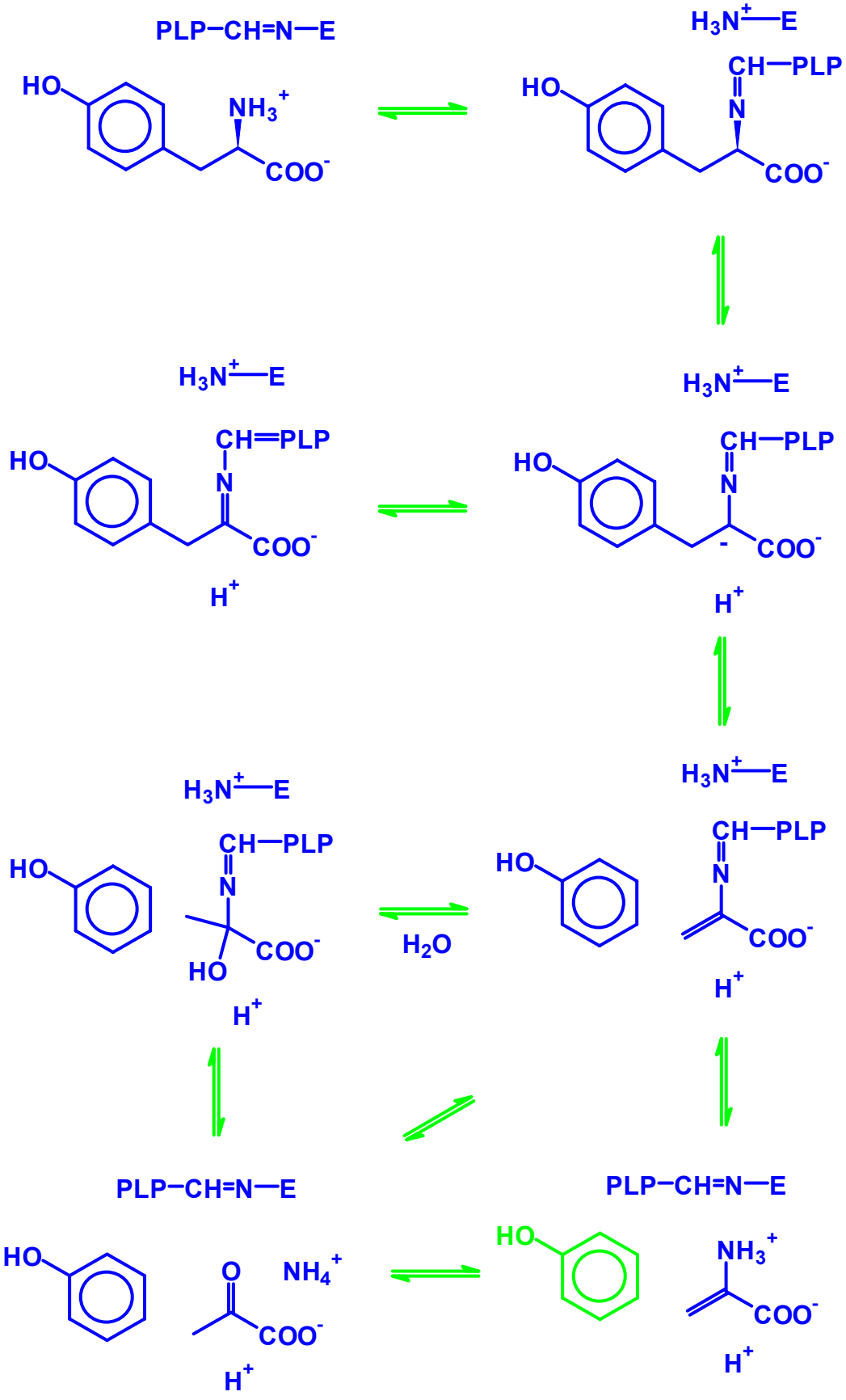
INTRODUCTION

In the living cells, the enzyme L-tyrosine phenol lyase (TPL, EC 4.1.99.2) catalyses decomposition of L-tyrosine (L-Tyr) to phenol, pyruvate and ammonia.

TPL also participates in the formation of many important derivatives of L-tyrosine such as L-dopa, variously halogenated L-tyrosine (starting from various phenol derivatives) or 3-methyl-L-tyrosine (very important kinase inhibitor)[2].

