SYNTHESIS OF TRITIUM LABELLED TRH-LIKE PEPTIDES USING ASYMMETRIC ANHYDRIDE COUPLING

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SUMMARY

A method is presented for the preparation of [^{3}H]-labelled TRH-like tripeptides. The synthesis involves preparation of the appropriate dipeptide by the solid phase procedure using Fmoc-protection and the [^{3}H]-labelled pyroglutamic acid is then coupled as a mixed anhydride with Fmoc-phenylalanine. Conditions suitable for the conversion of [^{3}H]-glutamine to [^{3}H]-pyroglutamic acid are also described. The [^{3}H]-labelled tripeptides are obtained in a high state of purity and with efficient incorporation of radiolabel.

Keywords: tritium labelling, TRH-like peptides, synthesis, asymmetric anhydrides.

INTRODUCTION

Recent studies have shown that many of the thyrotropin releasing hormone (TRH) immunoreactive peptides that occur in mammalian tissues (1,2) differ from TRH in that they possess a neutral or acidic residue in place of histidine (3,4). The 'TRH-like' peptides have been shown to be present in the male reproductive system and in certain regions of brain (5,6). In order to carry out further studies of these peptides, in particular to investigate the possible occurrence of specific receptors, we have synthesised a

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number of [^{3}H]-labelled tripeptides in which the histidine of TRH is replaced by a neutral or acidic amino acid and we present here a simple method for their synthesis using very small quantities of tritiated glutamine.

The synthesis of the isotopically labelled TRH analogues is carried out by the Fmoc procedure using a resin support which allows the formation of peptide amides (6) and the final step, coupling of the tritiated pyroglutamic acid, is carried out using a mixed anhydride derived from Fmoc-phenylalanine and tritiated pyroglutamic acid. We have also examined experimental conditions suitable for the conversion of [^{3}H]-glutamine to [^{3}H]-pyroglutamic acid.

MATERIALS AND METHODS

4-(2',4'-dimethoxyphenyl-Fmoc-aminomethyl)phenoxy resin(100-200 mesh, 0.3-0.6 mmol.g^{-1} substitution), N-α-Fmoc-proline-pentafluorophenylester (pfp), N-α-Fmoc-valine-pfp, N-α-Fmoc-phenylalanine-pfp, N-α-Fmoc-phenylalanine, 1-hydroxybenztriazole monohydrate (HOBt) and N,N'-dicyclohexylcarbodiimide (DCC) were obtained from Novabiochem Ltd. (UK); L-(G-^{3}H)glutamine (50 Ci.mmol^{-1}) from Amersham Int. (UK) and [^{3}H]-TRH were from New England Nuclear (U.S.A.). N,N-dimethylformamide (DMF) was from Aldrich Chemical Co. (UK); it was stored over 4A molecular sieve before use. Piperidine and pyroglutamic acid were from Sigma Chemical Co. (UK). High performance liquid chromatography (HPLC) was carried out on a Waters μBondapak C18 column (0.39 x 30 cm, dp 10μm, Waters Ltd.).

Conversion of [3H]-glutamine to [3H]-pyroglutamic acid

In order to determine the optimum pH for the conversion of [^{3}H]-glutamine to [^{3}H]-pyroglutamic acid, unlabelled glutamine was dissolved in 100mM sodium phosphate containing 2%

ethanol to a final concentration of 7.7mmole/l and the pH was adjusted to 4.2, 5.3, 6.3, 7.1, 8.0 or 8.9 by addition of hydrochloric acid. Incubations were carried out at 37°C for 20, 40, 60 or 100 h. At the end of each incubation period, aliquots of the mixtures were analysed by reverse phase HPLC. The amounts of pyroglutamic acid formed and unchanged glutamine remaining were determined by measurement of UV absorbance at 215 nm and the percentage conversion to pyroglutamic acid calculated on this basis.

The results showed that a suitable pH for conversion of glutamine to pyroglutamic acid is pH 5.3 (Figure 1). The procedure employed for the preparation of [^3H]-labelled pyroglutamic acid from [^3H]-glutamine was therefore as follows: 250 µCi of [^3H]-glutamine (5 nmole in 250 µl of 2% ethanol) was mixed with 10 µl of 100mM sodium phosphate at pH 5.3 and the mixture was incubated overnight at 37°C or for 4 h at 100°C.

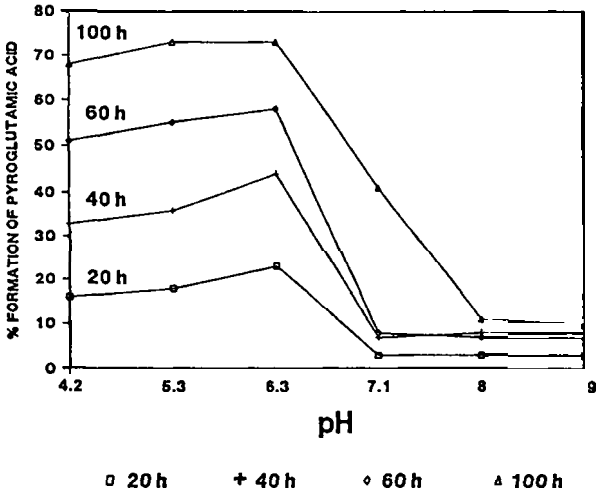

Figure 1: Conversion of glutamine to pyroglutamic acid. Incubations were performed at 37°C in 100mM sodium phosphate (adjusted to the required pH with HCl or NaOH) containing 2% ethanol. The % conversion to pyroglutamic acid was calculated after chromatography of an aliquot of the incubation mixture by reverse phase HPLC.

