Journal of Labelled Compounds and Radiopharmaceuticals
J. Labelled Cpd. Radiopharm. **44**, 295-304 (2001)
DOI: 10.1002/jlcr.461

SYNTHESIS OF TRITIUM LABELED [3R-^{3}H]-, AND [3S-^{3}H]-L-PHENYLALANINE

J. Jemielity, R. Kanski and M. Kanska*
Department of Chemistry, Warsaw University,
Pasteur 1 Street., 02-089 Warsaw, Poland

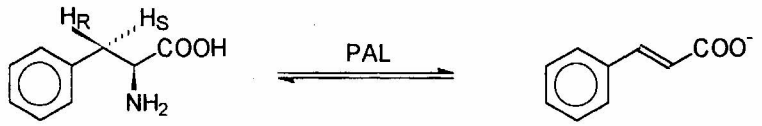
SUMMARY

The synthesis of two selectively labeled isotopomers of L-phenylalanine, (Phe), using chemical and enzymatic methods is reported. The [3R-^{3}H]-L-Phe isotopomer has been obtained from [3-^{3}H]cinnamic acid prepared from benzaldehyde and malonic acid using tritiated water as a source of radioactive label, and by addition of ammonia in the presence of enzyme PAL. The [3S-^{3}H]-L-Phe isotopomer has been synthesized by addition of ammonia to cinnamic acid in a buffered medium containing PAL and tritiated water.

Key words: tritium, labeling, optical isotopomer, cinnamic acid, phenylalanine, enzyme

INTRODUCTION

Enzyme phenylalanine ammonia lyase, PAL, EC 4.3.5.1, catalyses the elimination of ammonia and *pro-3S*-hydrogen from L-phenylalanine, L-Phe, leading to formation of (E)-cinnamate[1-5] according to Scheme 1:



Scheme 1

*To whom correspondence should be addressed:
(e.mail: mkanska@alfa.chem.uw.edu.pl)

Received 10 October 2000
Revised 4 December 2000
Accepted 8 January 2001

The above multistep reaction involves several intermediates, and therefore it is important to determine the structure of the active complex formed in the rate determining step. There is a rich literature on the enzyme PAL including the structure of the active center and its inhibitors, but up to now the mechanism of this reaction is still unclear[6-9]. The number of questions that arise can be minimized by determining kinetic isotope effects, KIE, of tritium in the 3R and 3S positions of L-Phe. Aforementioned studies require the use of the optically active form of L-Phe labeled with tritium in the 3R and 3S positions. The introduction of deuterium or tritium label in such specific positions by chemical methods is very tedious and sometimes even impossible. The experimental procedures described in the literature used for the preparation of isotopically labeled enantiomers of Phe [10-14] yielded products uniformly labeled or labeled specifically in unimportant positions. In the earlier reported studies on the synthesis of labeled L-Phe[15-19] the enzyme PAL was used to establish the stereochemical configuration of isotopic hydrogen in the side chain of the amino acid. This paper reports on the enzymatic synthesis of (3R)- and (3S)-tritium labeled isotopomers of L-Phe needed for KIE assays. We decided to utilize specific properties of the enzyme PAL for this synthesis. This enzyme catalyzes the elimination of ammonia from the *pro-S* position of L-Phe and leads, in the final step, to cinnamic acid, and this reaction has been well studied[20-21]. PAL, under proper conditions, also catalyzes the reverse reaction, that is addition of ammonia to (E)cinnamic acid resulting in formation of L-Phe.

RESULTS AND DISCUSSION

L-Phe undergoes smooth deamination catalyzed by PAL under mild conditions (equilibrium of this enzymatic elimination of ammonia in buffered conditions at pH 8.8 is already reached after 30-40 min at 30 °C) while the reverse reaction, i.e., the addition of ammonia to cinnamic acid does not proceed so readily. Therefore, certain preliminary kinetic studies had to be carried out to find the best reaction conditions for obtaining a high yield of the desired labeled product. We have chosen the UV-spectrometric technique for monitoring the progress of the addition reaction. By observing the absorbance of the (E)-cinnamate at 290 nm we have studied the

