LABELLED POLYPEPTIDES. V. SYNTHESIS OF α-MELANOTROPIN LABELLED WITH TRITIUM ON THE TYROSINE RESIDUE, VIA THE DIBROMO INTERMEDIATE

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Received on May 23, 1975
Revised on September 12, 1975

SUMMARY

A novel synthesis of α-melanotropin labelled with tritium on the tyrosine residue ($[Tyr\text{-}3,5\text{-}^{3}H^{2}]$-α-melanotropin) is described. First, a precursor peptide, containing 3,5-dibromotyrosine in position 2 ($[Dbt^{2}]$-α-melanotropin) is prepared, which can be converted into the labelled hormone by catalytic tritiation in the last step of the synthesis. By this procedure the tedious work with highly active labelled intermediates, and the storage of the tritiated compound, usually accompanied by significant autoradiolysis, can be avoided.

In an earlier paper we reported the synthesis of α-melanotropin labelled with tritium in the 3,5 positions of the tyrosine residue, starting from the labelled amino acid (1). This compound possessed a specific radioactivity of 2.8 Ci/mmole (1.56 Ci/mg), and we mentioned that significant radiolysis could be observed on storing the substance for a longer time. In many cases biological experiments may require products of still higher

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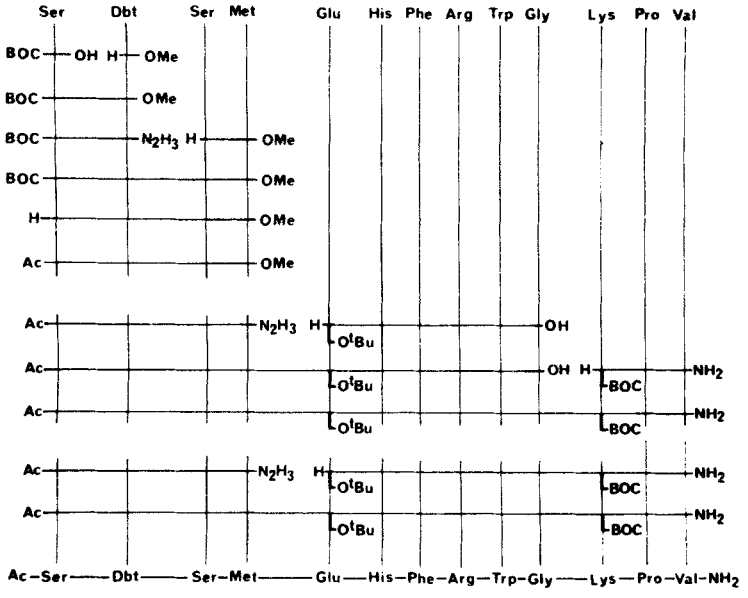
specific activity, where the danger of autoradiolysis is much more probable, and the synthetic work with the highly active intermediates can be even more tedious and dangerous. Although labelled α-melanotropins synthesized previously (1,2) were found suitable for various binding experiments, we have elaborated a new procedure for the preparation of the tritium-labelled hormone; this allows the introduction of the radioactive label into the molecule containing a suitable precursor amino acid in the last step of the chemical synthesis. Thus storage of large amounts of the highly radioactive hormone can be avoided and the required quantity of the labelled peptide can be prepared right before application, by a relatively simple operation.+

As the precursor amino acid 3,5-dibromotyrosine (Dbt) was selected. The outlines of the synthesis of α-melanotropin containing 3,5-dibromotyrosine in position 2 ($[Dbt^2]$-α-melanotropin), effected in two alternative ways, are shown in the Figure.



Scheme of synthesis of $[Dbt^2]$-α-melanotropin

+ Parts of the synthetic work detailed in this paper were presented at the Gasteiner Internationales Symposion, Bad Gastein, January 9, 1974. Symbols and abbreviations are used according to the rules of the IUPAC IUB Commission on Biochemical Nomenclature, J.Biol.Chem. 247:977 (1972). All amino acid derivatives mentioned in this paper are of the L-configuration.

