SYNTHESIS OF ^{3}H-D-ALA^{2}-LEU-ENKEPHALIN-CHLOROMETHYL KETONE OF HIGH SPECIFIC ACTIVITY

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SUMMARY

The enkephalin affinity reagent /^{3}H/-Tyr-D-Ala-Gly--Phe-Leu-CH_2Cl //^{3}H/-DALECK/ was synthesized by fragment condensation. /^{3}H/-Boc-Tyr-D-Ala-Gly was prepared by catalytic tritiation of protected, iodinated tripeptide. The protected tritiated tripeptide and the Phe-Leu-CH_2Cl dipeptide were condensed by the mixed anhydride method. The protecting group was removed by HCl-methanol. The /^{3}H/-DALECK had a specific activity of 34,2 Ci/mmole /1,27 TBq/mmole/.

Key words: affinity reagent, tritium labelling, enkephalin chloromethyl ketone.

INTRODUCTION

The chloromethyl ketone derivatives of amino acids and peptides are suitable for the identification of the functional groups of proteolytic enzymes /1/. Upon binding to the respective proteins these compounds can bind covalently

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under specific reaction conditions. Since the binding is irreversible, the identification, isolation and characterization of the receptor molecules can be facilitated.

Enkephalin chloromethyl ketone derivatives have been developed for the study of opiate receptors. These derivatives were able to bind in vitro to these receptors with high affinity /2,3,4,5/. The irreversible labelling of these receptors was demonstrated by using radiolabelled /^{3}H/-DALECK /6/. In this paper we describe in detail the synthesis of this radiolabelled compound.

Labelling of peptides with tritium can usually be accomplished by reacting a suitable precursor peptide with tritium gas /7,8/. Peptides containing tyrosine residue/s/ are initially iodinated and the iodine is then exchanged with tritium by catalytic dehalogenation. This procedure was therefore chosen for the tritiation of the DALECK. The synthesis route followed is shown in Figure 1.

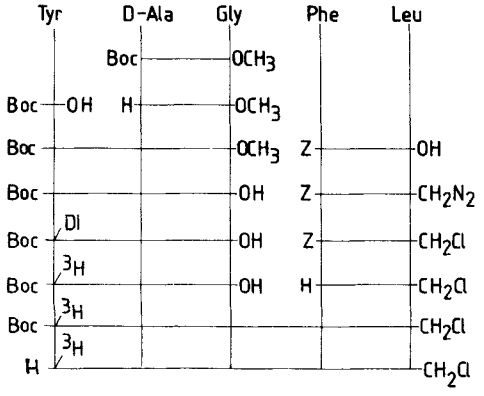

Figure 1.
Synthesis route of ^{3}H-DALECK

A similar method was described by E.L. Newman and E.A. Barnard /9/ but we modified and scaled it down about 200-fold. The specific activity of their product was

2,64 Ci/mmole /97,7 GBq/mmole/ more than 10 times less than ours.

MATERIALS AND METHODS

Tritium gas /Technabexport, USSR/ was stored in the form of uranium tritide. PdO catalyst and DC-Fertigplatten Kieselgel 60 thin layer chromatographic /TLC/ sheets were from Merck. N-methylmorpholine and chloroformic acid isobutylester were purchared from Aldrich. Nonradioactive Boc-Tyr-D-Ala-Gly-Phe-Leu-CH_2Cl and Leu-enkephalin chloromethyl ketone were synthesized according to Husztiné-Szécsi et al /4/.

Chemical concentrations of the radioactive peptides were determined by UV spectroscopy /Zeiss UV VIS spectrophotometer/, using the standard solutions of nonradioactive peptides as references. Radioactivity was measured in a Liquidfluor-Triton X-100-toluene scintillation coctail using a Searle Delta 300 instrument. The distribution of radioactivity on the TLC plates was assessed by a Packard Model 7201 Radiochromatogram Scanner. For autoradiography Medifort-RP films were used.

The purity of the radioactive DALECK was controlled by HPLC /Waters apparatus, μ-Bondapack C_{18} coloumn, 1,2 ml/min., eluent: 50 % methanol in 1 per cent trifluoracetic acid. Retention time was 13,7 min./.

Hydrogenation and tritiation were performed in a glass-vacuum apparatus as described earlier /7/.

EXPERIMENTAL

1./ Phenylalanyl-leucyl-chloromethylyketone

The compound was synthesized according to Huszthyné-Szécsi

/4/ from Z-Phe-Leu-OH by first preparing the respective di--azo-ketone, treating it with dry HCl gas to yield the chloromethyl ketone and removing the protecting group by HBr--acetic-acid

2./ t-Butyloxycarbonyl-tyrosyl-D-alanyl-glycine

Prepared by the method of Huszthyné-Szécsi /4/ following the strategy outlined in Figure 1.

3./ t-Butyloxycarbonyl-3,5-diiodotyrosyl-D-alanyl-glycine

1 g /2,44 mmole/ of Boc-Tyr-D-Ala-Gly-OH tripeptide-acid was dissolved in a 30 ml H_2O + 20 ml 1 M $NaHCO_3$ mixture. To this solution 0,97 g /5,85 mmole/ KI in water solution was added. 1,2 g /5,85 mmole/ chloramine-T in 10 ml water was added dropwise in 10 min and the solution was stirred at room temperature for 1 hour. Following ether extraction and acidification by acetic acid to pH 6, the mixture was stored at $4^{o}C$ overnight. The precipitated material was filtered, dissolved in ethyl acetate, washed with water and 30 % NaCl solution and dried over Na_2SO_4. After drying the residue was recrystalized from ether. TLC: ethyl acetate, pyridine, acetic acid, water 120:20:6:11. R_f= 0,58. Yield: 1,1 g /68 %/.