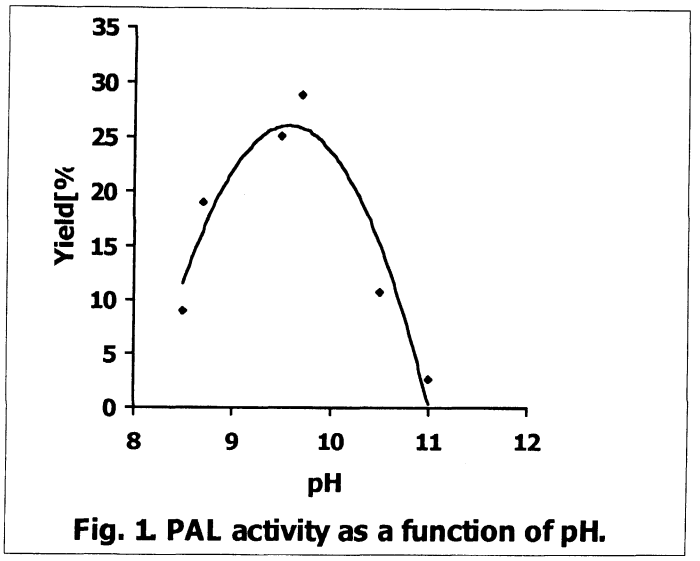


Fig. 1. PAL activity as a function of pH.

dependence of reaction yield (at 6 h reaction time, 30 °C) on the pH in the 8.5 - 11 range and found that the highest yield of product, i. e., L-Phe is achieved at pH 9.7 (Fig.1). This is characteristic of many enzymatic reactions.

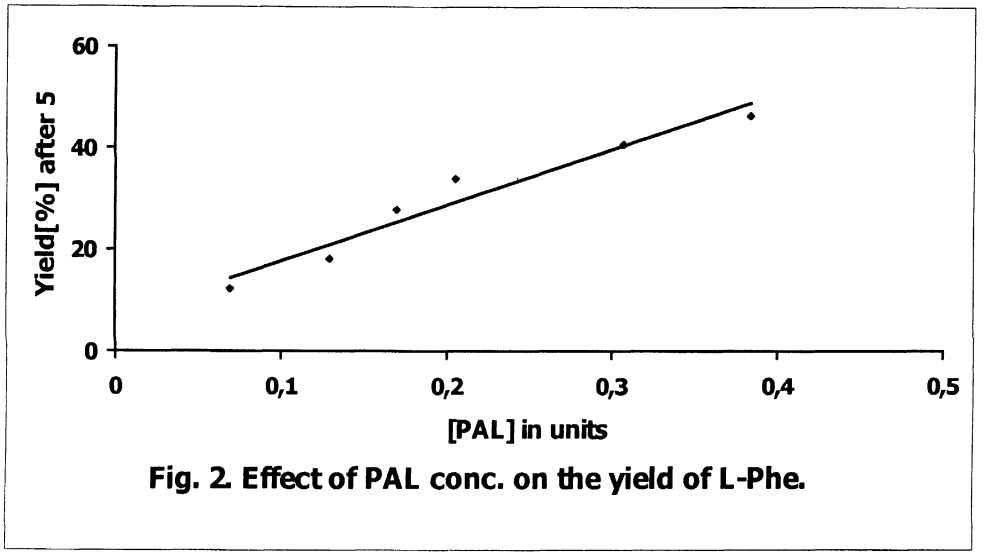


Fig. 2. Effect of PAL conc. on the yield of L-Phe.

Finally a linear dependence of the reaction yield on the enzyme concentration was observed between the 0.07 – 0.4 (enzyme unit) interval, which is in agreement with

the central role played by the enzyme in the catalytic addition, involving the formation of an enzyme-substrate complex PAL (Fig 2).

The virtually constant yield of L-Phe in the reversible addition reaction of ammonia to E-cinnamate at pH 10, as measured by detecting the decrease of the absorbance at 290 nm, was reached after 5 h at 30 °C. The influence of concentration of ammonia buffer on the yield and reaction time dependence has also been studied in the 0.8 – 3.3 M range. The addition rate decreased with an increase in the concentration of the ammonia buffer, but the final yield of L-Phe was highest for the 3.2 M ammonia buffer; here the equilibrium was not reached even after 7 h as it did in the case of 0.8 and 1.6 M buffers (Fig. 3). (At high concentration of ammonia the equilibrium is shifted to the right, i.e. to the product, L-Phe.).

