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Note

Enzymatic synthesis of tritium-labelled isotopomers of L-DOPA

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Summary

The synthesis of two isotopomers of L-DOPA labelled selectively with tritium is reported. In the intermediate step [3S-^{3}H]-, and [3′,5′-$^3H_2$]-L-tyrosine, have been obtained using a combination of chemical and enzymatic methods. The labelled isotopomers of L-tyrosine, L-Tyr, have been converted into, [3S-^{3}H]-, and [5′-^{3}H]-L-DOPA using the enzyme mushroom tyrosinase (monophenol oxidase, EC 1.14.18.1) from *Neurospora crassa*. Copyright © 2005 John Wiley & Sons, Ltd.

Key Words: DOPA; enzyme; labelling; tritium; tyrosine

Introduction

L-DOPA (3′,5′-L-dihydroxyphenylalanine) and its precursor L-Tyr are important in a variety of biological functions.[1] The former is a starting point in the synthesis of melanins (natural humans and animals pigments), in addition it is involved in the synthesis of catecholamines i.e., adrenaline, noradrenaline and dopamine. L-Tyr is one of the most important neurotransmitters in the nervous system[2–4] (Scheme 1).

L-DOPA is a drug used in pharmacotherapy as the precursor of dopamine. Deficiency in dopamine causes the disruption of proper brain functions, leading to many health problems such as Parkinson's disease.[5,6] L-DOPA, specifically labelled with β^+-emitters, is used in PET diagnostics to monitor the synthesis and decomposition of dopamine in the brain.[7–10] Selectively labelled L-DOPA can also be used to elucidate the reaction mechanisms associated with the formation of dopamine (Scheme 1). Despite numerous

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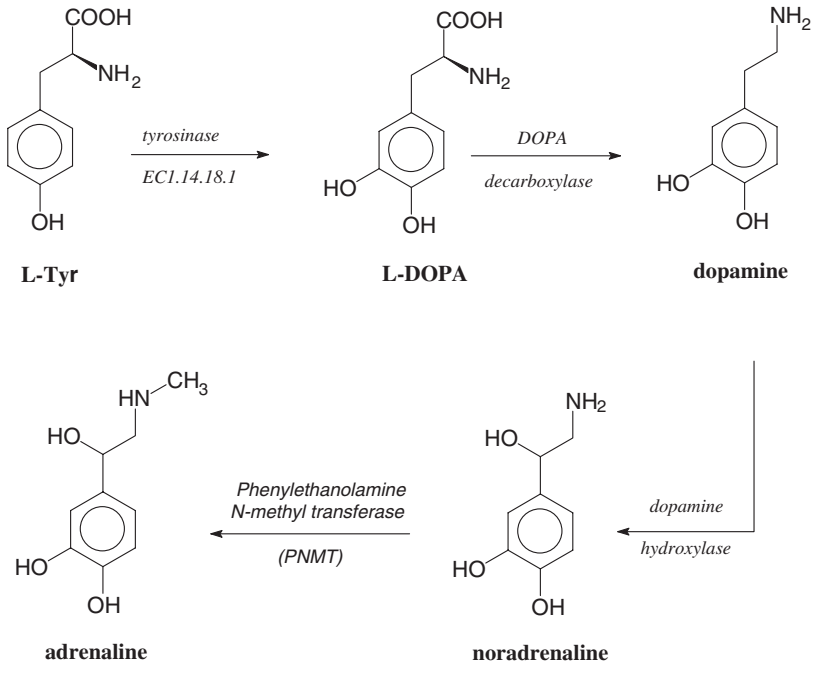


