Journal of Labelled Compounds and Radiopharmaceuticals—Vol. XXIV, No. 4

SYNTHESIS OF TRITIUM LABELED O-ETHYL-D-TYROSINE AND PHENYLALANINE

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Summary: Two amino acids, t-BOC-O-ethyl-D-tyrosine and t-BOC-phenylalanine, have been synthesized in tritium labeled form via exchange with tritium gas over catalyst on an unlabeled precursor and dehydrohalogenation on an iodinated precursor respectively. The tyrosine derivative has been obtained in specific activities of 4 - 7 Ci/mmol; tritium NMR indicates that at least 80% of the tritium resides in the benzylic position of the molecule. The phenylalanine has been synthesized with a specific activity of greater than 35 Ci/mmol. Tritium NMR confirms specific tritium/iodine replacement in the position.

Key Words: Tritium, Phenylalanine, Tyrosine

INTRODUCTION

Biological and pharmaceutical research in biotechnology has grown rapidly in the last few years. One area which has emerged from this research has been the development of drugs based on endogenous peptides. As a direct result, the demand for high specific activity radiolabeled peptides will continue to increase. One particularly effective means for the specfic introduction of a label is through the direct incorporation into the peptide of a specifically labeled amino acid. Our work on the synthesis of the cyclic octapeptide, SK&F 101926 (1),

0362-4803/87/040389–08$05.00

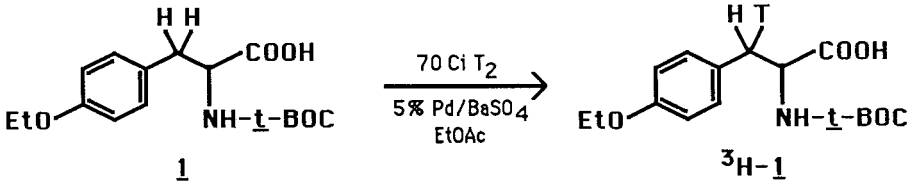
Received July 24, 1986
Revised September 10, 1986

necessitated the synthesis of two tritium labeled amino acids, t-BOC-O-ethyl-D-tyrosine and t-BOC-phenylalanine.

RESULTS AND DISCUSSION

LABELING OF t-BOC-O-ETHYL-D-TYROSINE. The synthesis of tritiated t-BOC-O-ethyl-D-tyrosine (^{3}H-1, SK&F D-101711) was carried out by exposing the amino acid to an excess of tritium gas over 100 weight percent catalyst as shown in Scheme I. Yields of the tritiated amino acid

Scheme I



are greater than 1 Ci with a specific activity of 4 - 7 Ci/mmol. Radiochemical purities of the crude amino acid are 80% or better. The remaining 20% consists of material which has been deprotected along with a small amount of unidentified nonpolar impurity.

Care must be taken to avoid any traces of acid either in solvent or in the glassware during the labeling due to the lability of the t-BOC protecting group. Otherwise complete deprotection can occur rapidly, necessitating an additional reprotection step with di-t-butyldicarbonate.

This exchange labeling of an amino acid is based on a method developed by Evans (3) for the labeling of molecules containing benzylic protons. Evans was able to label L-phenylalanine in the benzylic position (>92%) at pH 7 using excess PdO/$BaSO_4$ without racemization. Our studies with deuterium labeling of t-BOC-O-ethyl-D-tyrosine employing these and similar conditions based on Evans' work are summarized in Table 1. The data clearly show that the catalyst of choice is 5% Pd/$BaSO_4$ in ethyl acetate where deuterium incorporation of 15 to 23% is observed by NMR and mass spectrum analysis respectively. The predicted specific activity

Table 1. Exchange labeling of t-BOC O-Ethyl D-Tyrosine with deuterium.

catalyst	solvent	%D-NMR	%D-Mass Spectrum
5% $Pd/BaSO_4$	EtOAc	15%	23%
5%$Pd/CaCO_3$	EtOAc	11%	13%
5% $Pd/BaSO_4$	0.1M NaH_2PO_4[a]	8%	ND[b]

All reactions were carried out with 25 mg amino acid and 25 mg catalyst in 2.5 mL solvent for 18 hours.

a. A few drops of THF were added to solubilize the sample.

b. Not determined.

is therefore 4.4 - 6.7 Ci/mmol, which is precisely what is observed.

Tritium NMR (Figure 1) of the crude material obtained directly from the exchange indicates that about 80% of the tritium label resides in the benzylic position; no tritium is detected on the alpha carbon. Thus, within our limits of detection, epimerization is not observed. It is interesting to note that exchange of the benzylic protons is not equal; in fact a 1.6:1 tritium ratio is observed in the nonequivalent benzylic protons. The Newman projection shown in Figure 1 indicates a likely cause: one proton is more sterically hindered (due to COOH and NH-t-BOC gauche interactions) than is the other proton (H and NH-t-BOC gauche interactions). Thus, one would predict the less hindered proton to undergo tritium exchange more readily leading to the observed absorbance difference.