By catalytic hydrogenation $[Dbt^2]$-α-melanotropin can be converted into the hormone with the natural sequence, containing tyrosine in position 2. The similar reaction, using tritium instead of hydrogen gives the radioactive hormone derivative, containing tyrosine labelled with tritium in the 3,5 positions ($[Tyr\text{-}3,5\text{-}^3H^2]$-α-melanotropin).

Reproduction of the direct bromination described for the synthesis of 3,5-dibromotyrosine (3) resulted in an inhomogeneous product. Pure dibromo compound could be obtained by using excess bromine at elevated temperature. 3,5-Dibromotyrosine was esterified by the methanol-thionyl chloride procedure. Coupling with t-butyloxycarbonyl-serine azide (4) led to the protected dipeptide ester, which was converted into the hydrazide and azide and condensed with seryl-methionine methyl ester (5). The resulting BOC-Ser-Dbt-Ser-Met-OMe tetrapeptide ester was deprotected by trifluoroacetic acid, acetylated with p-nitrophenyl acetate, and transformed into the hydrazide.

The azide prepared from the BOC-Ser-Dbt-Ser-Met-N_2H_3 tetrapeptide hydrazide was coupled with the H-Glu(OtBu)-His-Phe--Arg-Trp-Gly-OH hexapeptide described earlier (6,7), and the resulting decapeptide with the C-terminal H-Lys(BOC)-Pro-Val-NH_2 tripeptide amide (8)(Route A). In Route B first the H-Glu(OtBu)--His-Phe-Arg-Trp-Gly-Lys(BOC)-Pro-Val-NH_2 nonapeptide was prepared (9) and acylated with the N-terminal acetyl-tetrapeptide azide. The latter procedure usually gave more homogeneous products and better yields.

The protected dibromo-α-melanotropins prepared by either way proved to be identical in every respect. Hydrogenation in the presence of $Pd/CaCO_3$ catalyst gave, after chromatography on carboxymethyl cellulose, pure α-melanotropin. Using tritium gas instead of hydrogen yielded in the same procedure $[Tyr\text{-}3,5\text{-}^3H^2]$-α-melanotropin with 2.6-4 Ci/mmole specific activity, depending on the dilution of the tritium used.

EXPERIMENTAL

Thin-layer chromatography was carried out on Kieselgel G (Merck) plates, chromatograms were developed with the following solvents (volumes), using ninhydrin and chlorine-tolidine reagents for detection:

Solvent 1 Ethyl acetate-pyridine-acetic acid-water 30-20-6-11
Solvent 2 Ethyl acetate-pyridine-acetic acid-water 60-20-6-11
Solvent 3 Ethyl acetate-pyridine-acetic acid-water 120-20-6-11
Solvent 4 Acetone-toluene 1-1

3,5-Dibromotyrosine

To a stirred suspension of 36.2 (0.2 moles) L-tyrosine in 150 ml acetic acid a solution of bromine (96.0 g, 30.8 ml, 0.6 moles) in 130 ml acetic acid was slowly added, resulting in a clear, red solution, warming spontaneously to 55-60°C. The stirring was continued at this temperature for two additional hours, the solution allowed to cool, and the crystalline precipitate (3,5-dibromotyrosine hydrobromide) was filtered off and washed with ether, yielding 33.3 g product. On evaporating the mother liquor and treating the residue with ether a second crop was obtained; total yield 68.5 g (81.8%) hydrobromide.

For purification the substance was dissolved in 350 ml water, treated with charcoal, filtered and adjusted to pH 6 with concentrated ammonium hydroxide solution. The precipitate was filtered off and washed with water.

Yield 50.6 g (74.5%). R_F^1 0.50. $[\alpha]_{588}^{30} = +4.49^\circ \pm 0.07^\circ$ (c= 0.84, in 1 n HCl). Calcd. for $C_9H_9O_3NBr_2$ (339.0): C 31.9, H 2.7, N 4.1, Br 47.2; found: C 31.7, H 2.6, N 4.1, Br 47.3%.

