

## Synthesis of carbon-14 labeled [1-<sup>14</sup>C]-, and [2-<sup>14</sup>C]-L-tyrosine

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### Summary

Two labeled isotopomers of L-tyrosine, L-Tyr, have been synthesized using specific properties of the enzymes phenylalanine ammonia lyase, PAL, and L-phenylalanine hydroxylase. In an intermediate step [1-<sup>14</sup>C]-, and [2-<sup>14</sup>C]-L-phenylalanine, L-Phe, have been obtained from [1-<sup>14</sup>C]-, and [2-<sup>14</sup>C]cinnamic acid, prepared from potassium [<sup>14</sup>C]cyanate or [2-<sup>14</sup>C]malonic acid, and by addition of ammonia in the presence of enzyme PAL. The labeled isotopomers of L-Phe have been converted into [1-<sup>14</sup>C]-, and [2-<sup>14</sup>C]-L-Tyr using the enzyme L-phenylalanine hydroxylase. Copyright © 2001 John Wiley & Sons, Ltd.

**Key Words:** carbon-14; labeling; optical isotopomer; L-phenylalanine; L-tyrosine; enzyme

### Introduction

L-Tyrosine, L-Tyr, is important in a variety of biological functions. Its metabolism plays a central role in relationships that occur in many processes of living cells. In particular many studies have been concerned with explaining the mechanism of the reversible reaction given in Scheme 1. The enzyme  $\beta$ -tyrosinase (tyrosine phenol-lyase, EC. 4.1.99.1) catalyzes the conversion of L-Tyr to phenol, pyruvate and ammonia.<sup>1–4</sup> In some conditions this enzyme also participates in the reverse reaction

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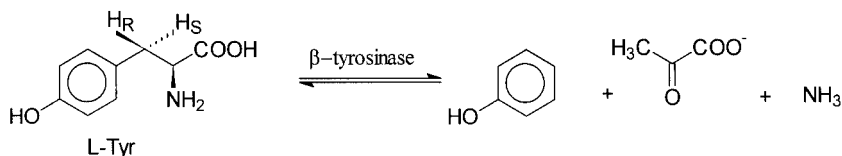
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**Scheme 1.**

leading to the formation of L-Tyr. The two mentioned reactions are multistep and some intrinsic details of the process are unclear.<sup>5–9</sup>

The goal of our planned research is directed at investigating the mechanism of this reaction by applying the kinetic isotope effect (KIE) method and determining KIE for each atom involved in the postulated rate determining step.<sup>10</sup> The studies require the synthesis of one *para*-derivative of L-Phe, i.e., L-Tyr labeled with <sup>14</sup>C in a particular position of the side chain. In this paper, a synthesis of two isotopomers of [1-<sup>14</sup>C], and [2-<sup>14</sup>C]-L-tyrosine is presented.

In the literature, there are several methods describing the synthesis of selectively or uniformly [<sup>14</sup>C]-labeled DL-Tyr<sup>11–18</sup>. [3-<sup>14</sup>C]-L-Tyr was separated by diluting the racemic form with L-Tyr and recrystallizing the product.<sup>18–19</sup> Recently, the extensive use of positron emission tomography has created a demand for [<sup>11</sup>C]-labeled L-Tyr. The described synthesis of [<sup>11</sup>C]-L-Tyr used a combination of chemical and enzymatic methods to achieve the desired L-enantiomer in the shortest possible time.<sup>20–23</sup> In the literature, there are reports of the synthesis of the different isotopomers of L-Tyr doubly labeled with <sup>3</sup>H and <sup>14</sup>C obtained from labeled racemates by removing one enantiomer enzymatically.<sup>24–25</sup> The chemical and chemo-microbiological methods have been also used to obtain the isotopomers L-Tyr, labeled with stable isotopes <sup>2</sup>H and <sup>13</sup>C, which have then been used for spectroscopic and metabolic studies.<sup>26–28</sup>

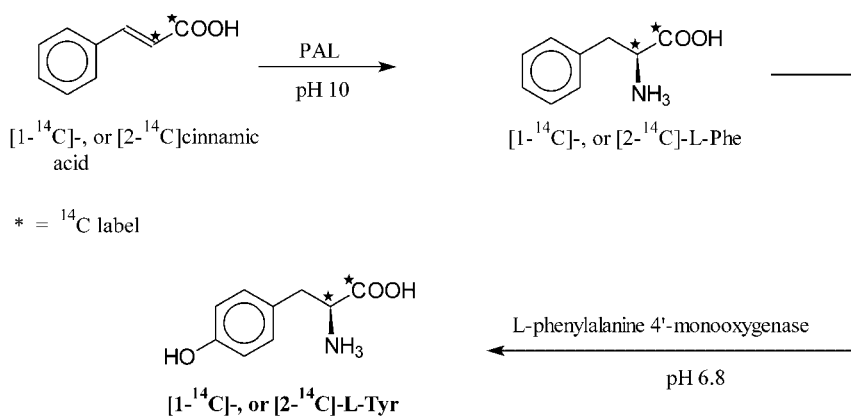
## Results and discussion

The above-mentioned routes for the synthesis of specifically labeled L-Tyr lead to the formation of racemates. The isolation of the L-enantiomer has been achieved by resolution by chemical or enzymatical methods. As a result half of the radioactive compound is wasted. To avoid this loss we applied enzymes in the key steps of the synthetic

pathway, i.e. stereospecific reduction of a double bond and oxidation of intermediate L-Phe to L-Tyr.

