LABELLED POLYPEPTIDES. III. SYNTHESIS OF α-MELANOTROPIN LABELLED WITH TRITIUM ON THE TYROSINE RESIDUE.

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SUMMARY

The synthesis and purification of α-melanotropin labelled with tritium on the tyrosine residue is described. The hormone possesses a specific radioactivity of 2.8 Ci/mmole, which enables its application in studying transport processes, metabolism and mechanism of hormone action.

α-Melanotropin† (α-MSH) is a polypeptide hormone isolated from the pituitary, possessing characteristic melanocyte stimulating (melanin dispersing) activity. Its structure resembles the first tridecapeptide sequence of the adrenocorticotropic hormone except that the N-terminal is acetylated and the molecule does not contain a free C-terminal carboxyl group, that is, the terminal valine residue is amidated. α-Melanotropin with exactly the same structure can be found in the hypophysis of different species (3):

Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2

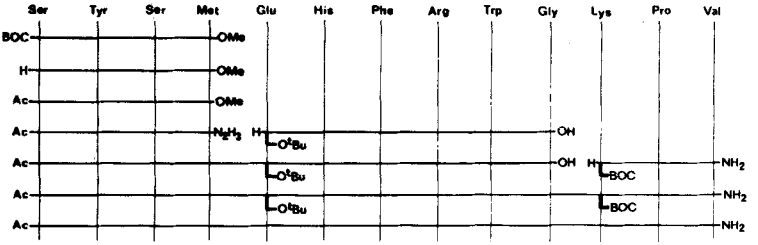
α-Melanotropin (α-MSH)

† For the nomenclature of melanotropins, see (1,2).

Although its melanocyte stimulating activity in amphibians is well established and thoroughly investigated and there are a great number of experiments for the elucidation of the biological role of this hormone in mammals, an unequivocal interpretation of its physiological significance is still lacking. Whatever this biological role may turn out to be, the hormone exerts it in very small amounts: as little as 0.02 ng of α-MSH per ml gives a positive effect in the skin darkening reaction (3) and melanocyte stimulating substances can be found in the blood at a concentration of 1-2 units per ml (4), which value corresponds to about 0.1 ng of α-MSH.

Because of the very small physiological doses, like in the case of other polypeptide hormones, transport processes, metabolism and the mechanism of action of α-MSH can be advantageously studied by the use of hormone preparations labelled with highly active radioisotopes. Recently, in a short notice we reported the synthesis of α-MSH labelled with tritium on the tyrosine residue (5); the present paper gives a detailed account on this synthesis which resulted in the pure hormone possessing a specific activity of 2.8 Ci/mmole.

Various syntheses of α-MSH have been described in the literature (6,7,8). Having prepared numerous intermediates for the synthesis of the labelled N-terminal decapeptide of the ACTH (9), which can be used for the preparation of α-MSH as well, we decided to follow the general route described by Schwyzer and co-workers (7) with slight modification, as it is shown in the scheme below:



Scheme for the α-MSH Synthesis

Tritium-labelled BOC-Ser-Tyr-Ser-Met-OMe* was synthesized as described in (9), with a specific radioactivity of 3.4 Ci per mmole. The t-butyloxycarbonyl protecting group was removed by 90% trifluoroacetic acid. The crystalline trifluoroacetate (m.p. 199-202°) was homogeneous on thin-layer chromatography (TLC) in a number of solvents, but resolved in Solvent 1 (see Experimental) into two spots with similar intensity. This phenomenon was apparently caused by some ion equilibrium, since transformation into the acetate led to a product which was homogeneous even in Solvent 1.

Acetylation and conversion to the hydrazide gave a product with unchanged specific radioactivity and with physical constants similar to those given in the literature (7). To avoid greater losses of radioactive product, the substances were recrystallized only once from the appropriate solvent; we did not strive to obtain analitically pure derivatives with the highest melting points recorded in the literature.

Synthesis of the partially protected H-Glu(OtBu)-His-Phe--Arg-Trp-Gly-OH hexapeptide was first published by Schwyzer and Kappeler (10); we followed the modified procedure as given in (9). The hexapeptide was acylated with the azide prepared from the Ac-Ser-Tyr-Ser-Met-N_2H_3 tetrapeptide hydrazide according to Schwyzer *et al.* (7), and the resulting labelled acetyl decapeptide was crystallized from 50% aqueous t-butanol.

Coupling this compound with the tripeptide amide H-Lys(BOC)-Pro-Val-NH_2 using dicyclohexyl carbodiimide and pentachlorophenol in dimethylformamide (DMF) as solvent yielded the protected acetyl tridecapeptide amide, which, in turn, gave the desired labelled Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys--Pro-Val-NH_2 (α-MSH) after deblocking with 90% trifluoroacetic acid.

