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Kinetic and solvent isotope effects on biotransformation of aromatic amino acids and their derivatives

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Aromatic amino acids such as L-phenylalanine, L-tryptophan, 3′,4′-dihydroxy-L-phenylalanine (L-DOPA), and their derivatives 3′,4′-dihydroxyphenylacelaldehyde (DOPAL) and 3′,4′-dihydroxyphenylethanol (DOPET), play an essential role in human metabolic processes. Incorrect or slow biotransformation of these compounds leads to some metabolic dysfunctions and in some cases to some neurodegenerative diseases. Therefore, studies of the biotransformation mechanisms of these metabolites draw biochemists' and medical researchers' attention. This study investigates the mechanisms of biotransformation of the aforementioned compounds using kinetic (KIE) and solvent (SIE) isotope effect methods. The overview presents the results and the numerical values of KIE and SIE methods, obtained in the study of biotransformation of L-phenylalanine, 5′-chloro-L-tryptophan, and L-DOPA, catalyzed by the enzymes from the lyases group (phenylalanine ammonia lyase, tryptophan indole-lyase, and tyrosine decarboxylase). Deuterium KIE was also determined during the deamination of 2′-chloro-L-phenylalanine in the presence of the enzyme L-phenylalanine dehydrogenase, as well as in the conversion of DOPAL into DOPET catalyzed by the enzyme alcohol dehydrogenase. The values of KIE and SIE have been determined using a noncompetitive spectrophotometric and a competitive (combined with internal radioactivity standard) radiometric methods.

Keywords: DOPAL; enzymes; isotope effects; L-DOPA; L-phenylalanine; L-tryptophan

Introduction

The mechanistic studies of reactions catalyzed by some enzymes from the groups of lyases (phenylalanine ammonia lyase, tyrosine decarboxylase, and tryptophan indole-lyase), and dehydrogenases (L-phenyloalanine dehydrogenase, and alcohol dehydrogenase) using kinetic isotope effect (KIE) and solvent isotope effect (SIE) methods. The numerical values of KIE and SIE are very helpful for clarifying the mechanistic intrinsic details or for distinction between the alternative mechanisms.[1]

Phenylalanine ammonia lyase, PAL, (EC 4.3.1.5), an enzyme commonly found in the higher plants and in some species of fungi, plays an important role in the metabolism of plants. The conversion of L-phenylalanine, L-Phe, into cinnamic acid is the first step in the biosynthesis of many important biological phenylpropanoid derivatives such as lignins, flavonoids, isoflavonoids, some of alkaloids, furanocoumarins, and the plant hormones. It is also involved in the biosynthesis of flavonoid pigments and of compounds protecting plants against harmful pathogenic factors (e.g., UV) and defending against herbivores.[2] PAL is used in experiments related to the treatment and diagnosis of phenylketonuria[3] and producing L-Phe from (*E*)-cinnamic acid by addition of ammonia *in vivo*.[4] Generally, PAL catalyzes the reversible elimination of ammonia from L-Phe yelding (*E*)-cinnamic acid, Scheme 1.

The mechanism of ammonia elimination from L-Phe has gained much attention and has been discussed by many research groups. This publication refers only to the ones,[5–7] being the most representative and useful in the discussion of this investigation of KIE and SIE isotope effects determined for the reaction catalyzed by PAL.

The tyrosine decarboxylase enzyme catalyzes the decarboxylation of 3′,4′-dihydroxy-L-phenylalanine (L-DOPA) to dopamine; see Scheme 2. Both these compounds, found in nervous systems of mammals, are important neurotransmitters and hormones.[8] The disturbance in the production and metabolism of dopamine and its derivatives leads to development of many pathologies, including the neurological disorders, such as Alzheimer's, Parkinson's diseases, and schizophrenia. The incorrect metabolism of L-DOPA induces the accumulation of some toxic metabolites, that is, 3′,4′-dihydroxyphenylacetaldehyd (DOPAL) and 3′,4′-dihydroxyphenylethanol (DOPET), which – combined with the

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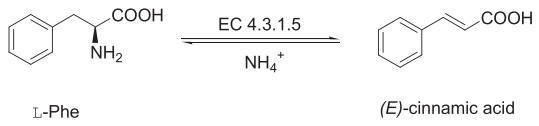
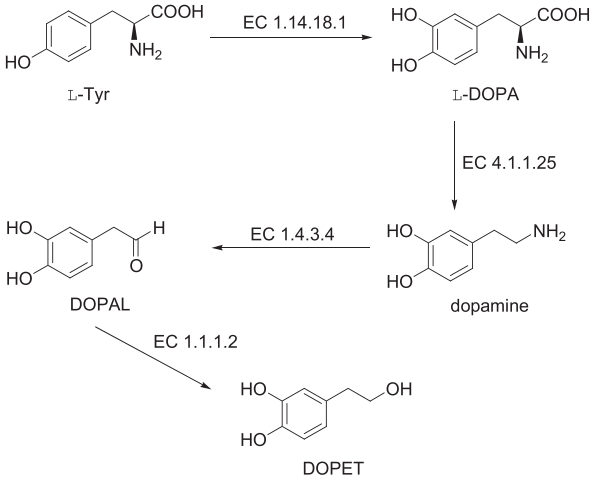


Scheme 1. The reversible reaction catalyzed by the enzyme phenylalanine ammonia lyase (EC 4.3.1.5).