3,5-Dibromotyrosine methyl ester hydrochloride

To 200 ml anhydrous methanol 37.7 ml thionyl chloride (0.52 moles) was added dropwise at -15°C, followed by the addition of 3,5-dibromotyrosine (44.0 g, 0.13 moles) under stirring, and keeping the temperature of the reaction mixture at -10°C. The stirring was continued for ten minutes, and the clear solution was allowed to stand at room temperature overnight. After evaporation the residue was dissolved in methanol, evaporated again, and crystallized from methanol-ether.

Yield 50.8 g (98%). M.p. 185-187°. R_F^3 0.65. $[\alpha]_D^{25} = +8.8^\circ$ (c=1.0 in methanol). Calcd. for $C_{10}H_{12}O_3NBr_2Cl$ (389.5): C 30.8, H 3.1, N 3.6, Br 41.0; found C 30.6, H 3.2, N 3.5, Br 40.8%.

t-Butyloxycarbonyl-seryl-3,5-dibromotyrosine methyl ester

To the solution of 6.60 g (30 mmoles) t-butyloxycarbonyl-

-serine hydrazide (8) in 50 ml dimethylformamide (DMF) chilled to -10°C, 15 ml (90 mmoles) cold 6 n hydrochloric acid was added, followed by the addition of a concentrated aqueous solution of 2.07 g (30 mmoles) sodium nitrite, under stirring at the same temperature. After 10 minutes a cold solution of 3,5-dibromo--tyrosine methyl ester hydrochloride (10.9 g, 28 mmoles) and triethylamine (12.4 ml, 90 mmoles) in 50 ml DMF was added, and the mixture was stirred at -5°C for two hours, then kept in an icebox overnight. Evaporation in vacuo, dissolving the residue in ethyl acetate, washing with citric acid and sodium bicarbonate solutions and repeated evaporation yielded after crystallization from ethyl acetate-petroleum ether 10.9 g (67.4%) dipeptide ester with m.p. 122-125°C, R_F^4 0.50.

Calcd. for $C_{18}H_{24}O_7N_2Br_2$ (540.2): C 40.0, H 4.5, N 5.2, Br 29.6; found C 40.1, H 4.5, N 5.3, Br 29.8%.

t-Butyloxycarbonyl-seryl-3,5-dibromotyrosine hydrazide

To a solution of BOC-Ser-Dbt-OMe dipeptide ester (10.8 g, 20 mmoles) in 45 ml methanol 7.0 ml 98% hydrazine hydrate was added and allowed to stand at room temperature for 24 hours. The precipitated substance was filtered off, washed with cold methanol and dried. For purification it was dissolved in the minimal amount of ice-cold 1 n hydrochloric acid, precipitated with a 10% solution of sodium carbonate and crystallized from 400 ml water.

Yield 7.50 g (69.4%), m.p. 197-199°C, R_F^4 0.52.

Calcd. for $C_{17}H_{24}O_6N_4Br_2$ (540.2): C 37.8, H 4.5, N 10.4, Br 29.6; found C 37.7, H 4.5, N 10.3, Br 29.6%.

t-Butyloxycarbonyl-seryl-3,5-dibromotyrosyl-seryl-methionine methyl ester

The solution of 5.40 g (10 mmoles) BOC-Ser-Dbt-N_2H_3 dipeptide hydrazide in 30 ml DMF was chilled to -10°C, 5 ml (30 mmoles) cold 6 n HCl and a concentrated aqueous solution of 690 mg (10 mmoles) sodium nitrite was added under vigorous stirring at -10°C. After 10 minutes the solution of 2.87 g (10 mmoles) of H-Ser-Met-OMe.HCl dipeptide ester hydrochloride and 4.14 ml (30 mmoles) triethylamine in 20 ml DMF was added, stirred at -5°C for two hours, and let stand at 4°C overnight. Evaporation in vacuo was followed by the usual washing procedure, the final residue was crystallized from methanol.

Yield 4.32 g (56.9%), m.p. 110-114°C, R_F^3 0.75.

