

Research Article

Synthesis of tritium labeled isotopomers of L-tyrosine

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Summary

The synthesis of four selectively labeled isotopomers of L-tyrosine, (L-Tyr), using chemical and enzymatic methods is reported. Four tritium labeled isotopomers of L-phenylalanine (L-Phe) – [2-³H]-, [2',6'-³H₂]-, [3R-³H]- and [3S-³H]- have been synthesized using a combination of chemical and enzymatic methods. The labeled isotopomers of L-Phe have been converted into [2-³H]-, [2',6'-³H₂]-, [3R-³H]-, and [3S-³H]-L-Tyr by using the enzyme L-phenyl-alanine 4'-monooxygenase. Copyright © 2002 John Wiley & Sons, Ltd.

Key Words: tritium; labeling; optical isotopomer; phenylalanine; tyrosine; enzyme

Introduction

The metabolism of L-tyrosine, L-Tyr, is a key step in many biological processes of living organisms. A number of questions cannot be answered without understanding the mechanism of the reversible conversion of L-Tyr to phenol, pyruvate and ammonia, a reaction that is catalyzed by the enzyme β -tyrosinase (tyrosine phenol lyase, EC

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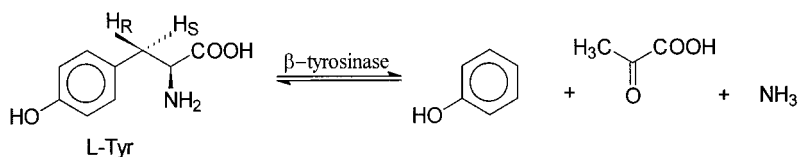


Figure 1. Decomposition of L-Tyr catalyzed by enzyme β -tyrosinase

4.1.99.2).¹⁻³ Under some conditions β -tyrosinase takes part in the formation of L-Tyr⁴⁻⁶ (Figure 1). The two mentioned reactions are multistep, involving several intermediates, and so complex that some details, particularly with respect of the timing of the bonding changes, are still unclear. The number of questions that arise can be minimized by determining the kinetic isotope effects (KIE) for each atom involved in the postulated rate determining step.^{7,8} The numerical values of the KIE can provide a useful tool for distinguishing between the alternative mechanisms. Such studies require different isotopomers of L-Tyr, specifically labeled with tritium in the desired positions.

In the literature, there are several methods describing the synthesis of tyrosine labeled with deuterium⁹⁻¹⁵ and tritium¹⁶⁻²⁰ yielding the different singly, multi and uniformly labeled isotopomers of L-Tyr. Most of them are not useful for our proposed KIE studies.

The synthesis developed by us to obtain the desired isotopomers of L-Tyr, specifically labeled with tritium consists of a combination of chemical and enzymatic methods. First, the key intermediate i.e. the corresponding tritium labeled isotopomer of L-phenylalanine, L-Phe, was obtained which in turn was converted to isotopomers of L-Tyr by using the enzyme L-phenylalanine hydroxylase (EC 1.14.16.1 – L-phenylalanine 4'-mono-oxygenase) (Figure 2).

Results and discussion

[2-³H]-L-Tyr, **1**, was synthesized from [2-³H]-L-Phe, **2**, obtained according to the reaction route shown in Figure 3. The tritium label was introduced into the methylene group of malonic acid, **3**, as a result of isotope exchange between malonic acid and tritiated water at elevated temperature.²¹ Knoevenagel condensation of [2-³H]malonic acid, **3**, with benzaldehyde leads to [2-³H]cinnamic acid,²² **4**, which in turn was converted²³ by the enzyme PAL (Phenylalanine ammonia lyase, EC 4.3.5.1) to [2-³H]-L-Phe. Finally, as shown in Figure 2, tritiated L-Phe