Synthesis

The resin dipeptides Fmoc-Phe-Pro-resin, Fmoc-Glu (t-butyl) Pro-resin and Fmoc-Val-Pro-resin were prepared on 4-(2',4'-dimethoxyphenyl-Fmoc-aminoethyl)phenoxy resin (Novabiochem) using Fmoc-amino acid pentafluorophenyl esters (7). The dipeptide resin (100mg, 42 µmole) was deblocked with 20% piperidine in DMF for 10 min. The resin was washed rapidly with DMF and dichloromethane and a freshly prepared solution of the mixed anhydride of Fmoc-phenylalanine and pyroglutamic acid was then added. Coupling was allowed to take place overnight at room temperature.

To prepare the asymmetric anhydride of Fmoc-phenylalanine and [^{3}H]-pyroglutamic acid, Fmoc-Phe (25µmole), [^{3}H]-pyroglutamic acid (5 nmole) and dicyclohexylcarbodiimide (DCC, 30 µmole) were dissolved in dichloromethane/DMF/9:1 (v/v) and the mixture allowed to stand at room temperature for 1 h. The dicyclohexyl urea formed was removed by filtration through glass wool in a pasteur pipette. The solution was evaporated to dryness and redissolved in DMF (1 ml).

The resin carrying the synthesised tripeptide was washed with DMF (x 6), resuspended in dichloromethane/TFA (98:2, 5 ml) and then was shaken for 2 h at room temperature to liberate the peptide amide from the resin. The resin was removed by filtration and the remaining peptide solution was evaporated to dryness. In the case of the glutamyl peptide, the t-butyl ester group was removed by dissolving in TFA/H_2O (95:5) and allowing to stand at room temperature for 2 h. After evaporation of the TFA, the residue was taken up in 10mM hydrochloric acid (0.9 ml) and the peptide was purified by chromatography on a Waters µBondapak C18 column. Elution was carried out in 10mM hydrochloric acid using a

methanol gradient (0.5% methanol min^{-1}) for 6 min, followed by 2% methanol min^{-1} for 30 min. Fractions (0.45 ml) were collected and the tritiated tripeptide was located by scintillation counting of 20µl aliquots.

Radioimmunoassay (RIA) was carried out using a sheep antiserum raised against synthetic TRH by Fraser (10). The final dilution of antiserum employed was 1/96,000. RIA was carried out according to the procedure described previously [5], except that separation of bound from free ligand was effected by the use of heat inactivated horse serum and 20% w/v of polyethylene glycol in place of activated charcoal.

RESULTS AND DISCUSSION

It has been reported previously that the cyclisation of glutamine to pyroglutamic acid is accelerated by the presence of phosphate and the reaction takes place maximally at pH 6.5 (8). In our experiments we observed that the cyclisation did not go to completion in 100 hr at 37°C. Furthermore our attempts to synthesise tritiated tripeptides using only 5 nmole of carrier free [^3H]-pyroglutamic acid by solid phase coupling were unsuccessful, since little or no incorporation of the tritiated pyroglutamic acid took place onto the resin peptide, possibly because of the difficulty of converting such a small quantity to the symmetrical anhydride.

We decided, therefore, to prepare a mixed anhydride of [^3H]-pyroglutamic acid with an unlabelled amino acid present in large excess. The choice of Fmoc-Phe for this purpose was dictated by the need to separate the phenylalanine containing tripeptide, formed as a by product, from the desired pyroglutamyl tripeptide. On reverse phase HPLC the aromaticity of the phenylalanyl peptide ensured that it was

strongly retained and this byproduct could be removed from the required pyroglutamyl tripeptide without difficulty. Under the conditions employed for HPLC of the reaction mixture, it was observed that the pyroglutamyl peptides eluted between 15 and 40% methanol (Figure 2) while the Fmoc-phenylalanyl peptides eluted at more than 50% methanol. Using the synthetic procedure described here, more than 90% of the tritiated pyroglutamic acid was incorporated into the tripeptide, indicating that coupling with the asymmetric anhydride proceeds almost entirely in the desired direction. RIA with TRH antibody showed that the tritium labelled peptides possessed the same degree of binding to the antibody as unlabelled tripeptides obtained by conventional resin synthesis using Fmoc protection.

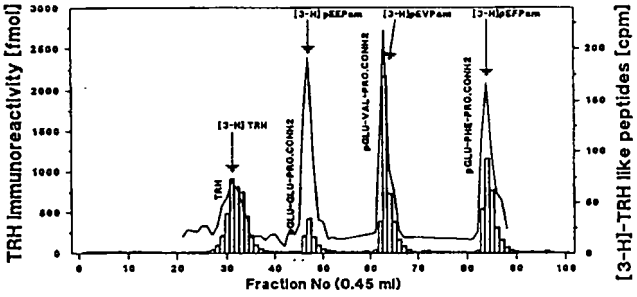

Figure 2. HPLC separation of TRH and TRH-like peptides. The separation of a mixture of peptides containing both tritiated and non-tritiated peptides was performed on a Waters μBondapak C18 column eluted with 10mM HCl using a methanol gradient of 0.5% min^{-1} for 6 min and then 2% min^{-1} for 30 min. Fractions (0.45 ml) were collected for RIA with a TRH antibody (solid bars) and radioactivity was determined by scintillation counting of 50μl aliquots of each fraction (continuous line).

The use of an asymmetric anhydride containing a suitable unlabelled amino acid as a separable carrier is thus seen to offer a simple and effective means of incorporating small quantities of radioactive labelled amino acids into a pep-

tide chain. The availability of tritiated peptides prepared by this method has allowed facile identification of the corresponding natural peptides which occur in mammalian tissues. In addition the tritiated analogues of TRH should prove valuable for in vitro receptor binding studies.

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