SYNTHESIS OF TWO S-(METHYL-^{3}H)-LABELLED ENKEPHALINS AND S-(METHYL-^{14}C)SUBSTANCE P

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SUMMARY

The synthesis of ^{3}H-labelled Met-enkephalin and Tyr-D-Ala-Gly-Phe-Met-NH_2 (DALA) and ^{14}C-labelled Substance P (SP) from previously described, fully protected intermediates is reported. The labelled peptides were prepared by methylation with (^{3}H)- or (^{14}C)methyl iodide of the sulphide anions formed on deprotection of the corresponding S-benzyl-homocysteine precursors with sodium in liquid ammonia. After purification by LC, the labelled peptides were obtained in radiochemical yields in the range of 9 to 24 % with a radiochemical purity higher than 97 %. The specific radioactivities of the ^{3}H- and ^{14}C-labelled products, corresponding to the labelled methyl iodides used, were 80 mCi/μmol and 60 μCi/-μmol, respectively.

Key words: S-(methyl-^{3}H)Tyr-Gly-Gly-Phe-Met, S-(methyl-^{3}H)Tyr-D-Ala-Gly-Phe-Met-NH_2, ^{3}H-labelled Met-enkephalin, ^{14}C-labelled Substance P, ^{3}H- and ^{14}C-labelled neuropeptides.

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INTRODUCTION

The radionuclide ^{3}H has been used extensively for labelling of peptides during the last three decades. Methods furnishing specifically labelled peptides have been developed.[1-3] Such methods as catalytic hydrogenation of olefinic bonds or hydrogenolysis of aromatic halogen compounds with ^{3}H-labelled hydrogen gas have been utilized, giving very low radiochemical yields. Besides, in some cases, the reactions have been shown to give non-specifically labelled products.[4,5]

The neuropeptides Met-enkephalin and Substance P have recently been labelled with ^{11}C[6,7] using an approach similar to that previously applied to the synthesis of S-[methyl-^{11}C]methionine.[8,9] Thus, methionine was substituted with S-benzyl-homocysteine in the protected precursor peptide. The corresponding homocysteine peptide anion was then generated by removal of the S-benzyl group with sodium in liquid ammonia and directly alkylated with labelled methyl iodide.

In this paper, the synthesis of two ^{3}H-labelled enkephalins, Tyr-Gly-Gly-Phe-Met (Met-enkephalin) and Tyr-D-Ala-Gly-Phe-Met-NH_2 (DALA), using (^{3}H)methyl iodide and essentially the procedure described previously, is reported. The synthesis of ^{14}C-labelled Substance P is also described. The labelled peptides were obtained in purities higher than 97 % and yields in the range of 9 to 24 %.

RESULTS AND DISCUSSION

The protected S-benzyl-homocysteine analogue of Met-enkephalin has recently been shown to be a suitable precursor for this neuropeptide. Using a twofold excess of methyl iodide, the unlabelled peptide was prepared in high yield without significant by-products.[9] This method has also been applied with success to the synthesis of ^{11}C-labelled Met-enkephalin,[6] and, with other suitable precursors, to the syntheses of three ^{11}C-labelled enkephalin analogues.[10] In continuation of the previous work, this method now has been applied to ^{3}H-labelling experiments.

The synthesis of the S-(methyl-^{3}H)labelled enkephalins proceeded in a high crude yield. However, problems were encountered in the purification step with a high loss of radioactivity, presumably to give tritiated water, as proposed by Stewart et al.[11] This loss was minimized after appropriate precautions had been taken, i.e., fast work-up of the crude product and storage of the purified labelled peptide in ethanol at -90 ^{o}C. The reported procedure then gave the labelled Met-enkephalin in 24 % yield and DALA in 10 % yield. Both peptides had a radiochemical purity higher than 97 %. Chromatograms of the crude and and purified products are shown in Figure 1.

The method discussed above has also been applied, with minor modifications, to the synthesis of ^{11}C-labelled Substance P.[7] The amino acid sequence of this peptide includes a Lys-Pro peptide bond. This bond is known to be particularly sensitive to the sodium/liquid ammonia reagent.[12] In the ^{11}C-labelling experiments, the C-terminal octapeptide was thus obtained as a major labelled by-product. Besides, when this method was used for synthesis of ^{3}H-labelled Substance P, the product turned out to be very sensitive to ^{3}H-^{1}H exchange during the purification procedures. Even when various recommended LC-conditions,[11] e.g., gradients of solutions of 0.005 % trifluoroacetic acid and 0.01 %