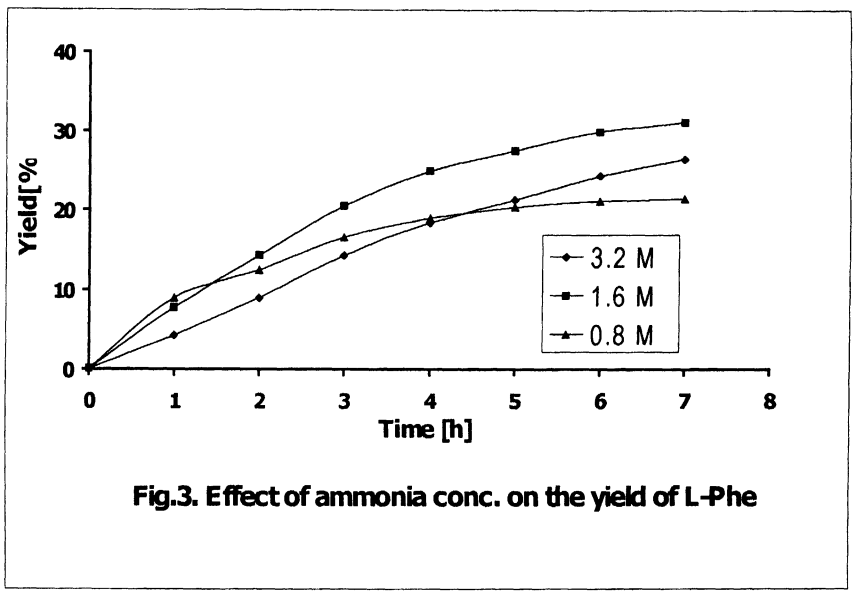
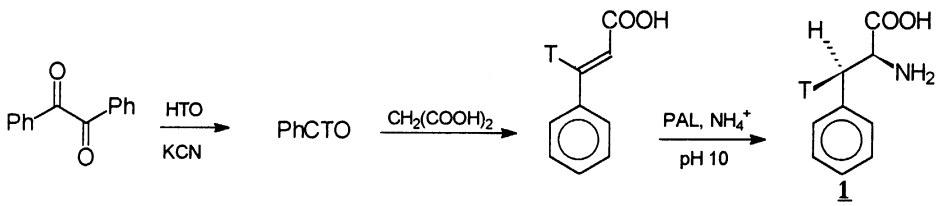


Fig.3. Effect of ammonia conc. on the yield of L-Phe

Thus, for this key reaction step the optimum parameters (pH, temp., reaction time, concentration of substrate, buffer solutions and enzyme) have been elaborated to achieve the highest possible yield of desired product.

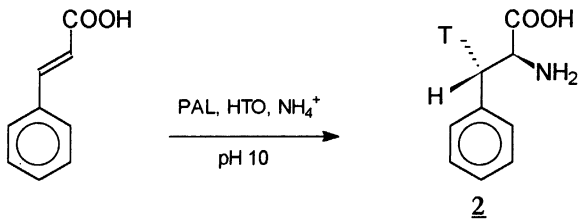
The synthesis of [3R-^{3}H]-L-Phe, **1**, has been carried out according to Scheme 2.



Scheme 2. Synthesis of [3R-^{3}H]-L-Phe.

The compound, **1**, was obtained by combining chemical and enzymatic methods. In the first step benzaldehyde labeled with tritium in the formyl group, PhCTO, was prepared. There are several synthetic protocols for obtaining the benzaldehyde, however, most of them require expensive reagents and long reaction time. The method chosen by us involved the steps in which benzil, $(PhCO)_2$, was cleaved by cyanide ion in tritiated water, HTO. The tritiated benzaldehyde was condensed with malonic acid in pyridine solvent using catalytic amounts of piperidine[22]. Addition of ammonia catalyzed by PAL to the [3-^{3}H]-cinnamic acid[23] leads to **1**.

