SYNTHESES OF CARBON AND HYDROGEN ISOTOPOMERS OF L-TYROSINE

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Summary

[3-^{14}C]-L-tyrosine was obtained via intermediate [3-^{14}C]-L-phenylalanine and subsequent oxidizing its using enzyme L-phenylalanine monooxygenase. Isotopomers of L-tyrosine specifically labeled with deuterium and tritium were synthesized using novel enzymatic methods. As a source of label a cheap commercial radiochemicals were used i.e., $Ba^{14}CO_3$, deuteriated and tritiated water.

Keywords: Carbon-14, deuterium, enzyme, L-tyrosine, labeling, tritium.

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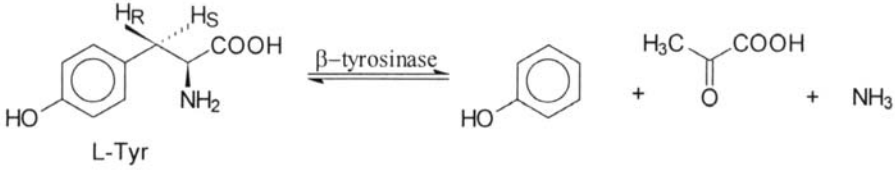
Introduction

The metabolism of L-tyrosine plays a important role in many processes that occur in living cells. In particular many studies have been concerned with explaining the mechanism of reversible reaction presented in Scheme 1. The enzyme β-tyrosinase (tyrosine phenol-lyase, EC. 4.1.99.2) catalyzes the decomposition of L-Tyr to phenol, pyruvate and ammonia[1].



Scheme 1. Reversible conversion of L-tyrosine catalyzed by enzyme β-tyrosinase

The mechanism of the above mentioned reaction[2-4] is very interesting since it occurs via abstraction of the α-proton and elimination of phenol moiety with quite rare bond rupture between aromatic and aliphatic carbon. The target of our planned research was to investigate the mechanism of above reaction using the kinetic isotope method (KIE) as a tool[5]. These kind of studies require the use only L-enantiomers specifically labeled

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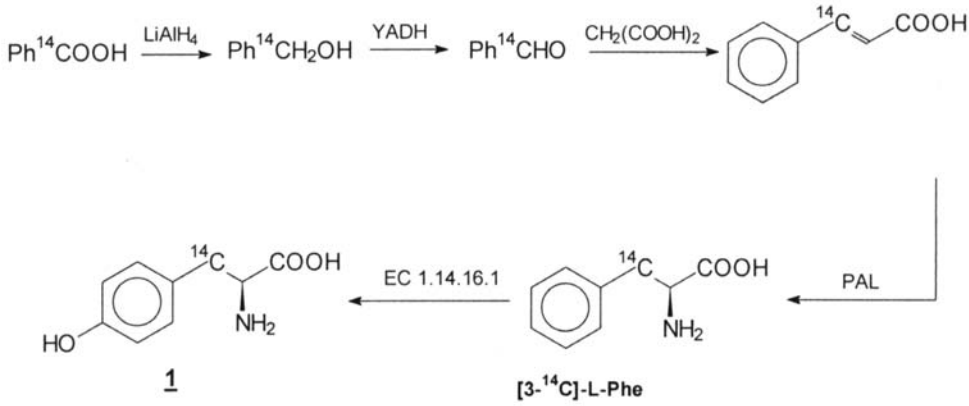
in desired positions. In our previous papers we reported combined (chemical and enzymatic) methods of syntheses of isotopomers of L-Tyr labeled with carbon-14 and tritium [6,7]. In this paper, a novel routes of syntheses of five isotopomers L-Tyr are presented.

Results and discussion

1. Synthesis of [3-^{14}C]-L-Tyr, **1**. This isotopomer was obtained using combined chemical and enzymatic approach. The key intermediate [3-^{14}C]-L-phenylalanine, L-Phe, was hydroxylated to **1** using the enzyme L-phenylalanine 4'-monooxygenase, EC 1.14.16.1, (Scheme 2). General procedure for this synthesis was described by us elsewhere[6].



Scheme 2. The combined chemical and enzymatic route synthesis of [3-^{14}C]-L-Tyr

In this case starting from [7-^{14}C]-benzoic acid via [7-^{14}C]-benzaldehyde as a intermediate [3-^{14}C]-cinnamic acid was obtained[8]. The enzymatic conversion of 100 mg (0.675 mmole) [3-^{14}C]-cinnamic acid of totalal activity 4.6 MBq via [3-^{14}C]-L-Phe[9,10] produced 33.5 mg of **1** with total radioactivity of 5.18 MBq and specific activity 2.8 MBq/mmol).

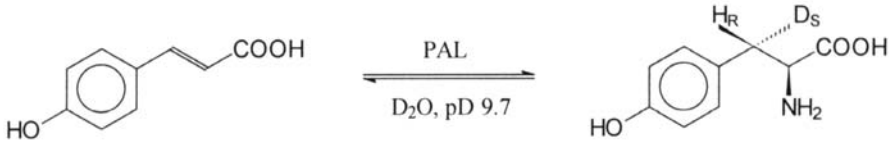
2. Synthesis of [2-^{2}H]-L-Tyr, **2**. This isotopomer of L-Tyr was synthesized using specific activity of the enzyme tryptophanase (EC 4.1.99.1) from *Escherichia coli.* This enzyme facilitates the liabilization of hydrogen bounded to α-carbon in side chain of L-tyrosine and subsequently accelerates its exchange with hydrogen from solvent. This is a simple way to introduce a label into α-position of L-Tyr.

