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Synthesis of ring labeled [l'-^{14}C]-L-tyrosine

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Abstract The synthesis of specifically ring labeled isotopomer of L-tyrosine, (L-Tyr), using chemical and enzymatic methods is reported. The carbon-14 labeled [1'-^{14}C]-L-Tyr has been synthesized by a 6-step conversion of [2-^{14}C]-malonic acid into [4'-^{14}C]-phenol and its subsequent condensation with *S*-methyl-L-cysteine using enzyme tyrosine phenol lyase from *Citrobacter freundii.*

Key words carbon-14 • labeling • tyrosine • enzyme • optical isotopomer

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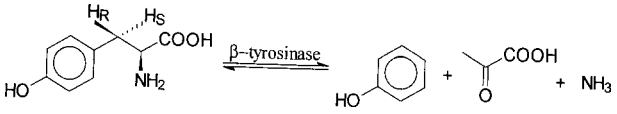
Introduction

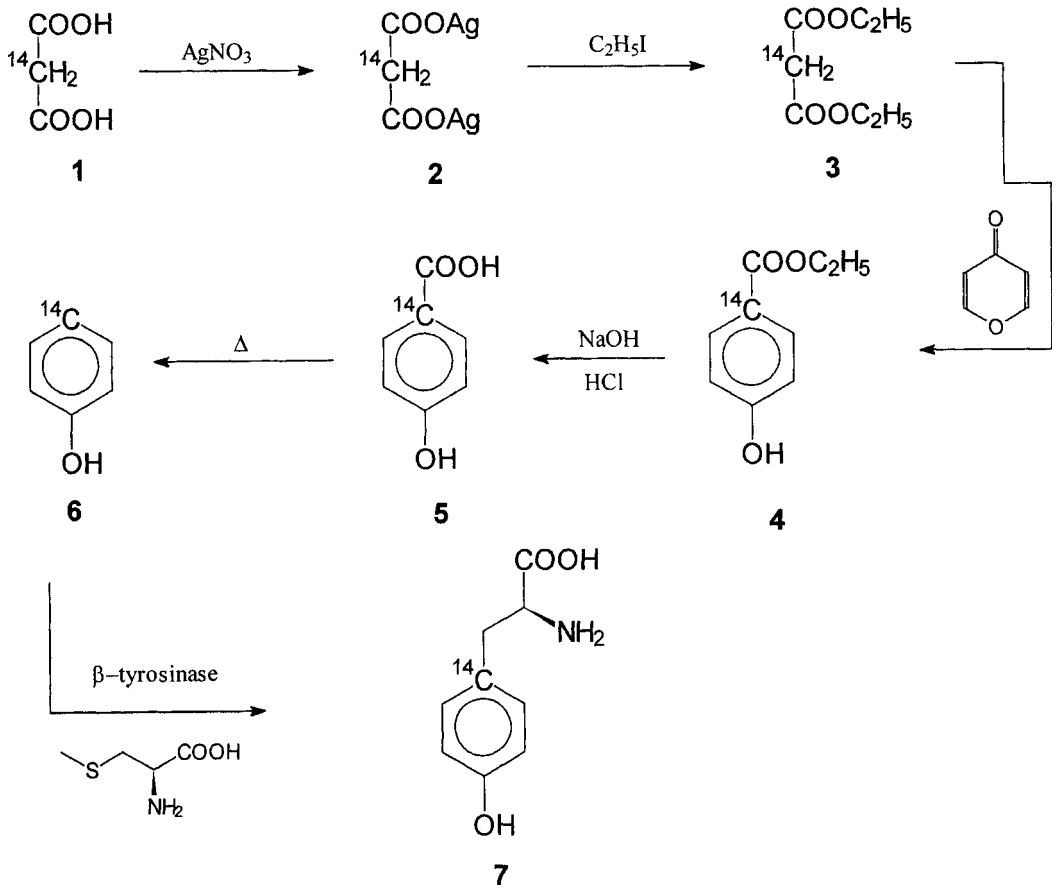
The enzyme β-tyrosinase (tyrosine phenol lyase, EC 4.1.99.2) has been shown [8, 9, 15] to catalyze the decomposition of L-Tyr to phenol, pyruvate and ammonia. Under some conditions, this enzyme also participates in the reverse reaction leading to formation [10, 12, 13] of L-Tyr (Scheme 1).