We adopted this exchange labeling approach because previously reported syntheses of tritiated tyrosine give low specific activity (4). Our first approach called for the halogenation of the O-ethyl tyrosine aromatic ring followed by tritium introduction via dehydrohalogenation. However, all attempts at iodinating the aromatic ring (ICl, NaI/H_2O_2, NaI/chloramine-T) failed, perhaps due to steric hindrance caused by the O-ethyl group. Alternatively, tyrosine itself could be iodinated.

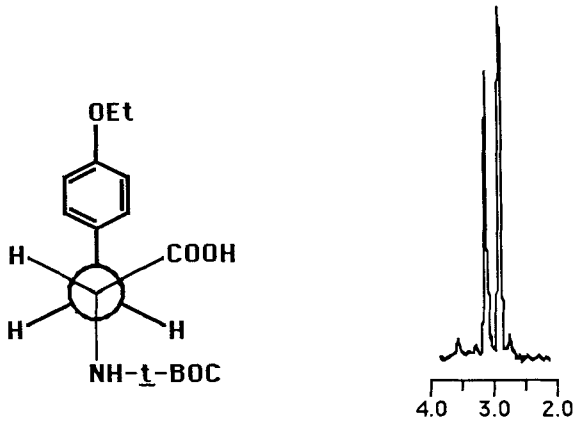
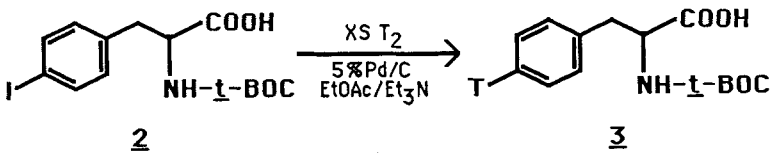


Figure 1. Newman projection of t-BOC-O-ethyl-D-tyrosine and its ^{3}H-NMR showing unequal benzylic tritium absorbances.

Unfortunately, the phenol on this iodinated tyrosine now could not be ethylated, probably due to steric hindrance, this time caused by the iodine. Thus, while it should be possible to sequentially iodinate tyrosine, tritiate via dehydrohalogenation and then ethylate the tritated tyrosine, in practice, the benzylic exchange labeling method proved to be much more direct.

LABELING OF t-BOC L-PHENYLALANINE. t-BOC-L-phenylalanine (3) was tritiated exclusively in the para position as shown in Scheme II via dehydrohalogenation (5) on t-BOC-para-iodo-L-phenylalanine (2). This is similar to the method used by Kovacs for the preparation of unprotected tritiated L-phenylalanine (6). Tritiation was performed on a one millimole scale with 40 weight percent 5% Pd/C in 3:1 ethyl acetate/triethylamine with 300 Ci of tritium gas. After 5 hours the labile tritium was removed, the residue reconstituted in ethyl

Scheme II



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acetate and extracted with 0.5M potassium hydrogen sulfate. The organic layer afforded ca. 20 Ci of product in greater than 98% radiochemical purity (by HPLC) and with a specific activity of 35.7 Ci/mmole. Tritium NMR shows tritium only in the para position of the aromatic ring. However, the signal-noise ratio is low. Since the specific activity is slightly greater than theoretical, a small amount of the label probably resides in the benzylic position. This is in agreement with the observations of Evans (2).

Deuterium model studies on the iodinated precursor indicate that the optimum catalyst is 5% Pd/C (versus 5% Pt/C or 5% $Pd/BaSO_4$). The reaction proceeds to 80% completion in ethyl acetate after 45 minutes while in acetone or THF the reaction is essentially complete. However, an acceptable rate is achieved only when 25% (by volume) triethylamine is added to the solvent. The use of less than this amount of base slows the reaction dramatically, presumably due to extensive catalyst poisoning by hydroiodic acid. Optimum concentration was found to be 80 - 100 mg/mL.

CONCLUSION

Two syntheses have been developed suitable for the labeling of t-BOC protected amino acids in moderate to high specific activity. Exchange labeling into the benzylic position of suitable amino acids in particular is attractive since no effort is required for obtaining the appropriate starting material and the product is obtained in high purity and moderate specific activity.