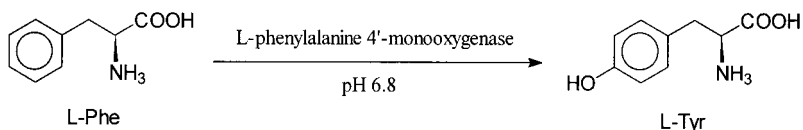


Figure 2. Enzymatic oxidation of L-Phe to L-Tyr

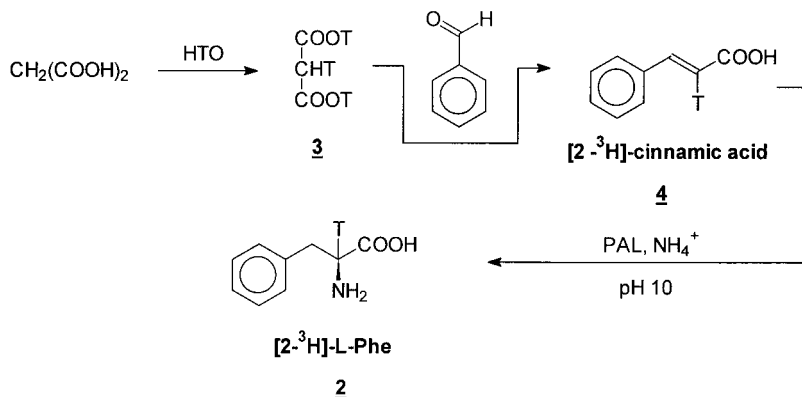


Figure 3. Synthesis of [2-³H]-L-Phe

incubated in a medium containing the enzyme L-phenylalanine 4'-monooxygenase (EC 4.1.99.2) from rat liver, produces [2-³H]-L-Tyr. The hydroxylation of L-Phe to L-Tyr was carried out in the presence of a cofactor and the enzyme catalase that protects L-Tyr from hydrogen peroxide formed during incubation. The general protocol of this step was described earlier.²⁴

The isotomers [3R-³H]-, 7, and [3S-³H]-, 8, of L-Phe, intermediates for synthesis of the corresponding isotomers of L-Tyr, were obtained as shown in Figures 4 and 5 according to the procedure described earlier.²³ These species, as well as [2',6'-³H₂]-L-Phe, 9, (commercial product) were converted (Figure 2) to [2R-³H]-L-Tyr, 10, [2R-³H]-L-Tyr, 12, and [2',6'-³H₂]-L-Tyr, 13, respectively.

The presence of tritium in the 3R position of 7 and 10 was confirmed by enzymatic transformation of 7 and 10 into [3-³H]cinnamic, 6, and *p*-hydroxy[3-³H]cinnamic, 11 acids, respectively, employing the reverse reaction catalyzed by the enzyme PAL (Figure 6). The obtained tritiated cinnamic acids were separated, purified and radio assayed. The molar activities of these labeled acids were the same as the molar activities of 7 and 10 used in the elimination reaction.

The same elimination reactions have been used to identify the presence of tritium in the 3S position of 8 and 11. The obtained

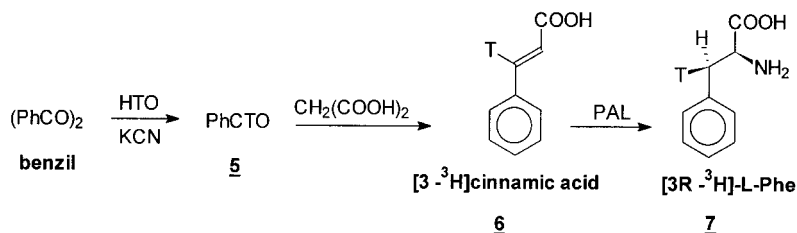


Figure 4. Synthesis of $[\text{3R-}^3\text{H}]\text{-L-Phe}$

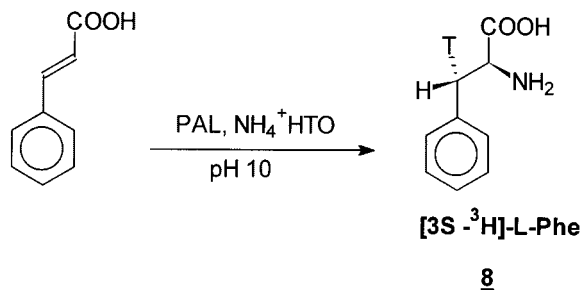


Figure 5. Synthesis of $[\text{3S-}^3\text{H}]\text{-L-Phe}$

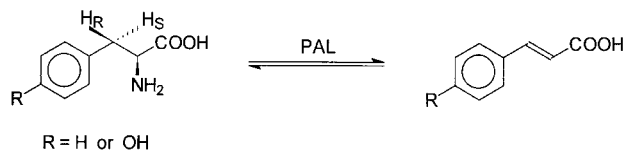


Figure 6. Reversible reaction catalyzed by enzyme PAL

cinnamic and *p*-hydroxycinnamic acids did not contain tritium. All the tritium radioactivity was found in the water.

In KIE experiments only substrates of high radiochemical purity should be used. To avoid contamination of starting tritiated L-Tyr with products of its self-radiation formed during storage, a fresh sample of the isotopomer of L-Tyr, sufficient for a single KIE run, was prepared.