As a metabolism of L-Tyr is an important process of the living cells, the mechanism of the above reaction draws a special interest among biologists. This mutlistep reaction involving hydrogen transport and formation or rupture of bond between C_1 ring and C_3 side chain carbon atoms is still not clear. Some mechanistic questions can be answered by determining kinetic isotope effect, ^{14}C KIE, [5, 6], for carbon atom in C_1 ring position. For this purpose, the L-enantiomer form of specifically ^{14}C-labeled isotopomer of tyrosine is needed. In the literature there are described several methods of synthesis of different isotopomers of tyrosine labeled with isotopes of carbon (^{11}C, ^{13}C and ^{14}C) using chemical and enzymatic routes. All these syntheses we described and discussed in our earlier paper [2] (see also references cited therein) dealing with obtaining of other ^{14}C-labeled isotopomers of L-Tyr.

This paper reports on the combined chemical and enzymatic synthesis of [1'-^{14}C]-L-Tyr, i.e., a specifically labeled isotopomer needed to perform such a kind of kinetic study. However, in this 6-step synthetic route the desired isotopomer, i. e., [1'-^{14}C]-L-Tyr was obtained



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



Scheme 2. Synthesis of [1-^{14}C]-L-tyrosine.

with a very low chemical and radiochemical yield (about 0.9%). Fortunately, this quantity of compound is sufficient to carry out KIE assays planned. The thermal decomposition of [1'-^{14}C]-*p*-hydroxybenzoic acid to [4-^{14}C]-phenol with an about 6% chemical yield is the crucial step, responsible for the overall low yield of final product. On the other hand, it is the easiest way to obtain [4-^{14}C]-phenol required for enzymatic coupling with *S*-methyl-L-cysteine leading to the formation of L-enantiomer of tyrosine labeled with ^{14}C in 1' ring position. Also the intermediates as ethyl [1'-^{14}C]-*p*-hydroxybenzoate, **4**, and [1'-^{14}C]-*p*-hydroxybenzoic acid, **5**, were synthesized with good radiochemical yields (34 and 32%, respectively).

Results and discussion

[1'-^{14}C]-L-tyrosine, specifically labeled with ^{14}C in 1' position in the ring, has been prepared in 6 step reaction sequence using a combination of chemical and enzymatic methods. For this synthesis, as a starting substrate and a source of ^{14}C label, [2-^{14}C]-malonic acid, **1**, was used. It was converted [11] via its silver salt, **2**, in diethyl [2-^{14}C]-malonate, **3**. The ring closure reaction [12] of **3** with 4*H*-pyran-4-one afforded ethyl [1'-^{14}C]-*p*-hydroxybenzoate, **4**, which was hydrolyzed [4] to [1'-^{14}C]-*p*-hydroxybenzoic acid, **5**. Its thermal decomposition yielded [4-^{14}C]-phenol [1], **6**. In turn, **6** was coupled [7] with *S*-methyl-L-cysteine by the enzyme β-tyrosinase from *Citrobacter freundii* giving desired [1'-^{14}C]-L-Tyr, **7** (Scheme 2).

Experimental

Materials

[2-^{14}C]-malonic acid was purchased from ICN Pharmaceutical Inc., Irvine, CA, USA. Scintillation cocktail was obtained from Rotiszint (Germany). Silica gel TLC plates, 60 F_{256}, were from Merck. The enzyme β-tyrosinase (EC 4.1.99.2) from *Citrobacter freundii* has been kindly gifted by Prof. R. S. Phillips from the University of Georgia, Athens, GA, USA. Cofactor, i.e., pyridoxal 5-phosphate, (PLP), *S*-metyl-L-cysteine, mercaptoethanol were from Sigma.

Methods

The presence and purity of L-Tyr and phenol was checked qualitatively by TLC using silica gel plates and developing solvents: acetonitrile: water – 4:1 (v/v) for tyrosine, and heptane: ethyl acetate – 2:1 (v/v) for phenol. The concentration of L-Tyr was determined spectrophotometrically as described earlier [2, 3] using a reproducible method developed for the assay of tyrosine in biological media [14]. In the preliminary studies with inactive compounds, the optical rotation

of tyrosine was checked on the polarimeter (P 3002 KRÜS Optronic, Germany). The radioactivity of all the samples was determined using liquid scintillation counting (LISA LSC PW470, Germany).