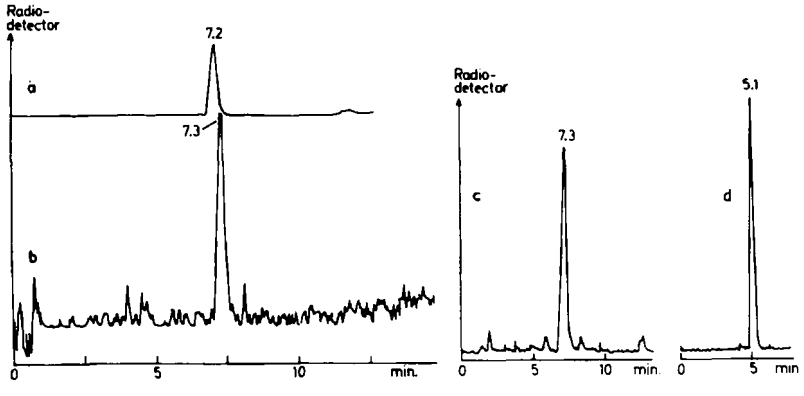


Figure 1: Chromatograms of crude (a) and purified (b) S-(methyl-^{3}H)-Met-enkephalin and crude (c) and purified (d) S-(methyl-^{3}H)DALA

mercaptoethanol in water and methanol or acetonitrile, were used, such exchange occurred when the LC fractions were evaporated and dissolved in absolute ethanol. The solution of the purified product thus contained 10 to 20 % of total radioactivity, which eluted in the void volume of the LC analysis, most likely corresponding to tritiated water.

When S-(methyl-^{14}C)Substance P was prepared using (^{14}C)methyl iodide and the same reaction conditions as in the ^{3}H-labelling of substance P, these by-products were not formed. This implies that ^{3}H-^{1}H exchange is important in the ^{3}H-synthesis and that the by-products are not formed by peptide degradation. Since the C-terminal octapeptide is of pharmacological interest, it was also collected during the LC purification. No radioactivity eluted in the void volume of the LC analyses of the purified octa- and undecapeptide. Small amounts of the corresponding labelled sulphoxides were detected. The ^{14}C-labelled Substance P was obtained in 97 % radiochemical purity and 9 % radiochemical yield. Chromatograms of the crude product (a) and the purified octa- (c) and undecapeptides (b) are shown in Figure 2.

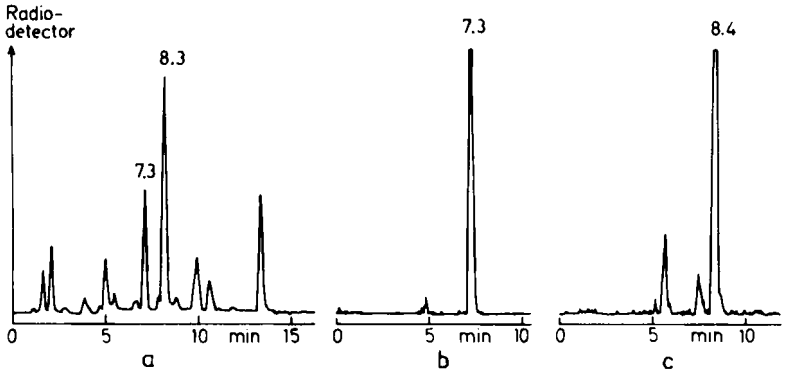
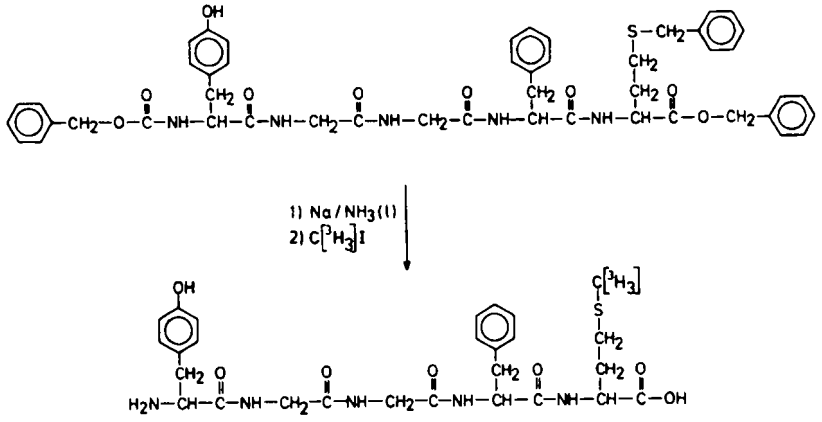


Figure 2. Analysis of crude product (a) and purified S-(methyl-^{14}C)SP (b) and SP_{4-11} (c).

