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Research Article

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

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Summary

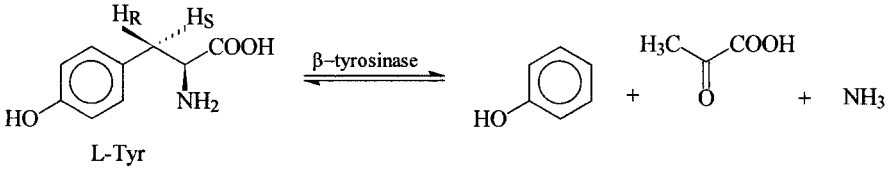
The synthesis of a specifically ring labelled isotopomer of L-tyrosine, (L-Tyr), using a combination of chemical and enzymatic methods is reported. The tritium labelled [2′,6′]-L-Tyr has been synthesized *via* catalytic exchange of phenol with tritiated water in the presence of K_2PtCl_4, reverse acid 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*.

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.



L-Tyr

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

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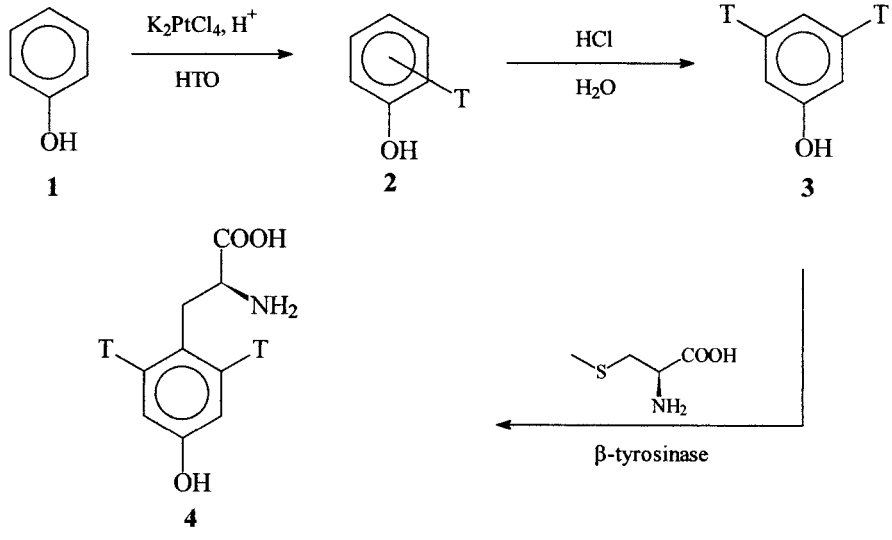
This multistep reaction involving hydrogen transport and formation or rupture of the bond between the C_1 ring and C_3 side chain carbon atoms is still not clear. Some mechanistic questions can be answered by determining kinetic isotope effects (KIE) for each atom involved in the postulated rate determining 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 deuterium[9–15] and tritium[16–20] labelled tyrosine. Unfortunately most of them are not useful for our proposed KIE studies. In our earlier paper[21] we described the synthesis of [2′,6′-$^3H_2$]-L-Tyr by hydroxylation of commercial [2′,6-$^3H_2$]-L-phenylalanine using the enzyme L-phenylalanine hydroxylase.[21]

Results and discussion

The new route to the synthesis of [2′,6′-$^3H_2$]-L-Tyr developed by us consists of a combination of chemical and enzymatic methods. First, the key intermediate i.e., [3,5-$^3H_2$]-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 data[22–26] 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′-$^3H_2$]-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 ^{1}H 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-$^2H_2$]-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 removes[26] the tritium label from the *o*- and *p*-positions of **2** leading to the [3,5-$^3H_2$] 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 ($HSCH_2CH_2OH$) 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 spectrophotometrically[27] using a sensitive and reproducible method for assaying of tyrosine in biological media.[28] Under appropriate conditions tyrosine reacts with l-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 ^{1}H 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-^{3}H]-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 HC1 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-$^3H_2$]-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′-$^3H_2$]*-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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