Experimental

1. *Materials:* Tritiated water was obtained from INC Pharmaceutical Inc., USA. Scintillation cocktails for aqueous and non-aqueous samples were obtained from Rotiszint (Germany) and Sigma,

respectively. Microcentrifuge filters NM-WL 10.000 and Amberlit IR-120 (25–50 mesh) were purchased from Sigma and Aldrich, respectively. Preparative TLC plates, PEI-Cellulose F, were obtained from Merck. The enzymes, i.e. PAL (Phenylalanine ammonia lyase, EC 4.3.1.5 from *Rhodotorula glutinis*) and catalase (EC 1.11.1.6 from bovine liver) were obtained from Sigma.

2. *Methods:* L-Phenylalanine-4'-monooxygenase (EC 1.14.16.1) was isolated from rat liver according to the described procedure.²⁵ The obtained enzyme had an activity of 0.55 U/ml. The concentration of L-Phe was determined indirectly by measuring the concentration of its derivative, i.e. cinnamic acid, spectrophotometrically. This general protocol was given earlier,²³ and it consists of incubation of L-Phe in the presence of enzyme PAL. The concentration of cinnamic acid formed was determined spectrophotometrically by measuring the absorbance at 290 nm ($\epsilon_{290} = 10.000$ for cinnamic acid) on the UV-VIS spectrometer (Shimadzu-UV-102 CE-LV). The concentration of L-Tyr was also determined spectrophotometrically²⁴ using a sensitive and reproducible method developed for assay of tyrosine in biological media.²⁶ Under appropriate conditions tyrosine reacts with 1-nitroso-2-naphthol to yield a stable yellow product, the concentration of which can be determined by measuring the absorbance at 450 nm.

The identities and purity of L-Tyr and L-Phe were also verified by TLC on cellulose and silica gel, respectively. In the preliminary studies with inactive compounds the optical rotation of phenylalanine and tyrosine were checked on the polarimeter (P 3002, A. KRÜS Optronic, Germany). The radioactivity of all the samples (aqueous and non-aqueous) was determined using liquid scintillation counting (LISA LSC PW470 – Raytest, Germany).

3. *Synthesis of [2-³H]-L-Tyr, **1**:*

- (a) *[2-²H]Malonic acid, **3**.* This compound was synthesized by isotope exchange²¹ between malonic acid and tritiated water. To the glass ampoule 480 mg (4.1 mmol) of malonic acid and 1 ml of tritiated water with a total activity of 5.55 GBq were added. The ampoule was frozen with liquid nitrogen, degassed and sealed, and thermostated at 80°C for 8 h. Water was removed by lyophilization and crude the **3** obtained was used for the synthesis of cinnamic acid.
- (b) *[2-³H]Cinnamic acid, **4**.* Knoevenagel condensation^{22,26,27} of the crude **3** with benzaldehyde (5 mmol) yielded 318 mg (2.2 mmol) of **4**

with a total radioactivity of 15.7 MBq, this compound was used directly for the synthesis of [2-²H]-L-Phe.

- (c) [2-³H]-L-Phe, **2**. The general procedure for the enzymatic synthesis of L-Phe from cinnamic acid in ammonia buffer containing an enzyme PAL has been early described previously.^{23,24,27,28} In this case 50 mg of **4** (0.34 mmol) of 2.42 MBq total radioactivity with 0.45 ml of enzyme PAL (4.4 U/cm³) were added to 9 ml of 1.6 M ammonia buffer (pH 9.8) and the mixture incubated at 30°C for 5 days. As a result a sample of 22.9 mg (0.138 mmol) of **2** was isolated with a total radioactivity of 0.98 MBq and used in the next step to synthesize tritiated tyrosine. The concentration of **2** was determined spectrophotometrically as described in *Methods*.
- (d) [2-³H]-L-Tyr, **1**. The general method of enzymatic oxidation of L-Phe to L-Tyr, and procedure for its isolation and purification has been reported previously.²⁴ The stoppered glass vial with the reaction mixture containing the obtained sample of **2** (0.138 mmol), 4 mg of DL-6-methyl-5,6,7,8-tetrahydropterine (cofactor), 10.2 mg of DL-dithiothreitol (HSCH₂(OH)CH₂(OH)CH₂SH), 500 μl of enzyme catalase (EC 1.11.1.6) from bovine liver (300 000 U/ml) and 3 ml of the enzyme L-phenylalanine 4'-monooxygenase (0.55 U/ml) in 0.1 M phosphate buffer (pH 6.8) of 20 ml total volume was incubated at 25°C for 3 days. After isolation and purification²⁴ a 17 mg (0.1 mmol) sample of **1** was obtained with total radioactivity of 0.715 MBq (specific activity 7.15 MBq/mmol). The concentration of **1** was determined spectrophotometrically as described in *Methods*.
4. *Synthesis of [3R-³H]-L-Tyr, **10***: The intermediates, tritiated benzaldehyde, **5**, [3-³H]cinnamic acid, **6** and [3R-³H]-L-Phe, **7**, were obtained (Figure 4) according to procedures described earlier.²³
- (a) [3R-³H]-L-Phe, **7**: The enzymatic conversion²³ of **6** to **7** proceeded as described in (3c). As a result, from 50 mg (0.3 mmol) of sample of **6** (total radioactivity of 1.6 MBq) 20 mg (0.13 mmol) of **7** was obtained (total radioactivity 0.61 MBq) and this was used for the synthesis of **10**.
- (b) [3R-³H]-L-Tyr, **10**: This product was synthesized as in (3d). Finally, 16.5 mg (0.094 mmol) of **10** was obtained with a total radioactivity of 0.436 MBq (specific activity 4.64 MBq/mmol).
- (c) *Confirmation of 3R position of the tritium atom in L-Phe, **7***: In a preliminary experiment the sample of **7** was deaminated to **6** in the presence of enzyme PAL (Figure 6). The conversion²³ was carried

