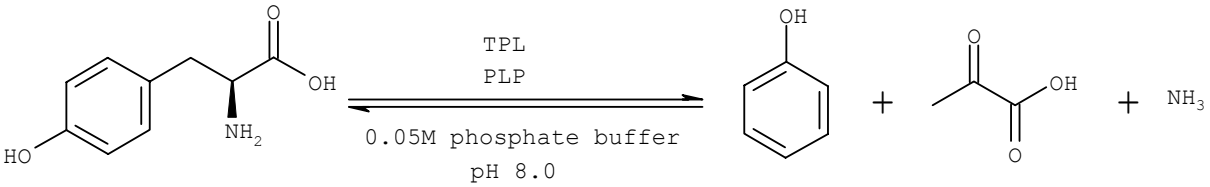
KINETIC ISOTOPIC EFFECTS IN ENZYMATIC DECOMPOSITION OF L-TYROSINE

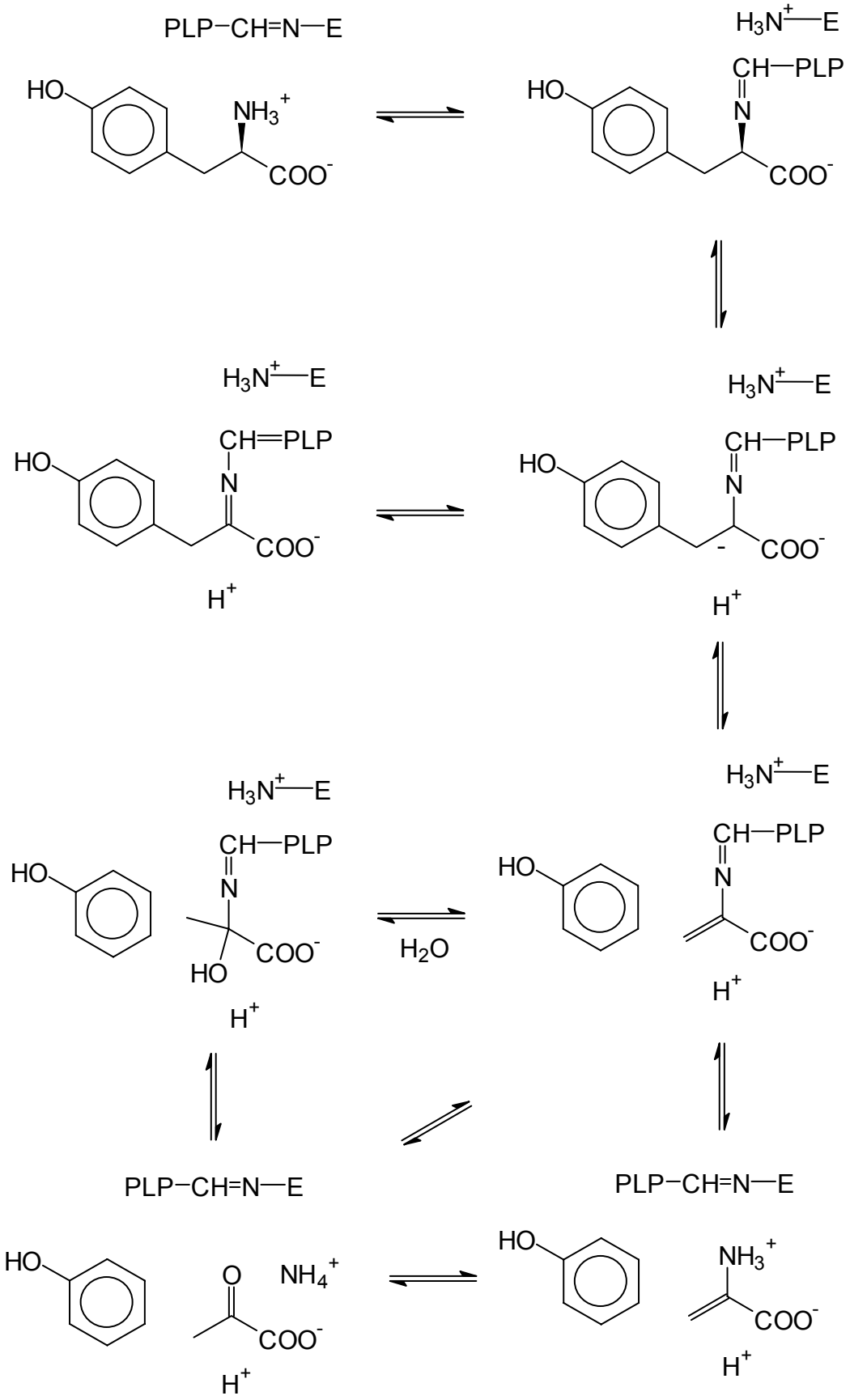
Extended abstract

L-tyrosine phenol-lyase (TPL, E.C. 4.1.99.2) is an enzyme which catalyses reversible decomposition of L-tyrosine to phenol, pyruvate and ammonia (scheme 1)[1].



Scheme 1. Reaction catalyzed by TPL.

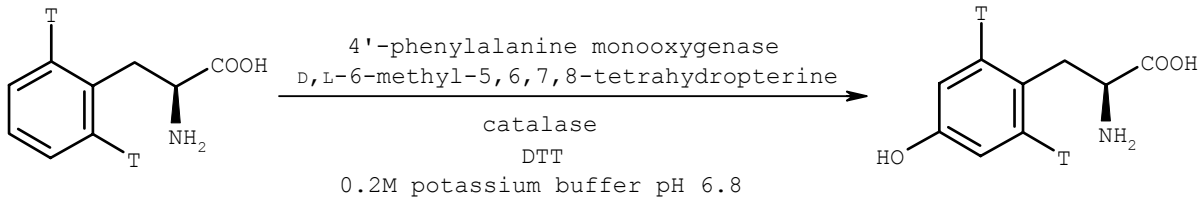
This enzyme is interesting to organic chemists, because it also catalyses formation of many important derivatives of L-tyrosine such as L-dopa, variously halogenated L-tyrosine (starting from various phenol derivatives) or 3-methyl-L-tyrosine (very important kinase inhibitor)[2]. Proposed mechanism (scheme 2)[3] of TPL action proceeds through binding of cofactor pyridoxal phosphate (PLP), which enables forming of carboanion and subsequent release of phenol ring with further rearrangement to pyruvate and liberation of PLP. This route was confirmed by many different experiments, especially with electron spectroscopy. Some isotope studies have already been performed[3], although there is no good, systematic evidence in the kinetic isotope effects area. It seems to be very interesting especially because of 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 labeled (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.



Scheme 2. Mechanism of decomposition of L-Tyr catalyzed by enzyme TPL.

Syntheses of differently labeled isotopomers of L-tyrosine.

