

Research Article

Synthesis of tritium labelled [2',6']-L-tyrosine

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Summary

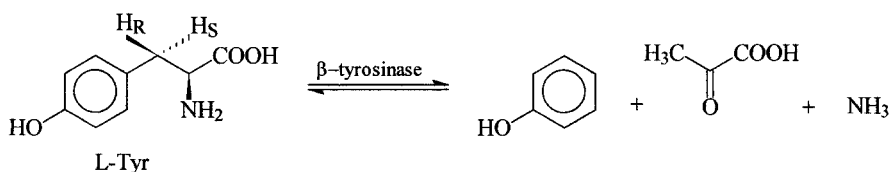
The synthesis of a specifically ring labelled isotopomer of L-tyrosine, (L-Tyr), using a combination of chemical and enzymatic methods is reported. The tritium labelled [2',6']-L-Tyr has been synthesized *via* catalytic exchange of phenol with tritiated water in the presence of K_2PtCl_4 , reverse acid catalysed removal of tritium from the *o*- and *p*-positions of phenol, and subsequent condensation of the resulting [3',5- 3H_2]-phenol with *S*-methyl-L-cysteine using the enzyme β -tyrosinase from *Citrobacter freundii*. Copyright © 2004 John Wiley & Sons, Ltd.

Key Words: tritium; deuterium; tyrosine; enzyme; optical isotopomer

Introduction

The enzyme β -tyrosinase (tyrosine phenol lyase, EC 4.1.99.2) has been shown^{1–3} 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^{4–6} of L-Tyr (Scheme 1).

As the metabolism of L-Tyr is an important process of living cells, the mechanism of the above reaction is of special interest, particularly biologists.



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

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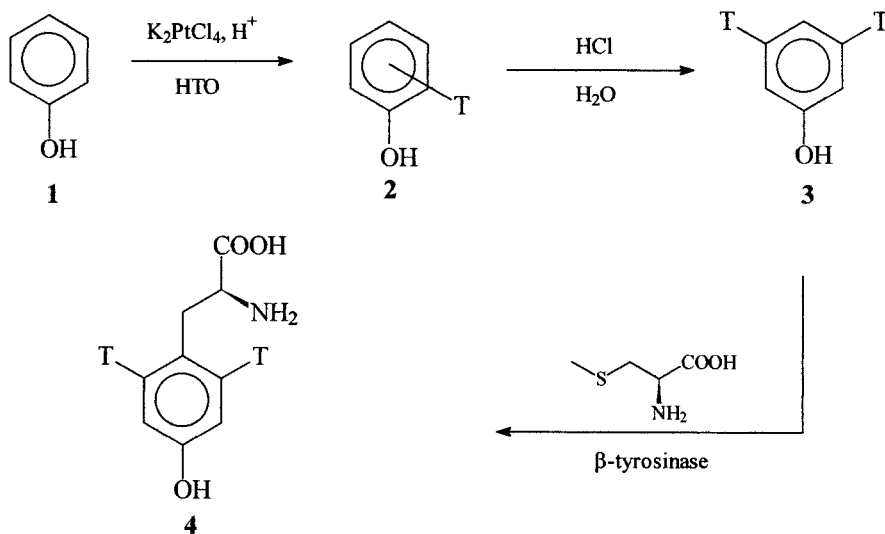
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This multistep reaction involving hydrogen transport and formation or rupture of the bond between the C₁ ring and C₃ side chain carbon atoms is still not clear. Some mechanistic questions can be answered by determining kinetic isotope effects (KIE) for each atom involved in the postulated rate determining step.^{7,8} For this purpose the L-enantiomer form of specifically tritium labelled tyrosine is needed. In the literature there are several papers which describe the synthesis of deuterium⁹⁻¹⁵ and tritium¹⁶⁻²⁰ labelled tyrosine. Unfortunately most of them are not useful for our proposed KIE studies. In our earlier paper²¹ we described the synthesis of [2',6'-³H₂]-L-Tyr by hydroxylation of commercial [2',6'-³H₂]-L-phenylalanine using the enzyme L-phenylalanine hydroxylase.²¹

Results and discussion

The new route to the synthesis of [2',6'-³H₂]-L-Tyr developed by us consists of a combination of chemical and enzymatic methods. First, the key intermediate i.e., [3,5-³H₂]-phenol was obtained as a result of *H/T* exchange between phenol and tritiated water. In turn it was condensed with *S*-methyl-L-cysteine using the enzyme β -tyrosinase from *Citrobacter freundii* (Scheme 2).

The literature data²²⁻²⁶ show that phenol can be catalytically exchanged with deuteriated or tritiated water selectively in the *o*- and *p*-positions or per labelled. By the reverse acid catalysed exchange of uniformly tritiated phenol, **2**, with water it is possible to prepare the 3,5-tritiated substrate, **3**, needed for the enzymatic synthesis of the final product **4** (Scheme 2).