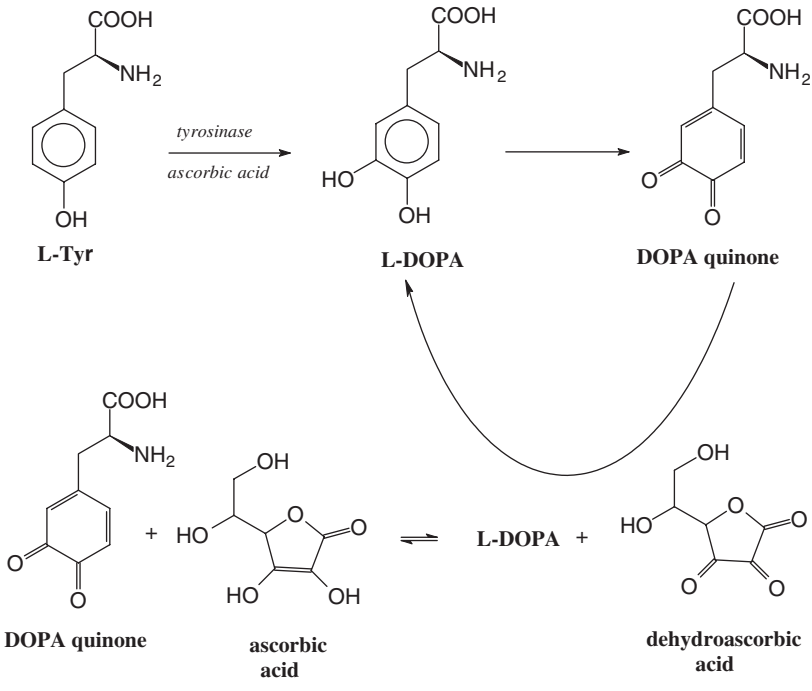
Scheme 1. Synthesis of catecholamines

literature reports, the mechanism of this reaction is not completely understood. Therefore, it would be interesting to investigate several reaction steps leading to the formation of dopamine, especially to characterize the active complex which is formed in the oxidation reaction of L-tyrosine to L-DOPA and in the decarboxylation of L-DOPA to dopamine. Thus, the kinetic isotopic effect method (KIE) can be used. This technique relies on obtaining the ratios of rate constants using the lighter and heavier isotope. The results obtained by this method will allow one to elucidate the rate determining step.[11]

In the literature there are several methods describing the enzymatic conversion of [^{3}H]-L-tyrosine to tritium-labelled L-DOPA. For this purpose the enzymes extracted from hamster melanoma,[3] adrenal glands[12] or rat brains[13] were used.

This paper reports on the enzymatic synthesis of two selectively tritium-labelled isotopomers of L-DOPA, i.e., [5′-^{3}H], and [3S-^{3}H]-L-DOPA from the appropriate isotopomers of L-Tyr.

Tyrosinase from mushrooms *Neurospora crassa* (EC 1.14.18.1) was the enzyme[14] used in this experiment. It catalyses the hydroxylation of L-Tyr to L-DOPA, and immediately mediates the oxidation of L-DOPA to dopaquinone.[15] However, in the presence of ascorbic acid, the oxidation of L-DOPA is a reversible process.[16] Ascorbic acid reduces dopaquinone to L-DOPA, and itself undergoes oxidation to dehydroascorbic acid (Scheme 2).



Scheme 2. Enzymatic conversion of L-tyrosine into L-DOPA

The intermediate substrate for the synthesis of labelled L-DOPA, i.e., isotopomer [3′,5′-$^{3}H_2$]-L-Tyr has been obtained by acid-catalysed H/T isotope exchange at elevated temperature. This synthetic step[17,18] has been tested by using deuterium as the label. The position and degree of incorporation of deuterium in the resulting product, i.e., [3′, 5′-$^{2}H_2$]-L-Try has been ascertained by proton NMR spectroscopy. The second intermediate needed, i.e., [3S-^{3}H]-L-Tyr was prepared in our research group by the procedure described elsewhere.[19,20]

Experimental

Materials

Tritiated water was purchased from ICN Pharmaceutical Inc., Irvine, CA, USA. Deuteriated water (99.9% deuterium) was obtained from Polatom (Poland). Scintillation cocktail was purchased from Rotiszint (Germany). Silica gel TLC plates, 60 F_{256}, were from Merck. The enzyme tyrosinase (EC 1.14.18.1) isolated from *Neurospora crassa*, and other chemicals, needed for the enzymatic synthesis and control experiments, i.e., L-tyrosine, L-DOPA, ascorbic acid were also obtained from Sigma.

Methods

The presence of tyrosine and DOPA was checked qualitatively by TLC using silica gel plates and developing solvents: (*n*-butanol–acetic acid–water – 4:1:1 v/v for tyrosine, and acetonitrile–water – 4:1 v/v for DOPA). The concentration of the above-mentioned compounds was determined spectrophotometrically as described elsewhere.[21–24] The extent of deuterium incorporation at 3′ and 5′ of phenyl ring in deuteriated L-Tyr was checked by measuring the proton NMR spectra on a Varian 500 MHz Unity-Plus spectrometer. The radioactivity of all samples was determined using an automatic liquid scintillation counter, LSC, (LISA LSC PW470 – Raytest, Germany).