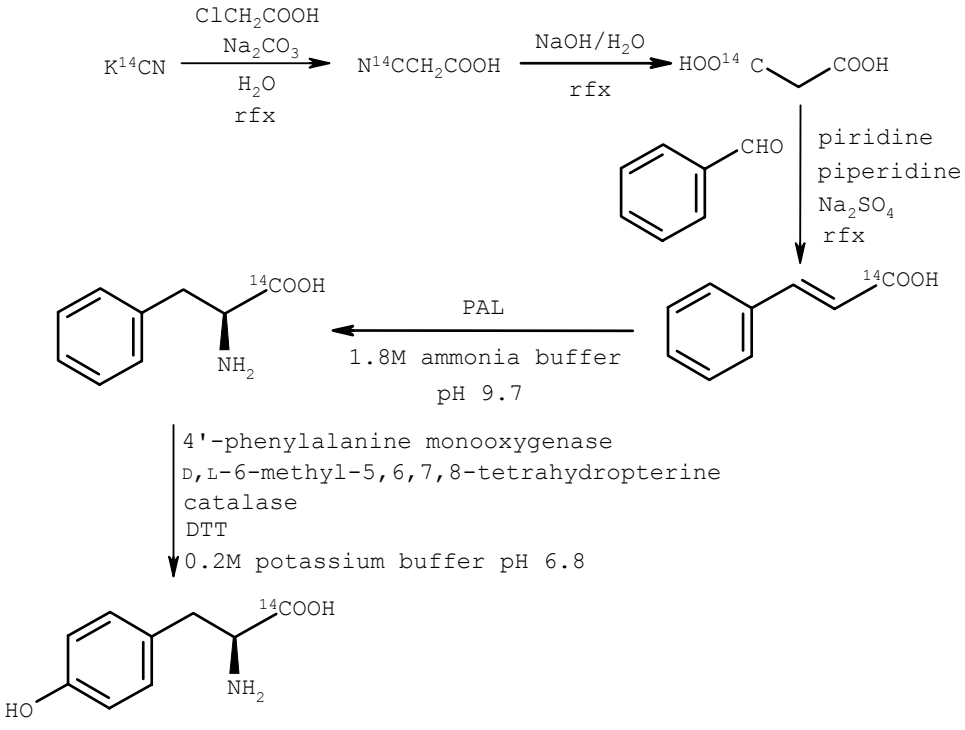
Synthesis of [2',6'-$^3H_2$]-L-tyrosine



Scheme 3. 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; scheme 3). The enzyme 4'-phenylalanine monooxygenase isolated from rat liver was used to perform the conversion. D,L-6-methyl-5,6,7,8-tetrahydropterine 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 a cellulose.

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



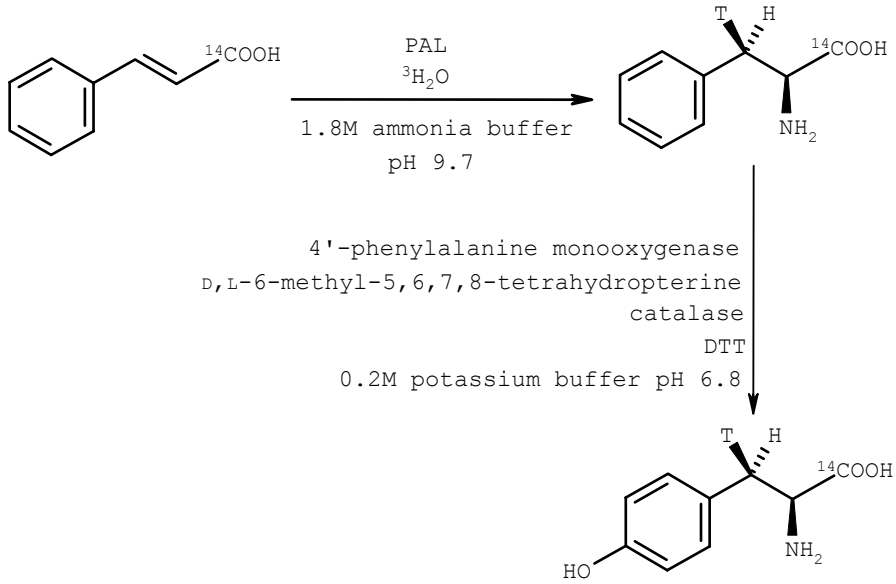
Scheme 4. Synthesis of [1-^{14}C]-L-tyrosine.

[1-^{14}C]-L-tyrosine was obtained starting from K^{14}CN (purchased from Polatom) in a few step procedure (scheme 4). At first, labelled cyanide was incorporated into

2-cyanoacetate by substitution of chlorine atom in aqueous solution. Formed [3-^{14}C]-2-cyanoacetate was hydrolyzed in harsh basic conditions (aqueous NaOH) to [1-^{14}C]-malonate with 48% yield in the 2 steps. Resulting [1-^{14}C]-malonate was converted to [1-^{14}C]-cinnamic acid using the Knoevenagel reaction in pyridine with piperidine with 77% yield (28MBq, 4.0mmol). A half of ^{14}C was lost in this reaction.

Obtained [1-^{14}C]-cinnamic acid was converted to [1-^{14}C]-L-phenylalanine by addition of ammonia catalysed by L-phenylalanine ammonia-lyase (PAL, E.C. 4.3.1.5) from *Rhodotorula glutinis* (Sigma) in ammonia buffer. After extraction of non-reacted [1-^{14}C]-cinnamic acid and separation of phenylalanine on Amberlit IR-120(H^+) – cation exchange resin – 37% yield of [1-^{14}C]-L-phenylalanine (226 kBq, 33 μmol) was obtained[4]. The low yield is quite usual, since it is caused by the reaction equilibrium. Obtained [1-^{14}C]-phenylalanine was converted to [1-^{14}C]-L-tyrosine (168kBq, 24μmol) with 68% yield[5] as previously described.