Calcd. for $C_{26}H_{38}O_{10}N_4Br_2S$ (758.5); C 41.2, H 5.05, N 7.4, Br 21.1; found: C 41.2, H 5.0, N 7.5, Br 20.9%.

Seryl-3,5-dibromotyrosyl-seryl-methionine methyl ester trifluoroacetate

BOC-Ser-Dbt-Ser-Met-OMe tetrapeptide ester (3.04 g, 4 mmoles), dissolved in 30 ml 90% trifluoroacetic acid (TFA) containing one drop of mercaptoethanol, was allowed to stand at room temperature for 30 minutes, evaporated to dryness, and 10 ml portions of methanol were distilled off from the residue several times. The final residue was powdered under peroxide-free ether and filtered.

The yield is 3.16 g, nearly quantitative, R_F^2 0.40. Crystallization from methanol gave a product which melted at 205-208°C.

N-Acetyl-seryl-3,5-dibromotyrosyl-seryl-methionine methyl ester

To a solution of H-Ser-Dbt-Ser-Met-OMe.TFA tetrapeptide ester trifluoroacetate (2.55 g, 3.3 mmoles) and triethylamine (1.37 ml, 9.9 mmoles) in 40 ml pyridine, 1.2 g (6.6 mmoles) p-nitrophenyl acetate was added, and the mixture was kept at 20°C overnight. The solvent was removed by distillation in vacuo, the residue dissolved in methanol, evaporated again and triturated with peroxide-free ether to remove excess p-nitrophenyl acetate. The product was crystallized from methanol, yielding 2.11 g (91.3%) acetyl-tetrapeptide ester, m.p. 248-252°C after repeated crystallization from the same solvent. R_F^2 0.75.

Calcd. for $C_{23}H_{32}O_9N_4Br_2S$ (700.4): N 8.0, Br 22.8; found: N 8.0, Br 22.5%.

N-Acetyl-seryl-3,5-dibromotyrosyl-seryl-methionine hydrazide

To a solution of Ac-Ser-Dbt-Ser-Met-OMe tetrapeptide ester (1.54 g, 2.2 mmoles) in 10 ml warm methanol 2.3 ml 98% hydrazine hydrate was added and allowed to stand at 50°C for four hours. The precipitated hydrazide was filtered off, washed with cold methanol and dried over sulphuric acid in vacuo. The dry material was thoroughly washed with water and crystallized from water to give 1.12 g (72.8%) pure hydrazide with a m.p. of

247-250°C; R_F^2 0.20.

Calcd. for $C_{22}H_{32}O_8N_6Br_2S$ (700.4): N 12.0, Br 22.8; found N 12.1, Br 22.5%.

N-Acetyl-seryl-3,5-dibromotyrosyl-seryl-methionyl-γ-t-butyl--glutamyl-histidyl-phenylalanyl-arginyl-tryptophyl-glycine

The solution of the acetyl-tetrapeptide hydrazide (700 mg, 1 mmole) in 5 ml DMF was chilled to -5°C, and under continuous stirring at the same temperature cold 6 n HCl (0.5 ml, 3 mmoles), sodium nitrite (76 mg, 1.1 mmoles) in the minimal amount of water, and after 10 minutes the solution of the hexapeptide H-Glu(OtBu)--His-Phe-Arg-Trp-Gly-OH (947 mg, 1 mmole) and triethylamine (0.41 ml, 3 mmoles) in 4 ml DMF were added. Stirring was continued at 0°C for two hours, and the mixture was kept at 4°C overnight. After evaporation of the solvents the residue was triturated with ether, ethyl acetate and acetone, filtered off and air-dried, yielding 1.58 g (~100%) crude decapeptide.

R_F^1 0.50, m.p. 208-219°C (decomp.) after crystallization from 50% aqueous t-butanol.

Calcd. for $C_{65}H_{86}O_{17}N_{16}Br_2S$ (1555.4): N 14.4, Br 10.3; found: N 14.2, Br 10.1%.