Scheme 2. Synthesis of [2',6'-³H₂]-L-tyrosine

To check these expectations we have investigated the *H/D* exchange between phenol and heavy water catalyzed with acid and a salt of divalent platinum. The degree of incorporation and removal of deuterium from the aromatic ring was determined using the ^1H NMR spectra of samples of exchanged phenol. Homogeneous, K_2PtCl_4 catalysed exchange gives phenol deuteriated in the *o*- and *p*-positions^{22,24–26} as well as the *m*-positions. Also the reverse acid exchange of uniformly deuteriated phenol with water does not remove label from the *m*-position, giving selectively labelled [3,5- $^2\text{H}_2$]-phenol.

Therefore, in the first step we prepared uniformly ring tritiated phenol, **2**, by *H/T* exchange with HTO in the presence of K_2PtCl_4 catalyst. In the next step reverse acid catalysed exchange of **2** with water removes²⁶ the tritium label from the *o*- and *p*-positions of **2** leading to the [3,5- $^3\text{H}_2$] product **3**.

Experimental

1. *Materials*: Tritiated water was obtained from INC Pharmaceutical Inc., USA. Deuteriated water was purchased from The Radioisotope Production and Distribution Center, Poland. Scintillation cocktails were obtained from Rotiszint (Germany). Amberlit IR-120 (2–50 mesh) and TLC plates were the products of Aldrich and Merck, respectively. Cofactor i.e., pyridoxal 5-phosphate, PLP, *S*-methyl-L-cysteine, mercaptoethanol ($\text{HSCH}_2\text{CH}_2\text{OH}$) were purchased from Sigma. The enzyme β -tyrosinase (EC 4.1.99.2) from *Citrobacter Freundii* was kindly given by Prof. R. Phillips from the University of Georgia, Athens, USA.
2. *Methods*: The concentration of L-Tyr was determined spectrophotometrically²⁷ using a sensitive and reproducible method for assaying of tyrosine in biological media.²⁸ Under appropriate conditions tyrosine reacts with 1-nitroso-5-naphthol to yield a stable yellow product, the concentration of which can be determined by measuring the absorbance at 490 nm using a Shimadzu-UV-VIS-102 CE-LV spectrometer (Japan). The catalyst i.e., K_2PtCl_4 , was prepared according to the literature.²⁹ In the preliminary deuterium labelling studies of phenol the degree and position of incorporation of the label was determined from ^1H NMR spectra using a Varian 500 MHz NMR spectrometer. For the tritium work the radioactivity was determined using a liquid scintillation counter (Lisa LSC470-Raytest, Germany).
3. [*G*- ^3H]-phenol, **2**. This intermediate was synthesized by isotope exchange between phenol and tritiated water catalysed by the salt of divalent platinum. To the glass ampoule 1 g (10.6 mmol) of phenol, 100 mg (0.24 mmol) of K_2PtCl_4 , 0.2 ml of concentrated HCl and 0.5 ml of tritiated water with a total activity 200 MBq were added. The ampoule was frozen with liquid nitrogen, degassed, sealed and heated at 100°C for 24 h. After

- cooling, the ampoule was opened and 5 ml of water was added to the post-reaction mixture. Phenol was separated from the catalyst by extraction (5×5 ml) with diethyl ether. Next, the combined organic layers were washed (5×10 ml) with water. The solvent from the organic layer was evaporated yielding 850 mg (about 9 mmol) of crude **2** of total radioactivity 36.8 MBq (about 85% chemical yield).
4. [$3,5\text{-}^3\text{H}_2$]-phenol, **3**. The above crude sample of **2** dissolved in 12 ml of 2M HCl was placed in an ampoule, degassed as above, sealed, and heated at 100°C for 24 h. The post reaction mixture was extracted with 5×5 ml of diethyl ether. Combined organic layers were washed with 2×5 ml of water. The solvent was evaporated off under reduced pressure. The phenol was separated from the residue by sublimation (110°C , about 1 Pa). As a result 675 mgs (7.18 mmol) of **3** of total radioactivity 4.5 MBq (627 kBq/mmol specific activity) were obtained (79.3% chemical yield).
5. [$2',6'\text{-}^3\text{H}_2$]-L-tyrosine, **4**. The enzymatic reaction was carried out in an encapped glass vial. To this 4 ml of 0.1 M phosphate buffer (pH 8), 29.8 mg (221 μmol) of *S*-methyl-L-cysteine, 0.1 mmol of PLP, 1 mmol of mercaptoethanol and 20 U of β -tyrosinase (EC 4.1.99.2) from *Citrobacter freundii* were added. To this vial the solution of 15.3 mg (163 μmol) of **3** dissolved in 0.5 ml of phosphate buffer was added in 5 equal portions at the start and subsequently after 17, 25, 41 and 49 h of the incubation. The incubation was carried out at 30°C for 5 days. The precipitated crystals of **4** were collected and washed with ethanol and diethyl ether. As a result 5.3 mg (29 μmol) of **4** of total radioactivity of 1.85×10^5 Bq (specific activity 627 MBq/mmol was obtained (18% radiochemical and chemical yield).

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