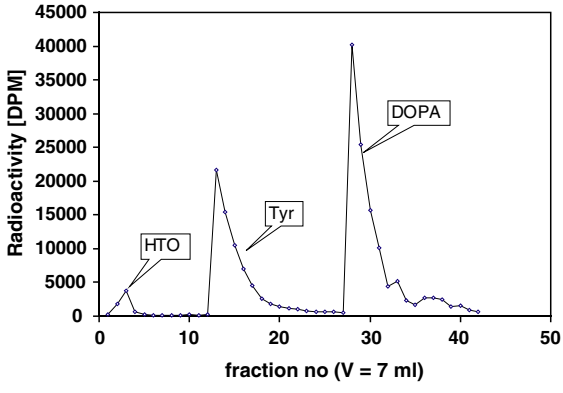
Synthesis of [3′,5′-$^3H_2$]-L-Tyr, **1**

To the glass reaction ampoule were added in turn: 200 mg (1.1 mmol) of L-tyrosine, 0.2 ml of conc. HCl, and 1 ml of tritiated water with total activity 5.2 GBq. The contents of the ampoule were frozen with liquid nitrogen, degassed under vacuum and the vial was sealed and thermostated at 130°C for 24 h. After cooling the ampoule has been opened and tritiated water was removed by lyophylization. The residue was dissolved in a small amount of water (about 1 ml) and loaded onto a column (Dowex 50WX-4-50 H^+ form). To remove the residual HTO and tritium incorporated into labile positions of tyrosine (-NH_2, -COOH groups) the column was washed with water up to the moment when the radioactivity of eluted fractions was steady and close to background. Next **1** was eluted with 0.5 M NH_3(aq) and collected as 3 ml fractions. For each fraction 10 µl samples were taken for the radioactivity assay. The fractions containing **1** were combined and evaporated under vacuum at 50°C and the purity checked by TLC. As a result a sample of 137 mg (0.75 mmol) of **1** was obtained with a total activity of 4.16 MBq (5.5×10^6 Bq/mmol specific activity) and chemical yield 75%.

Synthesis of [5′-^3H]-L-DOPA, **2**

To a vial containing 10 ml of phosphate buffer (pH 6.1), 22 mg (122 µmol) of **1** of total radioactivity 6.7×10^5 Bq, 44 mg (0.25 mmol) of ascorbic acid, and 0.5 ml (1250 U) of the enzyme mushroom tyrosinase (EC 1.14.18.1) from *Neurospora crassa* were added. The reaction mixture was incubated at room temperature for 20 min with aeration and stirring. The volume of post-reaction mixture was reduced to about 1 ml by lyophilization. The product obtained, **2**, was separated on the Dowex1X8 column (10×100) prepared as described elsewhere.[25] The column was eluted with water (about 100 ml) to wash out the buffer salts and tritiated water up to the moment when the radioactivity of eluted fractions was steady and close to background. First, unreacted

[3′,5′-$^3H_2$]-L-Tyr, **1**, was eluted with 0.2 M boric acid. Next, [5′-^{3}H]-L-DOPA, **2**, was eluted with 0.75 M HCl. The presence of L-DOPA was also checked using its colour reaction[24] with KIO_3. Radioactivity of the eluted fractions was determined using a liquid scintillation counter. Radiochromatogram from this synthesis is depicted in Scheme 3. As a result a 11.5 mg (63 μmol) sample of **2** was obtained with total radioactivity 1.7×10^5 Bq (sp. activity 2.7 MBq/mmol) and chemical yield 48%. The purity of **2** obtained was also checked by TLC.



Scheme 3. Radiochromatogram of the post-reaction mixture from synthesis of [5′-^{3}H]-L-DOPA

Synthesis of [3S-^{3}H]-L-DOPA, **3**

To the incubation vial containing 7 ml of phosphate buffer (pH 6.1), 18 mg (100 μmol) of [3S-^{3}H]-L-tyrosine of total radioactivity 3.1×10^7 Bq, 36 mg (0.2 mmol) of ascorbic acid and 0.1 ml (about 250 U) of the enzyme mushroom tyrosinase (EC 1.14.18.1) from *Neurospora crassa* were added. The reaction mixture was incubated at room temperature for 20 min with constant stirring, and aerating. The separation procedure and purification of **3** was the same as described in 'Methods' section. As a result a 10 mg (53 μmol) sample of **3** was obtained with total radioactivity of 1.6×10^7 Bq (specific activity 3×10^8 Bq/mmol) and radiochemical yield 53%.

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