The synthesis of [3S-^{3}H]-L-Phe, **2**, was performed according to scheme 3.



Scheme 3. Synthesis of [3-^{3}H] L-Phe

Addition of ammonia and the enzyme PAL to cinnamic acid was carried out in the buffer containing tritiated water, HTO, leading to formation of [3S- ^{3}H]L-Phe, **2.** The purity of the chiral compounds **1** and **2** was checked by TLC and an enzymatic method.

The presence of tritium in the 3R position of **1** was confirmed by enzymatically transforming **1** into [3-^{3}H]cinnamic acid employing the activity of the enzyme PAL (Scheme 1). The obtained [3-^{3}H]cinnamic acid was separated, purified and

radioassayed. The molar activity of the tritium labeled cinnamic acid was the same as the molar activity of **1** used in the elimination reaction.

The same elimination reaction has been used to identify the presence of tritium in the 3S position of **2**. The obtained cinnamic acid did not contain tritium. Its radioactivity, as measured with a liquid scintillation counter, was at the background level. All the tritium radioactivity was found in the water.

EXPERIMENTAL

1.Materials: Enzyme PAL (Phenylalanine ammonia lyase - EC.4.3.1.5) from *Rhodotorula glutinis* and Microcentrifuge filter NM-WL 10.000 were purchased from Sigma. Amberlite IR-120 (25-50 mesh) and cinnamic acid was obtained from Aldrich. Tritiated water was supplied from INC Pharmaceutical, Inc., USA. Scintillation cocktails for aqueous samples and non-aqueous samples were obtained from Rotiszint (Germany) and Sigma respectively.

2.Methods: The concentration of samples of L-Phe was determined indirectly by spectrophotometric means by measuring the concentration of its derivative , i. e., cinnamic acid. This procedure consists of converting L-Phe into cinnamic acid in the presence of enzyme PAL. The measured sample of L-Phe was introduced to a medium containing 0.2 M borate buffer (pH 8.8), enzyme PAL and incubated at 30 °C over 30 min. The concentration of cinnamic acid formed was determined spectrophotometrically by measuring the absorbance at 290 nm (ε_{290} = 10.000 for cinnamic acid) on the UV-VIS spectrometer (SHIMADZU-UV-102 CE-LV). The identities of L-Phe and cinnamic acid were also verified by TLC (silica gel, water-n-butanol acetic acid, 5:4:1 v/v) and by comparison of ^{1}H NMR spectra. For cinnamic acid the melting point was also determined. In the preliminary studies with inactive compounds the optical rotation of obtained phenylalanine was checked on the polarimeter (P 3002 –A. KRÜS Optronic– Germany).

The radioactivity of the all samples (aqueous and non aqueous) was determined using liquid scintillation counting (7 ml scintillation vials) on the automatic counter (LISA LSC PW470 – Raytest, Germany).

3. Synthesis of [3R-^{3}H]-L-Phe, **1**:

a) [1-^{3}H]-Benzaldehyde: A mixture of 2.1 g (0.01 mol) of benzil, $(C_6H_5CO)_2$, 5 ml of anhydrous freshly distilled 1,4-dioxane and 2 ml tritiated water of total radioactivity 4.36 GBq was placed in a two-necked 50 ml reaction flask equipped with magnetic stirrer and flushed with argon. To this solution in four portions 1 g of KCN (0.015 mol) was added with continuous stirring. After a few minutes the yellow coloured homogeneous mixture had become colourless and a precipitate of sodium benzoate was formed. This suspension was diluted with 20 ml of water and extracted with diethyl ether (2 x 10 ml). The combined ethereal solutions were subsequently washed with 10 ml of 5% Na_2CO_3, 20 ml of H_2O and 10 ml of a saturated solution of NaCl. The separated organic layer was dried over $MgSO_4$ and filtered. Next, the solution was placed in a 25 ml three necked conical flask (used in cinnamic acid synthetic step) and ether was removed under reduced pressure. The obtained crude [1-^{3}H]benzaldehyde was use in the following step without further purification.

