting from Boc-Ser-Tyr(I_2)-Ser-Met-Glu(OBu^t)-His-Phe-Arg-Trp-Gly-OH [10] (4) (201 mg, 48 μmol). Compound (4) was synthesised as described previously [10] but was obtained in 48% yield by two crystallisations from DMF–MeOH–H_2O (20 : 30 : 3, v/v). The *protected nonatriacontapeptide* (191 mg, peptide content 72%, yield 49%) was obtained after purification by counter-current distribution as described for compound (2). A total of 634 transfers of upper phase was required. The amino-acid analysis is given in Table 1.

Iodine-containing Free Nonatriacontapeptides.—Compound (2) (7.7 mg) was stirred at room temperature for 30 min with 90% aqueous trifluoroacetic acid (3 ml). The solution was evaporated, the residue was dissolved in water (1 ml) and passed through a column (0.5 ml) of Dowex 1 (acetate form) resin, and the column was washed with water (2 ml). The eluate was clarified by centrifugation and the

TABLE 3

Amino-acid analyses after acidic and enzymic hydrolysis, potency in the isolated adrenal cell bioassay, and specific radioactivity of the ^3H-labelled nonatriacontapeptides

Amino acid	$^3H_2$-Tyr^2-ACTH		^{3}H-Phe^7-ACTH		$^3H_2$-Tyr^{23}-ACTH	
	Acidic digest	Enzymic digest	Acidic digest	Enzymic digest	Acidic digest	Enzymic digest
Ala	3.01	3.00	3.00	3.08	2.97	2.46
Arg	3.01	2.93	3.00	2.97	2.98	2.97
Asn		0.91		0.88		3.24 [a]
Asp	2.03	1.01	2.08	0.94	2.07	0.34
Glu	5.08	4.93	5.15	4.83	5.00	3.93
Gly	3.02	3.00	3.02	3.16 [b]	3.07	3.04
His	0.97	0.95	1.01	0.97	1.00	0.99
Leu	0.96	0.99	0.96	1.01	1.00	0.92
Lys	4.09	3.91	4.03	4.02	3.96	3.90
Met	0.91	0.91	0.97	0.90	0.99	1.00
Phe	3.00	3.00	3.00	3.00	3.00	2.96
Pro	4.11	3.95	4.13	3.92	4.02	3.91
Ser	2.66	3.09	2.70	3.11	2.70	3.24 [a]
Trp	0.53	0.93	0.73	0.98	0.75	0.95
Tyr	1.99	2.00	2.02	2.00	1.93	1.89
Val	3.06	2.91	3.00	2.90	2.97	3.03
Potency [c]	106 (84—135)		100 (83—120)		106 (62—182)	
Specific activity (Ci mmol^{-1})	48.9		30.6		25.2	

[a] Combined value for Asn and Ser. [b] Combined value for Gly and Glu-Asp. [c] As defined in ref. 13.

supernatant solution was analysed for amino acids after acidic and enzymic hydrolysis. Compound (3) and the Dit[23]-containing protected nonatriacontapeptide [9] were similarly treated.

The analyses of the three peptides obtained are given in Table 2. This table also contains the values for the potencies of the peptides in the isolated adrenal cell bioassay.

[4-^{3}H-Phe[7]]-β-*Corticotrophin*-(1—39)-*nonatriacontapeptide* (5).—A solution of compound (2) (12.1 mg) in DMF (0.7 ml) was stirred with 98% $^3H_2$ gas (3.2 ml; 8 Ci) in the presence of 10% palladium–charcoal (12 mg) and 5%-rhodium–calcium carbonate (8 mg). After 45 min, the mixture was diluted with DMF and the catalysts were removed by filtration through a pad of cellulose powder (MN 300, Macherey, Nagel and Co.). The filtrate was evaporated to dryness and the residue was dissolved in 90% trifluoroacetic acid (3 ml) and kept at room temperature for 30 min. The mixture was evaporated to dryness and the residue was dissolved in water (1 ml) and passed through a column (0.5 ml) of Dowex 1 (acetate form) resin which was eluted with water (2 ml). The combined eluate and washings were evaporated to dryness and the residue was dissolved in water (0.5 ml). The solution was applied to a column (3 × 0.7 cm) of carboxymethylcellulose (Na^+ form) which was eluted with a linear gradient (40 ml total) of NaCl (0—0.125M) in a buffer of 25mM-sodium phosphate (pH 7.0). Fractions were monitored for radioactivity and those containing the peptide (tubes 12—15) were combined to yield the *product* (513 nmol, 39%). The amino-acid analysis, the specific radioactivity, the potency by bioassay, and the amino-acid analysis after enzymic hydrolysis are given in Table 3. Before enzymic hydrolysis, a sample (1 ml) of peptide solution was passed through a column (3.5 × 0.6 cm) of Dowex 1 (chloride form) resin contained in a Pasteur pipette and the column was eluted with water (2 ml). Radioactivity was distributed among the residues [10] in the ratio Phe : His = 91.8 : 8.2. The peptide solution was stored at −195 °C (liquid N_2) at a concentration no greater than 100 μg ml.$^{-1}$

[3,5-$^3H_2$-Tyr[2]]-β-*Corticotrophin*-(1—39)-*nonatriacontapeptide* (6).—This compound was prepared in 20% yield from compound (3) as described for compound (5). Analytical data are given in Table 3. Radioactivity was distributed among the residues in the ratio Tyr : His = 95.3 : 4.7.

We thank Dr. W. Rittel, CIBA–GEIGY AG., Basle for the gift of protected (11—39)-peptide, Mr. B. E. Evans for amino-acid analyses, Mrs. S. M. Garman for the assessment of distribution of radioactivity among the amino-acid residues, and Miss M. Chen for bioassay measurements.

[9/554 *Received*, 9*th April*, 1979]

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