4./ ^{3}H-t-Butyloxycarbonyl-tyrosyl-D-alanyl-glycine

3 mg /4,51 µmole/ of Boc-3,5-diiodo-tyrosyl-D-alanyl-glycine was dissolved in 1 ml of dimethylformamide and 1,3 µl triethylamine and 5,6 mg PdO catalyst was added. The reaction vessel was connected to the tritiating apparatus and the solution was frozen in liquid nitrogen. After removal of air from the apparatus by vacuum, tritium /about 15 Ci /555 GBq// was expanded into the reaction vessel. The solid was melted and stirred magnetically at room temperature

for two hours. The reaction mixture was frozen again and unreacted tritium was absorbed onto pyrophoric uranium. After melting, the catalyst was filtered through a Whatman GF/C filter, washed with methanol and concentrated. Labile tritium was removed from the residue by vacuum distillation of 3 x 15 ml water. The radioactivity of the crude product was 180 mCi /6,6 GBq/. Purity was checked by thin layer chromatography using the solvent systems chloroform-methanol-acetic acid /80:10:5/, pyridine-acetic acid-water--ethyl acetate /6,66:2:3,66:80/, cyclohexane ethyl acetate--methanol /3:1:0,5/. In all of these systems approx. 95 % of the radioactivity was found to chromatograph with the same R_f value as the standard, nonradioactive peptide. The monoiodinated precursor is the only possible contaminant.

5./ ^{3}H-t-Butyloxycarbonyl-tyrosyl-D-alanyl-glycyl-phenylalanyl-leucyl-chloromethyl ketone /^{3}H-Boc-DALECK/

90 mCi /3,33 GBq/ ^{3}H-Boc-Tyr-D-Ala-Gly-OH crude product was dissolved in 200 µl tetrahydrofuran /THF/. 0,62 µl /5,66 µmole/ N-methylmorpholine and 0,76 µl /5,66 µmole/ isobutyl chloroformate was added to the solution and the vessel was cooled to - 15°C. 2 mg /5,76 µmole/ HCl · H--Phe-Leu-CH_2Cl was dissolved in 200 µl THF and 0,64 µl /5,76 µmole/ N-methylmorpholine was added and cooled to - 15°C. This solution was mixed with the earlier prepared tripeptide solution and allowed to stand at -5°C - 0°C for 1 1/2 hours. The composition of the crude product was assayed by TLC as described above. More, than 90 per cent of the radioactivity co-migrated with the standard, nonradioactive Boc-DALECK. The ethyl acetate solution of the crude product was chromatographed in chloroform-methanol-acetic acid /80:10:5/ on 20 x 20 cm DC-Fertigplatten Kieselgel.

After mechanically removing the radioactive area corresponding to Boc-DALECK, the tritiated product was extracted from the silica with ethanol. Total radioactivity: 36,5 mCi /1,35 GBq/; specific radioactivity: 34,7 Ci/mmole /1,29 TBq/mmole/.

6./ ^{3}H-tyrosyl-D-alanyl-glycyl-phenylalanyl-leucyl-chloromethyl ketone /^{3}H-DALECK/

36,5 mCi /1,35 GBq/ ^{3}H-Boc-DALECK was dissolved in 0,2 ml methanol and allowed to react with 0,8 ml 1,8 N HCl in methanol for 45 min at room temperature. After concentration in vacuum the remaining HCl was removed under vacuum using additional /3 x 2 ml/ methanol. A minimal amount / 5 %/ of protected peptide could be detected. The residue was dissolved in 0,5 ml ethanol and purified by silica gel TLC using chloroform-methanol /90:24/ as the developing system. The tritiated product was extracted from the silica by 4 x 2 ml ethanol. Purity of the end product was checked in 2 TLC systems /chloroform-methanol 90:24; ethyl acetate--pyridine-acetic acid-H_2O 60:20:6:11/ and by HPLC as described in Materials and Methods. These assays have demonstrated the purity of these compounds. The total radioactivity was 22,7 mCi /0,84 GBq/. The chemical concentration /0,67 ,umole/ was determined using a standard curve based on the UV spectrum. Specific radioactivity: 34,2 Ci/mmole /1,27 TBq/mmole/.

DISCUSSION

The synthesis of /^{3}H/-DALECK required a number of modifications of the procedure previously used for the nonradioactive analogue. If the isobutyl chloroformate and the protected tripeptide were mixed at 1:1 molar ratio at the

2 /umole scale, the coupling took place at only 30 per cent. However if isobutyl chloroformate, was applied in two-fold molar excess the coupling was complete and the product proved to be homogeneous as shown by chromatography. This also means that the self-alkylating capacity of the excess chloromethyl dipeptide in negligible as compared to the coupling of dipeptide and tripeptide /4/.

After removing the protecting group extreme care should be taken in storing the tritiated DALECK. Storage of DALECK was best accomplished in H_2O-alcohol 1:1. Radioactive samples at 1 mCi/ml /37MBq/ml/ concentration were stored in liquid nitrogen. After one year of storage /^{3}H/-DALECK was of 90 % purity. Decomposition during storage can also be diminished by storing /^{3}H/-Boc-DALECK because of higher stability and the protecting group is removed just prior to use.
Purification prior to the biological assays was performed by TLC as described above.

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