Purification of the crude hormone was accomplished by ion exchange chromatography on carboxymethyl cellulose (Serva) or CM--Sephadex C 25 (Pharmacia) columns. The significant loss of α--melanotropin during the ion exchange chromatography reported in

*Symbols and abbreviations are used according to the rules of the IUPAC IUB Commission on Biochemical Nomenclature, e.g. J.Biol.Chem. 241; 527 (1966).

the literature (7) could not be observed when the above mentioned adsorbents were used, as shown by the weight of the substances recovered and by the radioactivity retained by the column. The pure product was tested for homogeneity by TLC, paper electrophoresis and amino acid analysis. Melanocyte stimulating activity was measured by the in vitro frog skin method of Shizume et al. (11), and found to be in the range of 1-2 x 10^{10} units per gram.

There was no loss in specific radioactivity through the different synthetic steps, except on treatment with trifluoroacetic acid (conversion of labelled BOC-Ser-Tyr-Ser-Met-OMe to H-Ser--Tyr-Ser-Met-OMe and deprotecting of the blocked α-MSH), where a 8-10 per cent decrease could be observed. Thus, the final product possessed a specific activity of 2.8 Ci/mmole (1.56 mCi/mg).

During storage for several months significant (up to 30%) autoradiolysis of the highly radioactive product took place, which could be detected both by changes in the chromatographic pattern and biological activity. To minimize radiolytic and secondary decomposition, labelled α-melanotropin was stored in sealed evacuated ampoules at -20°.

It is remarkable, how useful labelled compounds can be in evaluating peptide synthetic steps and identifying the by-products of the reaction (e.g. formation of acetyl decapeptide-dicyclohexylurea in the last coupling step). Experimental data on this topic will be reported later.

EXPERIMENTAL

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

Solvent 1	Ethyl acetate - pyridine - acetic acid - water 60-20-6-11 by vol.
Solvent 2	Ethyl acetate - pyridine - acetic acid - water 120-20-6-11 by vol.
Solvent 3	Methyl ethyl keton - pyridine - water 130-15-20 by vol.

Details for specific radioactivity determinations and TLC scanning were given previously (12). Scanning of TL chromatograms and paper electropherograms had to be made before staining with chlorine-tolidine, as this procedure was accompanied with a total loss of radioactivity.

Seryl-[tyrosyl-3,5-^{3}H]-seryl-methionine methyl ester trifluoroacetate

901 mg (1.5 mmoles) BOC-Ser-[Tyr-3,5-^{3}H]-Ser-Met-OMe tetrapeptide ester (9) (specific activity 3.4 Ci/mmole) was dissolved in 10 ml 90% trifluoroacetic acid, containing one drop of mercaptoethanol. The solution was kept at room temperature for 15 min., evaporated to dryness in vacuo, and the solid residue was crystallized from methanol. Yield 794 mg (86%), m.p. 199-202°. R_F^1 0.36 and 0.27 (tetrapeptide acetate R_F^1 0.27), R_F^2 0.06, R_F^3 0.70 (ninhydrin, chlorine-tolidine and radioactivity).

The product had a specific radioactivity of 3.2 Ci/mmole.

Acetyl-seryl-[tyrosyl-3,5-^{3}H]-seryl-methionine methyl ester

Seryl-[tyrosyl-3,5-^{3}H]-seryl-methionine methyl ester trifluoroacetate (790 mg, 1.29 mmoles) was dissolved in anhydrous pyridine (17 ml). After addition of triethylamine (0.53 ml) and p-nitrophenyl acetate (470 mg) the reaction mixture was kept at room temperature for 48 hours, the solvent was evaporated in vacuo and the residue treated with ether several times. The resulting yellow powder was recrystallized from ethanol, yielding 644 mg (92%) acetyl tetrapeptide methyl ester, m.p. 192-194°.

Homogeneous on TLC; R_F^1 0.73, R_F^2 0.54, R_F^3 0.77. Specific activity 3.2 Ci/mmole, unchanged.

Acetyl-seryl-[tyrosyl-3,5-^{3}H]-seryl-methionine hydrazide

Acetyl-seryl-[tyrosyl-3,5-^{3}H]-seryl-methionine methyl ester (640 mg, 1.18 mmoles) was dissolved in 4 ml methanol, and 100% hydrazine hydrate (1.25 ml) was added. After 4 hours at 50° the precipitated hydrazide was filtered and washed with cold methanol and water. Crystallization from hot water gave 562 mg (88%) acetyl tetrapeptide hydrazide, m.p. 239-245°.

TLC: R_F^1 0.30, R_F^3 0.66; there was a small, slower running radioactive contaminant (less than 3%, checked by quantitative radiochromatography). Specific radioactivity unchanged, 3.2 Ci/mmole.