b) [3-^{3}H]Cinnamic acid, **3**: To a 25 ml conical flask containing crude [1-^{3}H]benzaldehyde solution 1.25 g (0.012 mol) of malonic acid in 2 ml of freshly distilled pyridine and 50 μl of piperidine was added. The flask was equipped with a reflux condenser and gas-delivery tube. After flushing with argon the reaction mixture was heated under reflux at a temperature of 120 °C over 6 h with stirring and passage of a slow stream of argon. The post-reaction solution was acidified with conc. HCl. The precipitated crude product was filtered under reduced pressure on the Buchner funnel, dried and sublimed twice (85 °C and 1×10^{-1} mm Hg pressure) giving 0.63 g of tritiated cinnamic acid of total activity equal to 2.86×10^9 DPM ($4{,}7\times10^7$ Bq) and specific activity 6.8×10^{11} DPM/mol, i. e., 1.13×10^7 Bq/mmol. (After first and second sublimation the molar activities of cinnamic acid were the same.)

c) [3R-^{3}H]-L-Phenylalanine, **1** : In KIE experiments only substrates of high radiochemical purity should be used. Therefore, before each experimental KIE run we have prepared a fresh sample of **1** starting from a small sample of **3** to avoid contamination with self-radiation products formed during storage. Thus, for

example, 16 mg (0.108 mmol) of **3** with a total activity of 7.29×10^7 DPM (1.22×10^6 Bq) and 240 µl (1 unit) of enzyme PAL was dissolved in 5 ml of 1.6 M ammonia buffer (pH 9.7) and placed in a glass tube equipped with a cap and incubated at 30 °C for 120 h. The enzyme was removed by centrifugation using filter NM-WL 10.000. The filtrate was acidified to pH about 1 with 6 M HCl and extracted with diethyl ether to remove unreacted **3**. Extraction was carried out until the radioactivity of the organic layer had a constant value close to background. (Removed samples of **3** from other synthetic runs were combined and after purification used in the next experiments.) The water layer was loaded on a column (100×10 mm) of Amberlite IR-120(H^+ form), washed with water and then **1** was eluted with 0.3 M aqueous ammonia and collected as 3 ml fractions. From each fraction a 100 µl sample was taken for radioactivity assay. Fractions contained **1** were combined, concentrated under vacuum at 40-45 °C and the purity of **1** was checked by TLC and the enzymatic method. As a result a sample of 6.1 mg (0.037 mmol) of **1** dissolved in water (about 1 ml) with total activity 2.5×10^7 DPM (about 4.2×10^5 Bq) was obtained with a specific activity of 1.13×10^7 Bq (34,2% and 34.3% radiochemical yields respectively). The identity and amount of **1** was determined as described previously.

d) Confirmation of the position of the tritium label in **1**: In a separate experiment the sample of **1** was converted into **3** using the deamination reaction catalyzed by PAL according to Scheme 1. The reaction was carried out in a borate buffer solution at pH 8.8 at 30 °C over the course of 30 min. Under these conditions enzyme PAL stereospecifically removes the hydrogen from position pro-S leaving position pro-R unaffected. After reaction, **3** was extracted with diethyl ether, solvent was evaporated under reduced pressure and crude product was sublimed twice as described above in point 3c. The purified sample of **3** obtained after deamination has the same specific activity as the starting sample of **1** and the sample of **3** used for the synthesis **1**. This also means that during the PAL catalyzed addition of ammonia to cinnamic acid the hydrogen is directed stereospecifically into the 3S position.