The two isotopomers of L-Tyr, specifically labeled with  $^{14}\text{C}$  in the 1- and 2-position of the side chain, have been prepared using a combination of chemical and multienzymatic methods. For this synthesis we used, as an intermediate, an isotopomer of  $[1-^{14}\text{C}]$ - or  $[2-^{14}\text{C}]$ cinnamic acid, which has been converted into  $[1-^{14}\text{C}]$ - or  $[2-^{14}\text{C}]$ -L-phenylalanine, L-Phe, in the presence of the enzyme PAL.<sup>29–32</sup> In the next step labeled L-Phe was oxidized to L-Tyr using an enzyme L-phenylalanine hydroxylase (EC 1.14.16.1 – L-phenylalanine 4'-monooxygenase) from rat liver.<sup>16</sup> To stimulate the hydroxylation of L-Phe to L-Tyr the reaction was carried out in the presence of D,L-6-methyl-5,6,7,8-tetrahydropterine (cofactor) and D,L-dithiothreitol (1,4-dithiobutane-2,3-diol –  $\text{HSCH}_2(\text{OH})\text{-CH}_2(\text{OH})\text{CH}_2\text{SH}$ ). The medium also contained the enzyme catalase that protects L-Tyr from  $\text{H}_2\text{O}_2$  formed during the course of incubation. The general route for the synthesis of labeled L-Tyr is showed in Scheme 2.

Potassium cyanide,  $[^{14}\text{C}]\text{KCN}$ , and commercial  $[2-^{14}\text{C}]\text{malonic acid}$  were used as the source of  $^{14}\text{C}$ -label.  $[^{14}\text{C}]\text{KCN}$  was converted into  $[1,1'-^{14}\text{C}_1]\text{malonic acid}$  according to the literature<sup>33</sup> procedure. The required intermediates, i.e. two isotopomers ( $[1-^{14}\text{C}]$ - and  $[2-^{14}\text{C}]$ -) of cinnamic acid have been previously obtained by us by Knoevenagel condensation of benzaldehyde with labeled malonic acid.<sup>29–30</sup>



**Scheme 2.**

## Experimental

1. *Materials:* [2-<sup>14</sup>C]Malonic acid was supplied by Moravek Biochemical (USA). Potassium cyanide [<sup>14</sup>C]KCN was obtained from Polatom (Poland). Scintillation cocktails for aqueous and nonaqueous 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 livers according to a literature procedure.<sup>34</sup> The obtained enzyme had an activity equal to 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 procedure consists of converting L-Phe into cinnamic acid in the presence of enzyme PAL. The measured sample of L-Phe was introduced into a medium containing 0.2 M borate buffer (pH 8.8) and enzyme PAL and incubated at 30°C for 30 min. The concentration of cinnamic acid formed was determined by measuring the absorbance at 290 nm ( $\epsilon_{290} = 10^4$  for cinnamic acid) using a SHIMADZU-UV-102 CE-LV spectrophotometer. The concentration of L-Tyr was also determined spectrophotometrically using a sensitive and reproducible method developed for assay of tyrosine in biological media.<sup>35</sup> Under appropriate conditions tyrosine, like other substituted phenols, 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. (Standards for calibration were prepared by treating known amounts of tyrosine with nitrosonaphthol).

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 the obtained phenylalanine and tyrosine were checked on the polarimeter (P 3002 – A. KRÜS Optronic – Germany). The radioactivity of all samples (aqueous and nonaqueous) was determined using liquid scintillators (7 ml scintillation vials) on the automatic counter (LISA LSC PW470 – Raytest, Germany).

3. *Synthesis of [1-<sup>14</sup>C]-L-Tyr, 1*:

- (a) *[1,1'-<sup>14</sup>C] Malonic acid, 2*. This intermediate was synthesized from 0.85 g (13 mmol) of [<sup>14</sup>C]KCN, total activity 185 MBq, according to a literature<sup>33</sup> procedure. As a result 0.69 g (6.6 mmol, 94.3 MBq) of crude **2** was obtained in 51% radiochemical.
- (b) *[1-<sup>14</sup>C]Cinnamic acid, 3*. Knoevenagel condensation<sup>18,29,30</sup> of the crude **2** with benzaldehyde yielded 0.65 g (4.4 mmol) of cinnamic acid, **3**, with a total activity of 30.5 MBq. Crude **3** was used directly for the synthesis of [1-<sup>14</sup>C]-L-Phe.
- (c) *[1-<sup>14</sup>C]-L-Phe, 4*. To a glass vial equipped with a cap about 30 mg (0.2 mmol) of **3** of 1.4 MBq total activity was added and dissolved in 5.25 cm<sup>3</sup> of 1.6 M ammonia buffer (pH 9.8). The 0.25 cm<sup>3</sup> of enzyme PAL (4.17 U/cm<sup>3</sup>) was added and the mixture incubated at 30°C for 120 h. The enzyme was removed by centrifugation using NM WL 10.000 filter (Sigma). The filtrate was acidified to pH 1 with 6 M HCl and extracted with diethyl ether to remove unreacted **3**. Extraction was repeated until the radioactivity of the ethereal layer reached a constant value close to background. The water layer was loaded onto a column (100 × 10 mm) of Amberlite IR-120(H<sup>+</sup>). The column was washed with water and then **4** was eluted with 0.3 M NH<sub>3</sub>(aq) and collected as 3 ml fractions. From each fraction 50 μl samples were taken for radioactivity measurements. The fractions containing **4** were combined and evaporated under vacuum at 40°C and the purity checked by TLC (silica gel, acetic acid–*n*-butanol–water – 1:4:5, v/v/v; R<sub>f</sub>=0.2 and 0.7 for phenylalanine and cinnamic acid, respectively) and enzymatically. As a result a sample of 13.7 mg (0.083 mmole) of **4** was obtained with a total activity of 5.75 × 10<sup>5</sup> Bq (6.9 MBq/mmol specific activity), and used in the next step to synthesize labeled tyrosine. The concentration of **4** was determined spectrophotometrically as described in *Methods*.
- (d) *[1-<sup>14</sup>C]-L-Tyrosine, 1*: The reaction mixture containing 13.7 mg (0.083 mmol, 5.75 × 10<sup>5</sup> Bq) of **4**, 2.4 mg (10 μmol) of DL-6-methyl-5,6,7,8-tetrahydropterine, 6.16 mg (40 μmol) of DL-dithiothreitol, 330 μl of the enzyme catalase (EC 1.11.1.6) from bovine liver (300 000 U) and 2 ml (0.55 U/ml) of a solution of the enzyme L-phenylalanine 4'-monooxygenase (EC 1.14.16.1) in 0.1 M phosphate buffer (pH 6.8) of 20 ml total volume was incubated at 25°C for 3 days. The reaction was stopped by addition of 2 ml of 60%

HClO<sub>4</sub> and 1 ml of concentrated HCl. The mixture was then centrifuged to remove the precipitated proteins (7krpm, 10 min, 4°C), and supernatant was loaded onto a column (100 × 10 mm) of Amberlit 120(H<sup>+</sup>). The column was washed with water to remove the buffer salts, and then the amino acids (**1** and unreacted **4**) were washed out with 0.3 M NH<sub>3</sub>(aq). The fractions with high radioactivity were combined and evaporated under reduced pressure at 40°C. The residue was dissolved and applied to a preparative cellulose-coated glass plate (20 × 20 cm). The plate was developed by ascending chromatography with the solvent system composed of chloroform–methanol–25%NH<sub>3</sub>(aq)–water 58:32:8:2 (v/v/v/v; R<sub>f</sub> = 0.25, and 0.8 for tyrosine and phenylalanine, respectively). The plate was air dried and cellulose from the area corresponding to **1** was scraped off and washed with water. The water was then evaporated off under reduced pressure at 40°C and the residue again dissolved in 1 ml of water. The purity of **1** was tested on the analytical TLC cellulose plate using the same developing system as for a preparative assay. The concentration of **1** was determined spectrophotometrically as described in *Methods*. As a result 10.7 mg (0.057 mmol) of **1** with a total activity of 3.9 × 10<sup>5</sup> Bq was obtained (specific activity 6.8 MBq/mmol).

4. *Synthesis of [2-<sup>14</sup>C]-L-Tyr, **5*** :
- (a) *[2-<sup>14</sup>C]Cinnamic acid, **6***. The synthesis of **6** was carried out in a similar manner to that described in Section 3(b). As starting material 0.5 g (4.8 mmol) of [2-<sup>14</sup>C]malonic acid, with a total activity of 9.2 MBq, was used. As a result 0.6 g (4 mmol) of crude **6** with total activity 7.6 MBq was obtained (83% radiochemical yield), and this product was used to synthesize [2-<sup>14</sup>C]-L-Phe without further purification.
- (b) *[2-<sup>14</sup>C]-L-Phenylalanine, **7*** : The synthesis of **7** was carried out as described in 3c. To the same incubation medium 30 mg (0.2 mmol) of **6** of 3.8 × 10<sup>5</sup> Bq total activity was added. After chromatographic isolation 12.5 mg (0.076 mmol) of **7** was obtained with a total activity of 1.45 × 10<sup>5</sup> Bq (1.9 MBq/mmol specific activity) and used directly in the next step.
- (c) *[2-<sup>14</sup>C]-L-Tyrosine, **5*** : The enzymatic conversion of **7** to **5**, isolation and purification of product, was carried out in the same manner as described in Section 3(d) leading to **1**. To the incubation medium 12.5 mg of **7** was introduced and 9.1 mg (0.05 mmol) of **5** with a total activity of 9.4 × 10<sup>4</sup> Bq (1.88 MBq/mmol specific activity) was obtained.

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