Synthesis of [1'-^{14}C]-L-tyrosine, **7**

*Silver [2-^{14}C]-malonate, **2***

A 522 mg (5 mmol) sample of [2-^{14}C]-malonic acid, **1**, of total radioactivity of 9.25 MBq (specific activity 1.84 MBq/mmol) was added to 5 ml of water and neutralized with 1 M NaOH. To this 40 ml of 5% solution of $AgNO_3$ (11.8 mmol) was added. The grayish precipitate of **2** was filtered, washed with cold water, and dried. As a result 1.27 g (4 mmol) of **2** was obtained with 80% chemical yield.

*Diethyl [2-^{14}C]-malonate, **3***

To a crude 1.27 g sample of **2**, 7.8 ml (97.5 mmol) of ethyl iodide was added and the mixture was refluxed for 4 h. The residue was separated by filtration and washed with diethyl ether. Diethyl ether and the excess of ethyl iodide were removed by distillation leaving 385 mg (2.4 mmol) of crude **3** of 4.45 MBq total radioactivity (about 60% yield).

*Ethyl [1'-^{14}C]-p-hydroxybenzoate, **4***

To the above-mentioned crude sample of **3**, 660 mg of 4*H*-pyran-4-one dissolved in 12.5 ml of *t*-butanol was added. To the above refluxing mixture, a solution of 650 mg (5.8 mmol) of potassium *t*-butoxide in 15 ml of *t*-butanol was added dropwise during 4 h. Next, 9 ml of 1 M HCl (9 mmol) was added, and the mixture was refluxed for 1 h. Then, the solvent was evaporated, 30 ml of water was added to the residue, and the mixture was extracted with 4×15 ml of *t*-butyl methyl ether. Combined organic layers were washed with 2×10 ml of water. The *t*-butyl methyl ether was evaporated giving 276 mg (1.7 mmol) of crude **4** with total radioactivity 3.11 MBq (about 70% yield in this step, and 34% from the start of synthesis).

*[1'-^{14}C]-p-hydroxybenzoic acid, **5***

The above sample of **4** was dissolved in 10.5 ml of 2 M NaOH, and stirred for 21 h. Then, the mixture was acidified with 2 ml of conc. HCl, and extracted with 5×10 ml of *t*-butyl methyl ether. Combined organic layers were washed with 10 ml of 1 M HCl. After evaporation of the solvent, 219 mg (1.6 mmol) of crude **5** of 3 MBq total radioactivity was obtained (about 96% yield in this step, and 32% from the start of synthesis).

*[4-^{14}C]-phenol, **6***

A crude sample of **5** with 150 mg of Cu powder and 4 ml of quinoline were placed in a glass ampoule. The air was evacuated and the ampoule was sealed, then it was heated at 255°C for 80 min. After cooling, the ampoule was opened, and 15 ml of 30% solution of H_2SO_4 was added. At first, the post reaction mixture was extracted with 8×10 ml of diethyl ether. Next, the combined ether layers were extracted with 6×5 ml of 1 M NaOH. Finally, the combined water layers were extracted with 5×10 ml ethyl ether. The solvent from the combined ether layers from the final extraction was evaporated and the resulting (4-^{14}C)-phenol was purified twice by sublimation (120°C, 1×10^{-1} mm Hg) yielding 28.5 mg (0.3 mmol) of [4-^{14}C]-phenol with a total radioactivity of 0.555 MBq (specific activity 1.85 MBq/mmol). Radiochemical yield in this step was equal to 6%.

*[1'-^{14}C]-L-tyrosine, **7***

The reaction mixture contained 120 mg (0.9 mmol) of *S*-methyl-L-cysteine, 1 mmole of mercaptoethanol (to prevent of growth fungi), 0.2 mmole of PLP (cofactor) and 5 U of enzyme tyrosine phenol lyase from *Citrobacter freundii* dissolved in 0.5 ml of 0.05 M phosphate buffer of pH 8.0. This mixture was placed in an encapped vial and incubated at 30°C. Then, a solution of 28.5 mg (0.3 mmol) of [4-^{14}C]-phenol, **6**, dissolved in 1 ml of 0.05 M phosphate buffer (pH 8.0) was added in 4 equal portions at the start and after 17, 27, and 45 h of reaction course. After 3 days of incubation, the precipitate of [1'-^{14}C]-L-Tyr, **7**, was collected, washed with ethanol and diethyl ether, and recrystallized from hot water. As a result, 8.1 mg (45 mmol) of **7** was obtained of 82.5 kBq total radioactivity (specific activity 1.83 MBq/mmol) with 15% radiochemical and chemical yield. The overall radiochemical yield of **7** from this 6-step synthesis was equal to about 0.9%.

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