EXPERIMENTAL

General: (^{3}H)Methyl iodide (specific activity 87 mCi/μmol) and (^{14}C)-methyl iodide (specific activity 60 μCi/μmol) were obtained from Amersham. Preparative and analytical LC of the labelled peptides was performed on an HP 1090 instrument equipped with a 250 x 4.6 mm RP-18 5 μm column and a diode-array detector in series with a Raytest-Ramona-LS scintillation detector.



Scheme 1

S-(methyl-^{3}H)Met-enkephalin (I): (Scheme 1). To a three-neck septum-equipped reaction vial, 0.8 mg of N-Z-Tyr-Gly-Gly-Phe-Hcy(Bzl)-OBzl[9] and 0.5 mg of sodium were added and ammonia, passed through a drying tower containing sodium hydroxide, was condensed to a volume of 0.5 mL. After addition of 10 μL (1.25 nmol) of a toluene solution containing 10 mCi/mL of (^{3}H)methyl iodide, another septum-equipped vessel containing 5 mL of a 10 % solution of pyridine in toluene was connected as an outlet to the reaction vial. The drying tower was simultaneously removed from the reaction vial and connected to the second vessel (Figure 3). Dry nitrogen gas was slowly bubbled through the solution for 30 min at -50 ^{o}C. The solvents were then removed by gentle heating and increasing the nitrogen gas flow. Constant attention was paid to the flow in the pyridine/toluene solution to prevent escape of this solution through the drying tower.

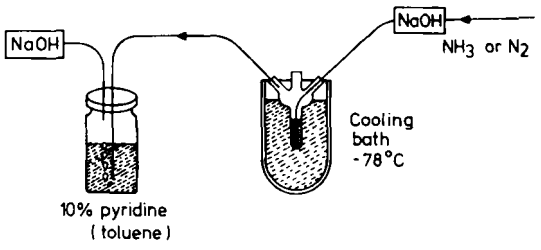


Figure 3. Experimental set-up used in the syntheses of ^{3}H- and ^{14}C-labelled peptides.

The solid residue obtained was dissolved in 300 µL MeOH/water (1/1, v/v); 220 µL of this solution was purified in 20 µL portions on the 1090 LC-system using solutions of 0.01 % mercaptoethanol and 0.005 % trifluoroacetic acid in water and in methanol/water (70/30, v/v, (A)) and the following program: Time 0-9, linear gradient %A 40-80; time 9-11, linear gradient to %A 95; time 11-13, %A 95; with a flow of 2.0 mL/min and a column temperature of 40 °C. Retention time of the product was 7.2 min (Figure 1). After evaporation, the purified product was dissolved in 3 mL of absolute EtOH with a trace of mercaptoethanol and stored at -90 °C under nitrogen, as recommended by Stewart et al.[11] Analysis of the purified product was performed using the conditions mentioned above in connection with the purification.

S-(methyl-^{3}H)Tyr-D-Ala-Gly-Phe-Met-NH_2 (II): N-Z-Tyr-D-Ala-Gly-Phe-Hcy(Bzl)-NH_2[10] (1.0 mg) and sodium (0.6 mg) were dissolved in liquid ammonia (0.5 mL) and the generated sulphide anion was reacted with (^{3}H)methyl iodide (300 µCi (3.75 nmol) in 30 µL of toluene) as described above. The purification procedures were also the same as in the synthesis of I. Retention time of the product was 7.3 min. Analysis of the purified product was performed with the system described above and an isocratic mixture of 70 % A, with a flow of 1.85 mL/min (Figure 1).

S-(methyl-^{14}C)Substance P (III) and S-(methyl-^{14}C)Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2 (IV): N-Z-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Hcy(Bzl)-NH_2 [7] (4.6 mg) was deprotected with sodium (2.2 mg) in liquid ammonia (0.5 mL) and the generated sulphide anion reacted with (^{14}C)methyl iodide (10 µCi (0.17 µmol) in 50 µL of toluene) as described above. The solid crude product obtained on removal of the solvents was dissolved in 0.5 mL of acetic acid (aqueous, 20 %) and purified in 150 µL portions on the 1090 LC-system using a gradient of aqueous sodium dihydrogen phosphate (0.1 M, pH 3) and acetonitrile (B): time 0-10, %B 17.5-35; time 10-12, %B 35-56; time 12-14, %B 56, with a flow of 2 mL/min and UV-detection at 210 nm. Retention times for III and IV were 7.3 and 8.3 min respectively (Figure 2). After evaporation of the appropriate LC fractions, each product was dissolved in acetic acid (20 %, 2.5 mL) containing 0.1 % (v/v) of mercaptoethanol and stored at -20 °C.

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