N-Acetyl-seryl-3,5-dibromotyrosyl-seryl-methionyl-γ-t-butyl-glutamyl-histidyl-phenylalanyl-arginyl-tryptophyl-glycyl-ε-t-butyl-oxycarbonyl-lysyl-prolyl-valine amide (protected dibromo-α-melanotropin)

A) 1-10 + 11-13 coupling

To a solution of the acetyl-decapeptide (807 mg, 0.52 mmoles) and pyridine hydrochloride (62 mg, 0.52 mmoles) in 15 ml DMF, H-Lys(BOC)-Pro-Val-NH_2.$CH_3C_6H_4SO_2OH$ tripeptide amide p-toluenesulphonate (638 mg, 1.04 mmoles), 0.145 ml (1.04 mmoles) triethylamine, 277 mg (1.04 mmoles) pentachlorophenol and dicyclohexyl-carbodiimide (214 mg, 1.04 mmoles) were added, and the mixture was stirred at room temperature for 48 hours. The DMF was removed by distillation in vacuo, the residue powdered under peroxide-free ether and ethyl acetate, filtered off, dried and washed thoroughly with water to give 1.2 g crude tridecapeptide, containing some dicyclohexyl-urea. The main component showed an R_F^1 value of 0.60.

B) 1-4 + 5-13 coupling

Ac-Ser-Dbt-Ser-Met-N_2H_3 tetrapeptide hydrazide (140 mg, 0.2 mmoles) was suspended in 0.4 ml DMF. After the addition of 0.2 ml water and 0.12 ml 6 n HCl a clear solution was obtained. To this mixture 15.2 mg sodium nitrite was added at -20°C and stirred for 5 minutes, keeping the temperature between -5°C and -10°C. The resulting azide was then reacted with H-Glu(O$\underline{t}$Bu)-His--Phe-Arg-Trp-Gly-Lys(BOC)-Pro-Val-NH_2 nonapeptide amide acetate (286 mg) and triethylamine (0.054 ml) in 0.6 ml DMF, kept in the refrigerator overnight and diluted with the mixture of 6 ml ethyl acetate and 2 ml water. The precipitated substance was filtered off and dried in vacuo, yielding 275 mg (79%) protected trideca-peptide amide with R_F^1 0.60.

N-Acetyl-seryl-3,5-dibromotyrosyl-seryl-methionyl-glutamyl-histi-dyl-phenylalanyl-arginyl-tryptophyl-glycyl-lysyl-prolyl-valine amide (dibromo-α-melanotropin, $[Dbt^2]$-α-melanotropin)

The protected dibromo-α-melanotropin (800 mg) was dis-solved in 15 ml 90% TFA containing one drop of mercaptoethanol, and the solution was kept at room temperature for 20 minutes. After evaporation the residue was dissolved in 15 ml water, some insoluble material was filtered off and the peptide was converted into the acetate using Amberlite IRA-400 ion exchanger in the acetate cycle. The lyophilisate weighed 408 mg and yielded an electrophoretically and chromatographically homogeneous product after carboxymethyl cellulose chromatography using an ammonium acetate buffer gradient with increasing pH and molarity.

R_F^1 0.15.

Acetyl-seryl-$[$tyrosyl-3,5-$^3H]$-seryl-methionyl-glutamyl-histidyl-phenylalanyl-arginyl-tryptophyl-glycyl-lysyl-prolyl-valine amide (tritium-labelled α-MSH)

Dibromo-α-melanotropin (38 mg) was dissolved in the mixture of 1 ml DMF and 1 ml water, and tritiated under magnetic stirring in the presence of 25 mg 10% palladium-charcoal catalyst and 25 mg $CaCO_3$ (or 25 mg 10% palladium-on-$CaCO_3$) for one hour in a specific tritiation equipment designed for this purpose (10). The catalyst was filtered off, the solution evaporated, the residue

applied onto a carboxymethyl cellulose column and fractionated as described in (1).

The specific radioactivity of the labelled hormone was 2.6 to 4 Ci/mmole, depending on the purity of the tritium gas used. The biological activity was determined by the in vitro frog skin assay of Shizume et al. (11) and found to be $1\text{-}2.10^{10}$ units per g.

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