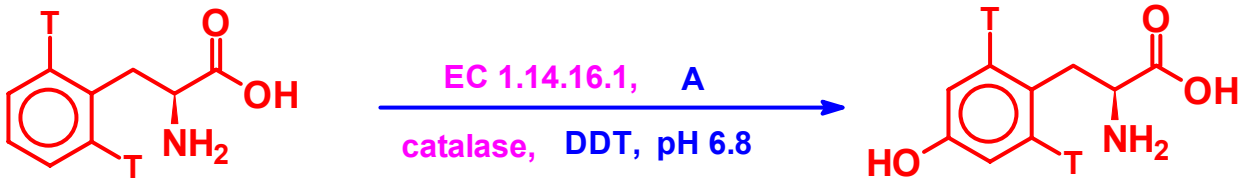
The proposed mechanism[3] of TPL action leads through binding of cofactor pyridoxal phosphate (PLP), which enables formation of carboanion and subsequent release of phenol ring with further rearrangement to pyruvate and liberation of PLP. This route was confirmed by several various experiments, especially with UV-VIS spectroscopy.

PROPOSED MECHANISM OF TPL ACTION



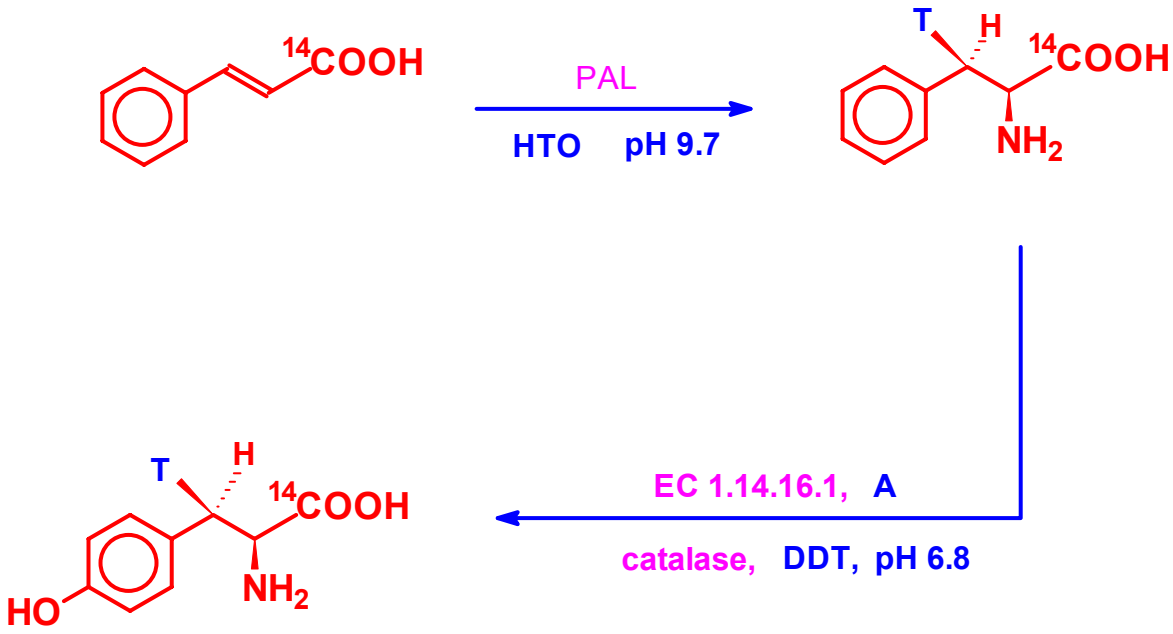
This study is directed towards investigating the mechanism of the reaction with kinetic isotope effect (KIE) method. Some isotope studies have already been performed[3], although there is no good, systematic evidence in kinetic isotopic effects area. It seems to be very interesting, especially due to the possibility of multiple hydrogen transfer, tunnelling effects and comparison of effects on 3*S* and 3*R* positions. Our goal is to synthesize variously labelled (with ^{3}H and ^{14}C) L-tyrosine and to use them in kinetic isotope effect investigations. Here, the syntheses of [2',6'-$^{3}H_2$]-L-tyrosine, [3*S*-^{3}H]-[1-^{14}C]-L-tyrosine and [1-^{14}C]-L-tyrosine; and their use in determination of $^{1}H/^{3}H$ kinetic isotope effects at 2',6' and 3*S* positions are reported.