4. Synthesis of [3S-^{3}H]-L-Phe, **2**:

a) [3S-^{3}H]-L-Phenylalanine, **2:** 1.6 M ammonium buffer (pH 9.7), prepared from NH_4Cl and NaOH, and 1.75 ml of tritiated water of total activity 4 GBq were placed in an incubation vial equipped with a cap. To this solution 15 mg of cinnamic acid (0.1 mmol) and 240 µl of enzyme PAL (1 unit) was added, and the mixture incubated for five days. After reaction enzyme PAL and unreacted cinnamic acid were removed as described in point 3c. The obtained **2** was separated chromatographically as described above (3c). First, the column was washed with water up to the moment when the radioactivity of the eluted fractions were close to background. Next, **2** was eluted with 0.3 M NH_3aq. and activity of each fraction was checked. To complete the removal of HTO the "peak" radioactivity fractions were combined, evaporated to 2 ml under reduced pressure, loaded on the column, washed with water, 0.3 M NH_3aq, and the fractions containing **3** were collected , combined and evaporated. This operation was repeated 3 times. As a result a sample of 6.3 mg (0.038 mmol) of **2** (as determined by spectrophotometric means) dissolved in 2 ml of water with a total activity of 2.12×10^8 DPM (3.53×10^6 Bq) was obtained. The specific activity of **2** was equal to 9.3×10^8 Bq/mmol, the chemical yield is 37.7% with respect to cinnamic acid.

b) Confirmation of the position of the tritium label in **2**: Addition of the tritium label in the 3S position of **2** only was confirmed in the same manner as described in (3d). A small portion of **2** diluted with inactive L-Phe was enzymatically converted to cinnamic acid. The sample of cinnamic acid obtained was inactive; all the radioactivity was found in the water layer.

ACKNOWLEGMENTS

This work was supported by grant KBN 3 T09A 046 17

REFERENCES

1. Havir E. A., Hanson K. R., *Biochemistry* **7:** 1904-1914 (1968)
2. Havir E. A., Hanson K. R., *Biochemistry* **14:** 1620-1626 (1975)

3. Pendharkar M. B., Nair P. M., *Ind. J. Biochem. Biophys.* **18:** 356-358 (1981)
4. Pometto III A. L., Crawford D. L., *Enzyme Microb. Technol.* 3: 73-75 (1981)
5. Hodgins D. S. *J. Biol. Chem.* **246:** 2977-2985 (1985)
6. Hermes J. D., Roeske C. A., O'Leary M. H., Cleland W.W., *Biochemistry* **21:** 5106-5114 (1982)
7. Hermes J. D., Cleland W.W., *Biochemistry* **24:** 2959-2967 (1985)
8. Gloge A., Langer B., Poppe L., Rétey J., *Arch. Biochem. Biophys.* **359:** 1-7 (1998)
9. Lewandowicz A., Jemielity J., Kanska M., Zon J., Paneth P., *Arch. Biochem. Biophys.* **370:** 216-221 (1999)
10. LeMaster D. M., Cronan Jr. J. E., *J. Biol. Chem.* **257:** 1224-1230 (1982)
11. Haldin C., Långström B., *J. Labelled Compd.Radiopharm.* **23:** 715-722 (1986)
12. Walker T. E., Matheny C., Storm C. B., Hayden H., *J. Org. Chem.* **51:** 1175-1179 (1986)
13. Hädener A., Tamm, Ch., *J. Labelled Compd.Radiopharm.* **24:** 1291-1305 (1987)
14. Tanimura K., Kato T. Waki M., Lee S., Kodera Y., Yzumiya N., *Bull. Chem. Soc. Jpn.* **57**: 2193- 2197(1984)
15. Ife R., Haslam E., *J. Chem. Soc (C),* 2818-2821 (1971)
16. Kirby G. W., Michael J., *Chem. Comm.,* 187-188 (1971)
17. Hanson K. R., Wightman R. H., Staunton J., Battersby A. R., *Chem. Comm.,* 185-186 (1971)
18. Wightman R. H., Staunton J., Battersby A. R., Hanson K. R, *J. Chem. Soc. Perkin I,* 2355-2364 (1972)
19. Bartl K., Cavalar C., Krebs T., Ripp E., Rntey J., Hull W. E., Günther E., Simon H., *Eur. J. Biochem.,* **72:** 247-250 (1977)
20. Hanson K. R., Havir E. A., *Arch. Biochem. Biophys.,* **141:** 1-17 (1970)
21. Hanson K. R. Havir E. A., *Recent Adv. Phytochem.,* **12:** 91-137 (1978)
22. Bukowski J., Szul J., Kanski R., Kanska M., *J. Radioanal. Nucl. Chem.* **243**: 635-638 (2000)
23. Jemielity J., Kanska M., Kanski R., *Isotopes Environ. Health Stud.* **34:** 33-339 (1998)