Acetyl-seryl-[tyrosyl-3,5-^{3}H]-seryl-methionyl-γ-tert.-butyl--glutamyl-histidyl-phenylalanyl-arginyl-tryptophyl-glycine

Acetyl-seryl-[tyrosyl-3,5-^{3}H]-seryl-methionine hydrazide (560 mg, 1.03 mmoles) was dissolved in 1.6 ml dimethylformamide (DMF) with the addition of 1.9 ml water and 0.68 ml 6N hydrochloric acid and chilled to -10°. Under stirring 73 mg (1.06 mmoles) of powdered sodium nitrite was added, after 5 min. following by the addition of the hexapeptide monoacetate H-Glu(OtBu)-His-Phe-Arg--Trp-Gly-OH.CH_3COOH (9)(966 mg, 1.02 mmoles) in 5 ml DMF containing 0.56 ml triethylamine. Stirring was continued for two hours at -5° (bath temperature), the mixture was then kept in the refrigerator overnight. The gelatinous reaction product was worked up according to the procedure described by Schwyzer et al. (7) for the inactive compound, filtered and crystallized from 50% tert.-butanol. Yield 1210 mg (84%), m.p. 218-221°.

TLC (R_F^1 0.10) shows less than 2% radioactive contamination (R_F^1 0.65). The crystalline substance had a specific radioactivity of 3.1 Ci/mmole.

Acetyl-seryl-[tyrosyl-3.5-^{3}H]-seryl-methionyl-γ-tert.-butyl--glutamyl-histidyl-phenylalanyl-arginyl-tryptophyl-glycyl-ε-tert.-butyloxycarbonyl-lysyl-prolyl-valine amide (Protected α-MSH)

In 25 ml DMF 1.20 g (0.86 mmoles) labelled acetyl decapeptide and 100 mg (0.86 mmoles) pyridine hydrochloride were dissolved, to the solution ε-tert.-butyloxycarbonyl-lysyl-prolyl--valine amide tosylate (985 mg, 1.6 mmoles) and triethylamine (0.224 ml) were added, followed by the addition of 427 mg pentachloro phenol and 330 mg (1.6 mmoles) dicyclohexylcarbodiimide. The mixture was kept at room temperature overnight, evaporated to a thick oil which was taken up in 8 ml methanol and precipitated with a large volume of peroxyde free ether. The precipitate was filtered, washed with ether, airdried, triturated twice with water, filtered and dried again. The crude product weighed

1140 mg (73% yield), but it was contaminated with small amounts of unchanged acetyl decapeptide, dicyclohexylurea and acetyl decapeptide-dicyclohexylurea derivative.

The main component's R_F^1 0.35, because of the inhomogeneity, specific radioactivity can be given on weight basis only: 1.6 mCi/mg.

Since silica gel column chromatography led to the pure product with significant losses, further purification and characterization of the protected, labelled α-MSH was abandoned, and the crude compound was used in the deblocking step.

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

The crude protected, labelled α-MSH described above (906 mg) was dissolved in 20 ml trifluoroacetic acid, containing 2.0 ml water and a small amount of mercaptoethanol. After 20 minutes at room temperature the solvent was distilled off, the residue dissolved in 6 ml water, and the small amount of insoluble substance (mainly dicyclohexyl urea) removed by filtration. The peptide trifluoroacetate was transformed into acetate by filtering through a 2x15 cm Amberlite IRA-400 (acetate cycle) ion exchanger column and the solution liophylised.

The resulting free peptide (710 mg) was dissolved in 9 ml 0.05 molar (pH 5.3) ammoniumacetate buffer and fractionated on a 1.6 x 25 cm carboxymethyl cellulose column equilibrated with the same buffer solution. Elutions were carried out with the following ammonium acetate buffers: 0.05 M, pH 5.3 (200 ml); 0.05 M, pH 5.5 (500 ml); 0.05 M, pH 5.7 (350 ml); 0.10 M, pH 6.2 (200 ml) and finally 0.06 M ammonium hydroxide (200 ml). 10 ml fractions were collected at the flow rate of 60 ml per hour, fractionation was followed by continuous measuring of absorbance at 280 nm. Fairly much substance has been found in the first fractions (167 mg, mainly decapeptide and derivative), pure α-MSH was collected from the 69-107 fractions (pH 5.7 buffer), yielding after liophylisation to constant weight 310 mg (35%, calculated to the crude, protected α-MSH). From the material applied to the column a total amount of 677 mg was recovered.

From smaller amount (193 mg) the fractionation of crude α-MSH was reproduced using CM-Sephadex C 25 ion exchanger column with the following ammonium acetate buffer solutions: 0.05 M, pH 5.7 (150 ml); 0.075 M, pH 6.2 (110 ml); 0.10 M, pH 6.5 (170 ml); 0.25 M, pH 6.9 (250 ml) and 0.05 M ammonium hydroxide (50 ml). 8 ml fractions were collected at a flow rate of 45 ml per hour, total amount recovered 171 mg, fractions 46-63 contained pure α-MSH, yield after liophylisation to constant weight 88 mg.

The purified, labelled α-MSH proved to be homogeneous on paper electrophoresis (1200 V, pH 3.8, 6.5 cm to the cathode); amino acid analysis (24 h. hydrolysis in 6N HCl, 105° in evacuated tube) gave the following results: Ser 2.09, Tyr 1.13, Met 1.0, Glu 1.09, His 0.98, Phe 1.02, Arg 0.96, Gly 1.10, Lys 0.95, Pro 1.09, Val 1.09, Trp was not determined. Specific radioactivity 2.8 Ci/mmole.

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