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



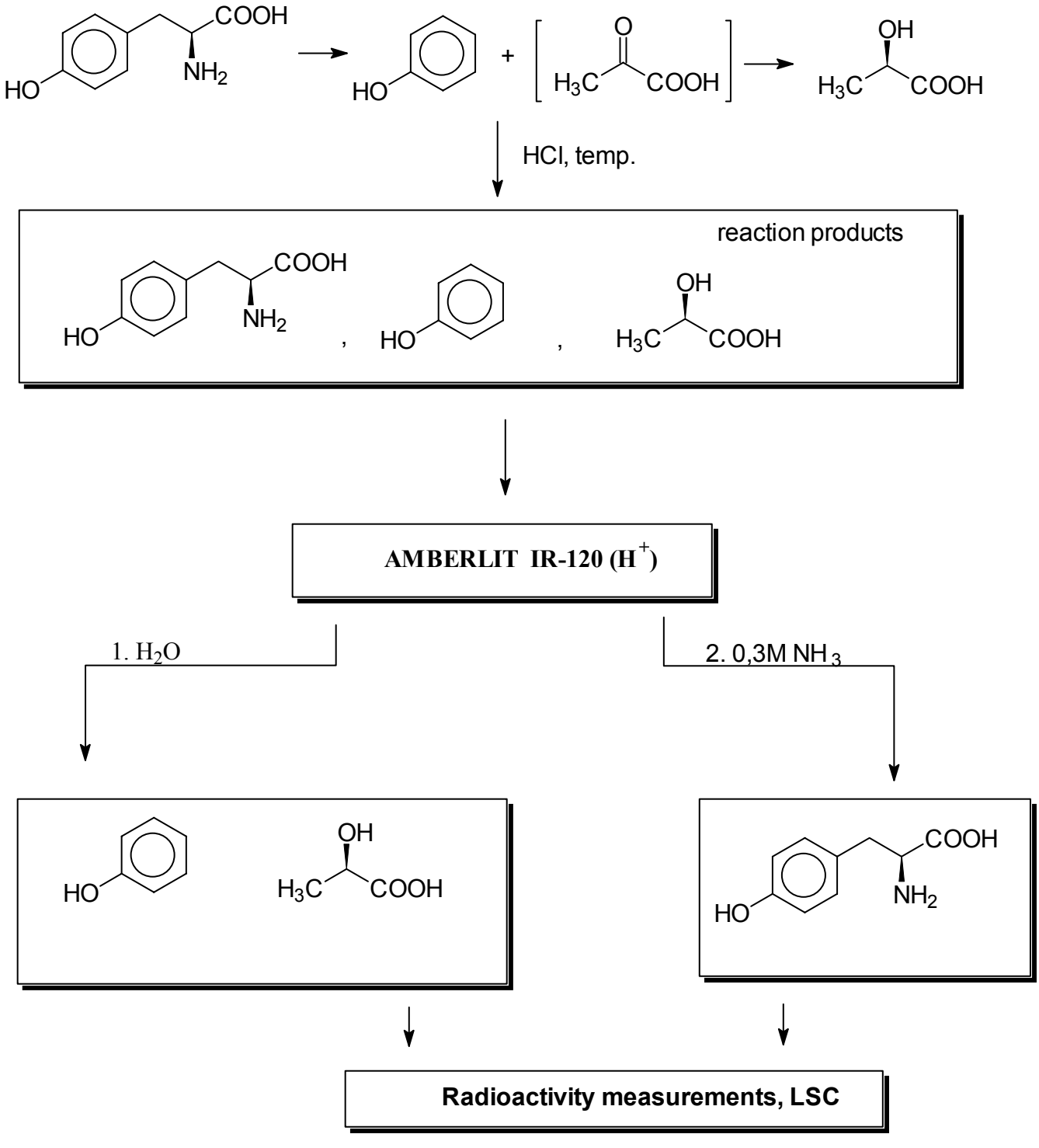
Scheme 5. Synthesis of [3*S*-^{3}H]-[1-^{14}C]-L-tyrosine.