EXPERIMENTAL

Tritiation was performed by Chemsyn Science Laboratories (Lenexa, KS), Amersham Corporation (Arlington Heights, IL) or was carried out at the Lawrence Berkeley Laboratory National Tritium Labeling Facility (Berkeley, CA). Tritium NMR was performed at New England Nuclear (for ^{3}H-1) and Lawrence Berkeley Laboratory (for 3) on a Bruker

AM 200. The t-BOC-p-iodophenylalanine was purchased from Bachem (Torrence, CA). Catalysts used were obtained from Engelhard (Newark, NJ). Di-t-butyldicarbonate was purchased from Aldrich Chemicals (Milwaukee, WI). All reagents were of analytical reagent grade or better. Ethyl acetate was dried over 4Å molecular sieves and filtered prior to use.

t-BOC-O-Ethyl-[3-^{3}H]-D-Tyrosine (^{3}H-1). A 20 mL round bottom flask was charged with 100 mg 5% Pd on $BaSO_4$ and the flask plus catalyst dried at 56° for 2 days at reduced pressure (ca. 0.1 mm). To this, at room temperature, was added 100 mg t-BOC-O-ethyl-D-tyrosine (1, 0.32 mmol) in 10 mL dry ethyl acetate. The solution was frozen in liquid nitrogen and evacuated. The solution was flushed and evacuated 2 times with nitrogen. To complete degassing the solution was warmed to room temperature and the freezing procedure repeated. To the frozen solution was added 70 Ci tritium gas. The mixture was warmed to room temperature and the pressure adjusted down to 740 mm by opening the system to a vacuum line. The reaction mixture was stirred 22 hours at room temperature. The solution was then refrozen in liquid nitrogen, the flask evacuated and refilled with nitrogen. This was repeated 2 times. The solution was concentrated to ca. 5 mL in vacuo. Labile tritium was removed by addition of EtOH, reconcentration to 5 mL and another addition of EtOH. The solution was not taken to dryness. The reaction was filtered and the flask rinsed 2 times with EtOH. The product was then lyophilized, reconstituted in EtOH and stored at -80° until use. Yields of labeled product are greater than 1 Ci at a specific activity of 4-7 Ci/mmol (based on results of 4 separate preparations). Analysis by TLC/radiochromatogram scanning indicates the crude purity to be 80% (silica gel 60, CH_2Cl_2 /MeOH 84:16, R_f =0.57), the remaining 20% is material which has been deprotected. The product may also be analyzed by HPLC (Waters Nova-Pak C-18, 48:48:4 H_2O/CH_3CN/HOAc, 1 mL/min, UV

at 275 nm, retention time = 4.3 min). The compound could be purified by preparative TLC. However, this was not necessary in our peptide syntheses (1).

Reprotection of O-Ethyl-D-Tyrosine. A 50 mCi portion of O-ethyl-[3-^{3}H]-D-tyrosine in EtOH was taken to dryness in vacuo. The amino acid was reprotected by dissolving the residue in 30 µL 1N NaOH and 0.67 mL H_2O. To this was added 25 µL di-t-butyldicarbonate and the mixture stirred one hour at room temperature. The mixture was then extracted with ethyl acetate and the remaining aqueous layer acidified with 40 µL 1N $KHSO_4$ giving a white precipitate. This was extracted with ethyl acetate and the organic layer dried (Na_2SO_4). The organic layer was taken to dryness in vacuo and dissolved in 1 mL anhydrous EtOH giving 33.9 mCi (68.7% radiochemical yield) of t-BOC-O-ethyl-[3-^{3}H]-D-tyrosine (^{3}H-1). TLC of the product shows it to be 95% radiochemically pure (silica gel 60, as above).

t-BOC-[4-^{3}H]-L-Phenylalanine (3). t-BOC-p-iodophenylalanine (2, 400 mg,1 mmol) was dissolved in 5 mL 25% triethylamine in ethyl acetate. To this was added 160 mg (40 weight percent) 5% Pd/C (oven-dried 18 hours). The solution was frozen in liquid nitrogen, evacuated and filled with nitrogen. This was repeated 2 times. To complete degassing, the solution was warmed to room temperature and the freezing procedure repeated. To the frozen solution was added 300 Ci tritium gas. The solution was warmed to room temperature and the pressure adjusted down to 730 mm via opening the system to a vacuum line. Uptake of tritium was rapid (pressure drop of 195 mm in 5 hours, 38 Ci). Exchangeable tritium was removed by washing with MeOH and vacuum transfer of solvent. The residue was reconstituted in 10 mL ethyl acetate and the catalyst removed via filtration. The ethyl acetate solution containing the crude tritiated product was extracted twice with 0.5M $KHSO_4$ (pH 1.4). The organic layer, containing 20 Ci of tritiated product, was diluted to a total volume of 100 mL with EtOH and

stored at -78° . Radiochemical purity was determined to be 98.6% by HPLC (Waters μ-Bondapak C-18, 59:40:1 MeOH/H_2O/HOAc, 1.5 mL/min, UV at 215 nm) and TLC (silica gel, 95:5 $CHCl_3$/HOAc). Reassay of the product by the TLC method after 14 days showed no decomposition (ie. only one peak).

ACKNOWLEDGEMENTS

We would like to thank Dr. Hiromi Morimoto of the National Tritium Labeling Facility for his help is carrying out the tritiations described. We would also like to thank Dr. Wil Kokke and Mr. Sid Levinson for their assistance in developing analytical procedures for the analysis of the phenylalanine.

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