General procedure: To a glass vial equipped with cap containing 42 ml of 0.05 M potassium phosphate buffer (prepared from heavy water, pD 8.3) with 80 mg (1.0 U) enzyme tryptophanase, 22.8 mg (0.126 mmole) of L-Tyr and 1.1 mg of 5'-pyridoxal phosphate, PLP, (cofactor) were dissolved. The incubation was carried out at 30°C for 8

days. The reaction was stopped with 2 ml of conc. DCl. Post-reaction mixture was loaded on a chromatografic column (1×10 cm, Amberlit IR 120 HP, H^+ form) and buffer's salts were washed out with water. Next the tyrosine was eluted with 0.3 M ammonia. The fractions containing **2** were combined and evaporated under reduced pressure. As a result 10.4 mg (57 mmol) sample of **2** was obtained with more than 95% incorporation of deuterium at the α-carbon position as determined by ^1H NMR (200 MHz Unity-plus spectrometer) and mass spectrometry (ESI method).

3. Synthesis of [2-^{3}H]-L-Tyr, **3**. This isotopomer was obtained in similar manner as **2**; the tritiated incubation medium was applied instead of deuteriated one.
Procedure: To a stoppered glass vial containing 0.75 ml of 0.1M potassium phosphate buffer (pH 8.3), 20 mg (0.26 U) of enzyme tryptophanase, 20 mg of PLP and 2.71 μmole of L-tyrosine, 0.75 ml tritiated water (total activity of about 30 GBq) was added. The reaction mixture was incubated at 30°C for 5 days. The reaction was quenched with 100 μl of conc. HCl and loaded on chromatographic column (1×10 cm, Amberlit IR HP, H^+ form). The buffer salts and excess of tritiated water were washed out. Resulting **3** was eluted with 0.3 M ammonia, and fractions containing **3** were combined, evaporated to 2 ml and once more chromatographed to remove the residual tritiated water. A a result a 2.66 μmol of **3** were obtained with total radioactivity of 0.95 MBq (specific radioactivity 3.58×10^8 Bq/mmol).

3. Synthesis of [3S-^{2}H]-L-Tyr, **4.** The enzyme Phenylalanine ammonia lyase, PAL, (EC 4. 1. 3. 5) from *Rhodotorula glutinis* was used for this synthesis. Under proper condition this enzyme catalyzes addition of ammonia and hydrogen into *pro-S* position of p-coumaric acid yielding L-Tyr.(Scheme 3).



Scheme 3. Enzymatic conversion of p-coumaric acid in [3S-^{2}H]-L-Tyr

General procedure: To an encapped glass vial containing 10.5 ml of 2.0 M ammonium buffer (heavy water, pD 9.70 48 mg (0.293 mmole) of p-coumaric acid and 0.20 ml (0.68 U) of enzyme L-phenylalanine ammonia lyase from *Rhodotorula glutinis* were added. The mixture was incubated at 30°C for 11 days. The reaction was quenched by adding 0,2 ml of conc. DCl. Non-reacted p-coumaric acid was extracted with 2×5 ml of diethyl ether. Then water fraction was loaded on chromatographic column (1×10 cm) filled with Amberlit IR 120 HP (H^+ form) and washed with water to remove buffer salts. Next, tyrosine was eluted with 0.3 M ammonia. The fractions containing tyrosine were combined, water was evaporated under reduced pressure. As a results 3.06 μmole of [3S-^{2}H]-L-Tyr was obtained with about 1% yield. Incorporation of deuterium at α-carbon

position (> 95%) was confirmed by ^{1}H NMR and mass spectrometry (ESI method). The yield of this synthetic route is very small; however it is the simplest way to obtain the sufficient amount of product needed for KIE assay.

4. Synthesis of [3S-^{3}H]-L-Tyr, **5**. The reaction was carried out in the similar manner as described in the synthesis of **4** with the difference that instead D_2O the tritiated water serve as medium solvent. To stoppered glass vial containing 16.2 mg (98.8 μmole) p-coumaric acid and 0.2 ml (0.68 U) of enzyme L-phenylalanine ammonia lyase from *Rhodotorula glutinis* dissolved in 0.75 ml of 3.6 M ammonium buffer (pH 9.7), 0.75 ml of tritiated water (total activity of about 30 GBq) were added. The mixture was incubated at 30°C for 14 days. The reaction was quenched by adding 0,2 ml of conc. HCl, and p-coumaric acid was extracted with 4×2.5 ml of diethyl ether. Water fraction was loaded on the chromatographic column (as above) and salts were removed by washing. The product was eluted with 0.3 M ammonia. Fractions containing radioactive L-Tyr were combined, water was evaporated under reduced pressure to 2 ml volume and once more chromatographed to remove residual tritiated water. After evaporation about 1 mg (5.8 μmole) of **5** was obtained with total radioactivity of 2.1×10^6 Bq (specific activity 3.7×10^8 Bq/mmol). Confirmation of tritium label in **5** was proven by elimination of ammonia and *pro-S* hydrogen catalyzed by enzyme PAL (scheme 3). The sample of **5** was incubated with enzyme PAL and radioactivity of recovered p-coumaric acid and reaction medium was checked using LSC method. All radioactivity found in water and its lack in p-coumaric acid clearly confirmed that tritium label was placed at *pro-S* position.

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