Synthesis of [2',6'-$^3H_2$]-L-tyrosine



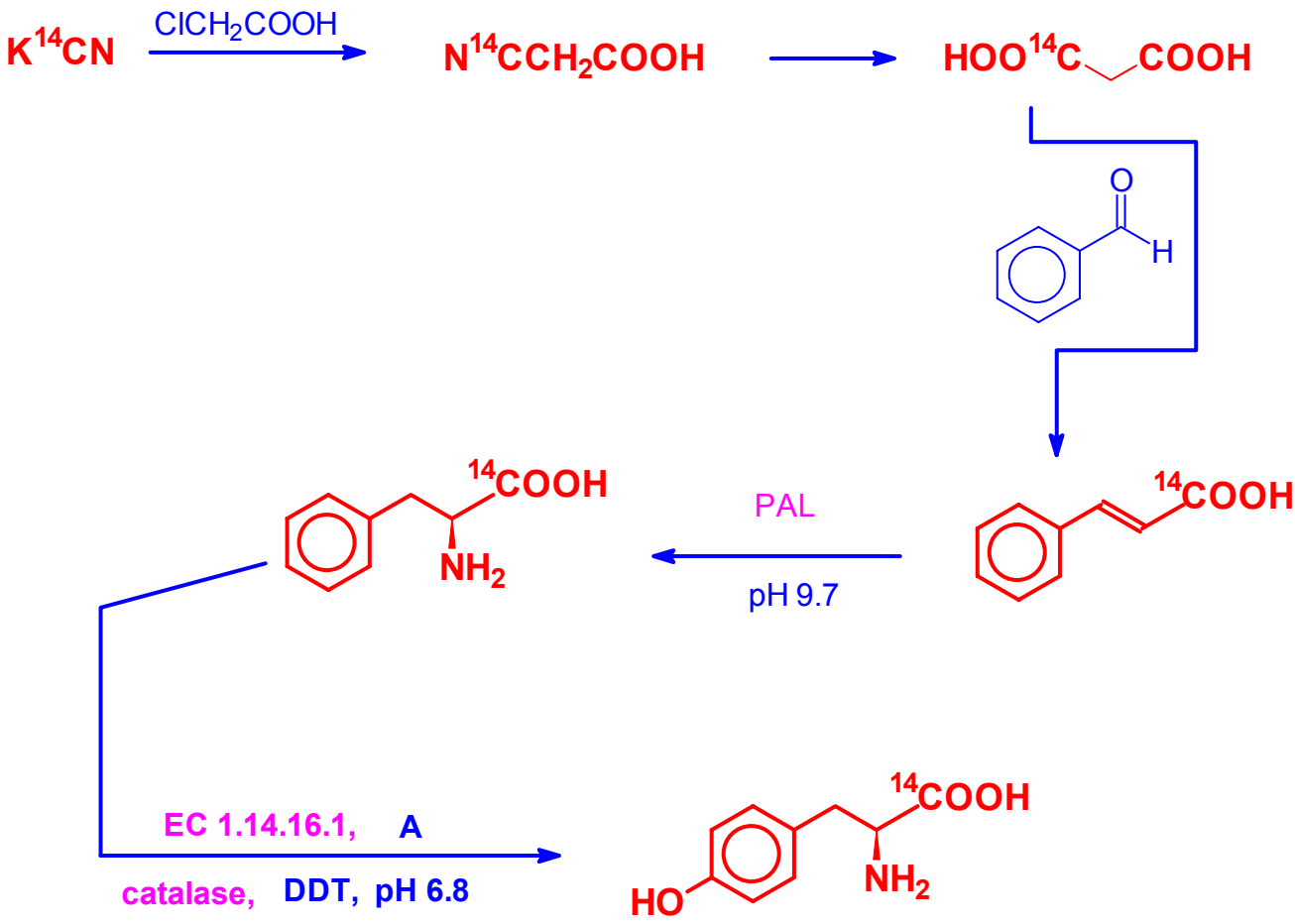
[2',6'-$^3H_2$]-L-tyrosine was obtained starting from [2',6'-$^3H_2$]-L-phenylalanine (purchased from Sigma). The enzyme 4'-phenylalanine monooxygenase (E.C. 1.14.16.1) isolated from rat liver was used to perform this conversion. D,L-6-methyl-5,6,7,8-tetrahydropterine (**A**) was applied as a cofactor, D,L-dithiotreitol (**DTT**) was used as an oxygen carrier and catalase from bovine liver (Sigma) was applied to avoid formation of hydrogen peroxide and further radical reactions. The reaction yielded 58% of [2',6'-$^3H_2$]-L-tyrosine (76kBq, 1.8μmol) after purification on the Amberlit IR-120 (H^+) – cation exchange resin – and preparative TLC on cellulose.