out in borate buffer solution at pH 8.8 at 30°C over the course of 30 min. Under these conditions enzyme PAL stereospecifically removes *pro-S* hydrogen from *pro-S* leaving the *pro-R* hydrogen unaffected.²⁹ The purified sample of cinnamic acid obtained after deamination of **7** has the same specific activity as a starting **7** and **6** used for synthesis of [3R-³H]-L-Phe. This also means that during the addition of ammonia to cinnamic acid the enzyme PAL introduces hydrogen into position 3S.

- (d) *Confirmation of 3R position of the tritium atom in L-Tyr, **10***: This was performed similarly as in (4e). After one day of incubation of **10** in the presence of PAL in borate buffer only about 50% of tyrosine was converted to *p*-[3-³H]hydroxycinnamic acid, **11**. The isolated and purified sample of **11** has the same specific activity as samples **7** and **10** and the sample of **6** used to synthesize of **7**.
5. *Synthesis of [3S-³H]-L-Tyr, **12***:
- (a) [*3R-³H*]-L-Phe, **8**, the key intermediate was obtained according to procedures described previously.²³ The synthesis was carried out as in (4c). The 50 mg (0.34 mmol) sample of cinnamic acid was dissolved in 1.6 M ammonium buffer (pH 9.8) prepared from NH₄Cl and NaOH and 6 ml of HTO of total activity 5 GBq in an incubation vial. To this solution 0.8 ml of enzyme PAL (4.4 U/ml) was added and the mixture incubated for 5 days. As a result, 21.6 mg (0.14 mmol) of **8** with a total radioactivity of 4.3×10^7 Bq (specific activity 3.07×10^7 Bq/mmol) was obtained.
- (b) [*3S-³H*]-L-Tyr, **12** was prepared from the above sample of **8** according to the protocol given in (3d). After incubation and purification a 19.2 mg (0.112 mmol) sample of **12** of total radioactivity 3.5×10^7 Bq (specific activity 3.1×10^8 Bq/mmol) was obtained.
- (c) *Confirmation of 3S position of the tritium atom in L-Phe, **8** and L-Tyr, **12***: The confirmation was performed in the same manner as described in (4c) and (4d). In this case the samples of cinnamic and *p*-hydroxycinnamic acids obtained after deamination reaction of **8** or **12** in the presence of enzyme PAL were inactive. The radioactivity was found in the water medium only.
6. [*2',6'-³H₂*]-L-Tyr, **13**: This isotopomer of L-Phe was prepared in the same manner as described in (3d). The starting substrate i.e. an aqueous solution of [*2',6'-³H₂*]-L-Phe, **9**, was a commercial product from SIGMA. (specific activity – 1.0 mCi/ml, 50 Ci/mmol). To the incubation vial containing 20 mg (0.13 mmol) of L-Phe (carrier) dissolved in the same reaction mixture as given in (3d), a 30 μl of

aqueous solution of **9** of total radioactivity of 1×10^6 Bq was added. The reaction mixture was incubated at 25°C for 3 days. After separation and purification 16.7 mg (0.098 mmol) of **13** containing 7.2×10^5 Bq (7.3 MBq/mmol) was obtained.

Acknowledgements

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