[3S-^{3}H]-[1-^{14}C]-L-tyrosine was obtained starting from [1-^{14}C]-cinnamic acid (scheme 5) that was prepared as described above. Starting substrate was similarly converted to [3*S*-^{3}H]-[1-^{14}C]-L-phenylalanine with PAL after addition of $^{3}H_2O$ to the reaction mixture. PAL stereospecifically incorporates hydrogen atom from water into 3*S* position. This reaction yielded 10% of [3*S*-^{3}H]-[1-^{14}C]-L-phenylalanine (69kBq ^{14}C, 147kBq ^{3}H, 10μmol). Resulting [3*S*-^{3}H]-[1-^{14}C]-L-phenylalanine was oxidized to the [3*S*-^{3}H]-[1-^{14}C]-L-tyrosine with 82% (19kBq ^{14}C, 40kBq ^{3}H, 2.7μmol) in similar manner as described previously.

Kinetic isotope effect investigations

$^{1}H/^{3}H$ kinetic isotope effects (KIE) were measured using dual label methodology. Compounds (L-tyrosines) were labelled with ^{3}H in target positions (2',6'-$^{3}H_2$ and 3*S*) as described previously. As a ^{14}C marker carbon atom of carboxyl group was used. We are convinced that this carbon atom is not involved in the mechanism of action of TPL. ^{14}C-labelling procedure was also described previously.

Labeled L-tyrosine was decomposed in 0.05M phosphate buffer pH 8.0 containing TPL (isolated from expression strain of *Escherichia coli* with pTXB4E plasmid after induction with L-tyrosine), PLP, lactate dehydrogenase (LDH, E.C. 1.1.1.27) from rabbit muscle (Sigma) to reduce formed unstable pyruvate to lactate; and NADH (cofactor of LDH). The reaction normally occurs with about 50% conversion. Reaction was stopped by acidifying and heating at different degrees of conversion. The products (phenol, pyruvate) were separated from non-reacted substrate (L-tyrosine) on the Amberlit IR-120(H^{+}) by washing with water, since the products do not contain any positively charged group (scheme 6). Then the non-reacted substrate was washed out with 0.3M aqueous ammonia solution (converting positively charged amino groups, which attached amino acid to the resin, into neutral amino group), which enabled washing out of L-tyrosine from the resin. The radioactivities of ^{3}H and ^{14}C of separated products and substrates were measured. There was no need to measure amounts of compounds separately, since they were already determined by the radioactivity of ^{14}C marker, which is a great advantage of dual label method. Kinetic isotopic effects were calculated using ratios of $^{3}H/^{14}C$ as equivalents of specific radioactivities and degrees of conversions calculated from activities of ^{14}C marker measured for separated products and non-reacted substrates.



Scheme 6. Separation procedure and KIE determination

Discussion

Measured $^{1}H/^{3}H$ kinetic isotope effects for 2',6' and 3*S* positions in enzymatic decomposition of L-tyrosine are both secondary ones, which means that bonds between those atoms and carbon atoms that they are attached to are not broken during rate-limiting, the slowest steps of the reaction. The effects are due to higher masses of ^{3}H atoms than ^{1}H. The masses of molecules are changed, thus they cannot have a great effect on the reaction rate and investigated kinetic isotope effects cannot be high. The changes in C-H (2',6'-H and 3*S*-H case) vibration frequencies do not impact the rate of reaction, because they are too far from the "hot centre" 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' and 3*S* positions, neither. We have not found any mechanism involving these hydrogen atoms.

The 2',6'-$^{1}H_2/^{3}H_2$ kinetic isotope effect (1.01) is an usual secondary effect connected with simply change of mass of the ^{3}H labeled L-tyrosine 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 centre" of L-tyrosine molecule (i.e. 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 always causes a little inverse secondary kinetic isotopic effect.

Next step that we are going to investigate are hydrogen ($^{1}H/^{3}H$, or $^{2}H/^{3}H$) kinetic isotope effects in positions 3*R* and 2. We also aim to measure $^{12}C/^{14}C$ effects in positions 2, 3, and 1'. Determination of all these kinetic isotope effects should result in the detailed analysis of the mechanism of enzymatic decomposition of L-tyrosine. Particularly, comparison of hydrogen effects in 3*S* and 3*R* positions and investigation of possible tunnelling effects should be very interesting.

References

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