Synthesis of [3*S*-^{3}H]-[1-^{14}C]-L-tyrosine



[3*S*-^{3}H]-[1-^{14}C]-L-Tyr was obtained starting from [1-^{14}C]-cinnamic acid. The starting substrate was converted to [3*S*-^{3}H]-[1-^{14}C]-L-Phe with the enzyme PAL after addition of HTO to the reaction mixture. PAL stereospecifically incorporates hydrogen atom from water into 3*S* position. Resulted [3*S*-^{3}H]-[1-^{14}C]-L-Phe was oxidized (enzyme E.C. 1.14.16.1) to [3*S*-^{3}H]-[1-^{14}C]-L-Tyr with 82% yield.

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



[1-^{14}C]-L-Tyr was obtained starting from K^{14}CN in a few step synthesis. An intermediate, i.e. [1-^{14}C]-cinnamic acid, was converted to [1-^{14}C]-L-Phe by addition of ammonia catalysed by L-phenylalanine ammonia-lyase (PAL, E.C. 4.3.1.5). Obtained [1-^{14}C]-L-phenylalanine[5] was converted to [1-^{14}C]-L-tyrosine as described above.

KIE ASSAYS

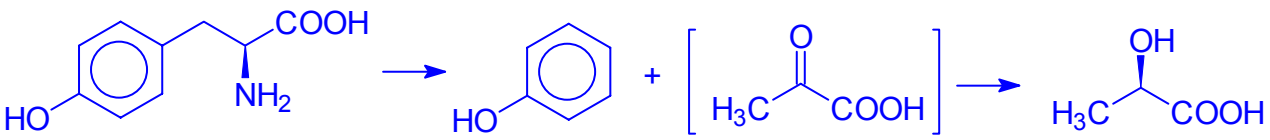
$^{1}H/^{3}H$ kinetic isotope effects (KIEs) were measured with internal radiometric standard, i.e. the ^{14}C atom of carboxyl group was used as a marker. We are convinced that this carbon atom is not involved in the mechanism of action of TPL.

$^{1}H/^{3}H$ kinetic isotope effects for [2',6'-$^{3}H_2$] and [3*S*-^{3}H] positions in enzymatic decomposition of L-Tyr are both secondary ones, which means that bonds between those atoms and carbon atoms that they are attached to are not broken during the rate-limiting steps of the reaction. The effects are due to higher masses of ^{3}H atoms than ^{1}H. Only the masses of molecules are changed, thus they cannot have a great effect on the rate of the reaction and the investigated kinetic isotope effects cannot be large. Also changes in C-H (2',6'-H and 3*S*-H case) vibrations frequencies do not impact the rate of the reaction, because they are too far from the „hot center" of the molecule. It is not surprising if we consider the mechanism of action of TPL. It does not involve hydrogen atoms in [2',6'-$^{3}H_2$] and [3*S*-^{3}H], neither. We have not found any mechanism involving those hydrogen atoms.

The 2',6' $^{1}H_2/^{13}H_2$ kinetic isotopic effect (1.01) is a secondary effect connected with the change of mass of the ^{3}H labelled L-Tyr molecule.

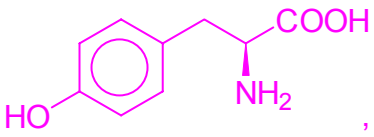
The 3*S* $^{1}H/^{3}H$] is also a secondary effect, and it is a little lower (1.00) than previous one, although 3*S* hydrogen is placed much closer to the „hot center" of L-Tyr molecule (the atom is attached to the 3-carbon atom, which forms the C-C bond broken in the reaction course). We would expect a little higher secondary effect, but it is probably decreased by hyperconjugation of the methyl group of formed pyruvate. Hyperconjugation causes a little inverse secondary kinetic isotope effect.

KIE EXPERIMENTAL PROTOCOL



HCl, temp.

reaction products



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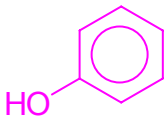

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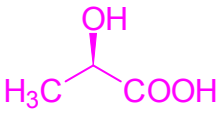
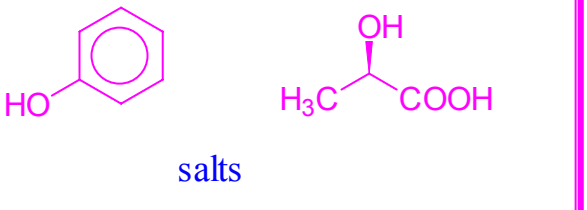


proteins, cofactors, salts

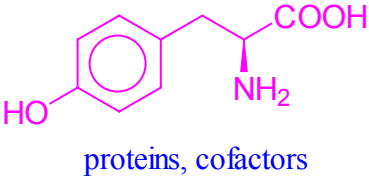
AMBERLIT IR-120 (H^+)

1. H_2O

2. 0,3M NH_3



salts



proteins, cofactors

RADIOMETRIC MEASUREMENT, LSC

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