Scheme 2. Incorrect metabolism of L-Tyr to DOPET.

imbalance of protective enzymes – may result in degeneration of dopamine neurons in the brain.

This research determines ^{14}C KIE for the 1-position of L-DOPA in its decarboxylation to dopamine, catalyzed by the tyrosine decarboxylase enzyme (EC 4.1.1.25); see Scheme 2. The deuterium KIE and SIE in reduction of DOPAL to DOPET in the presence of a cofactor nicotinamide adenine dinucleotide phosphate (NADPH), catalyzed by the alcohol dehydrogenase enzyme (ALDH, EC 1.1.1.2), have also been investigated; see Scheme 2.

The mechanism of action of halogen derivatives of amino acids and bioamines, labeled with the short-lived β^+-isotopes and used in medicinal diagnostics,[9] is not fully understood because the presence of halogen in their structure causes a change in the catalytic activity of enzymes involved in their degradation. Therefore, this research investigates the mechanism of their biodegradation using the KIE and SIE methods. The tryptophan indole-lyase enzyme (TPase, EC 4.1.99.1) catalyzes the chemical reversible reaction[10]; see Scheme 3.

This enzyme was used for determination of deuterium KIE and SIE in decomposition of 5′-chloro-L-tryptophan.

Phenylketonuria (PKU)[11] is a human genetic disease caused by a mutation in the gene responsible for coding of L-phenylalanine dehydrogenase enzyme (PheDH, EC 1.4.1.20), which converts L-Phe to L-tyrosine. The accumulated L-Phe is metabolized by enforced side reactions[12] to phenylpyruvate, phenylacetate, and phenyl-L-lactate; see Scheme 4. The excess of L-Phe in cells hinders the transport of other amino acids, causing a reduction in neurotransmitters and melanins synthesis. PKU leads to mental retardation, brain damage, and skin discoloration.

For this purpose, as mentioned earlier, the deuterium KIE and SIE for 2′-chloro-[(3*S*)-^{2}H]-L-Phe in the oxidative deamination reaction catalyzed by PheDH were investigated; see Scheme 5.

The results of studies of the KIE and SIE for the aforementioned enzymatic reactions, useful for explaining some details of their mechanisms, have not been published yet.

Experimental

Materials

The enzymes: PAL (EC 4.3.1.5) from *Rhodotorula glutinis*, Tyrosine Decarboxylase (EC 4.1.1.25) from *Streptococcus faecalis*, Tyrosinase (EC 1.14.18.1) from *Neurospora crassa*, TPase (EC 4.1.99.1) from *Escherichia. coli*, Lactate Dehydrogenase (EC 1.1.1.27) from rabbit muscle, PheDH (EC 1.4.1.20) from *Sporosarcina* sp., ALDH (EC 1.1.1.2) from *Thermoanaerobium brockii*, Malic Dehydrogenase (EC 1.1.1.40) from chicken liver, Glucose Dehydrogenas (EC 1.1.1.47) from *Pseudomonas* sp., as well as the cofactors: PLP (Pyridoxal 5′-Phosphate), NADH and NAD^+ (nicotinamide adenine dinucleotide – reduced and oxidized forms), NADPH and $NADP^+$ (nicotinamide adenine dinucleotide phosphate – reduced and oxidized forms) were from Sigma.

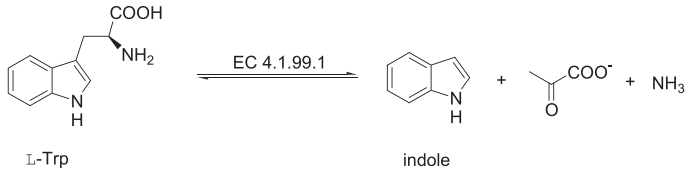
Radioactive preparations, that is, [^{14}C]KCN, [^{14}C]$BaCO_3$, and [1-^{14}C]-sodium acetate were supplied from Radioisotope Centre Polatom, Świerk, Poland. [1-^{14}C]- and [2-^{14}C]-malonic acids and ^3H_2O were purchased from NEN, Science Products Inc., Boston, USA. [1-^{14}C]-L-tyrosine and ^3H_2O were from Moravec Biochemical, USA. The producers of deuteriated substrates used in our experiments are listed as follows: Heavy water (99.9% D) (Sigma, St. Louis, MO, USA); [1-^{2}H]-D-glucose (Aldrich); concentrated $^2HCl/^2H_2O$, 85% $^2H_3PO_4/^2H_2O$, and 30% $KO^2H/^2H_2O$ (POLATOM).

Liquid scintillations cocktails were produced by Rotiszint Eco Plus Roth, Germany.

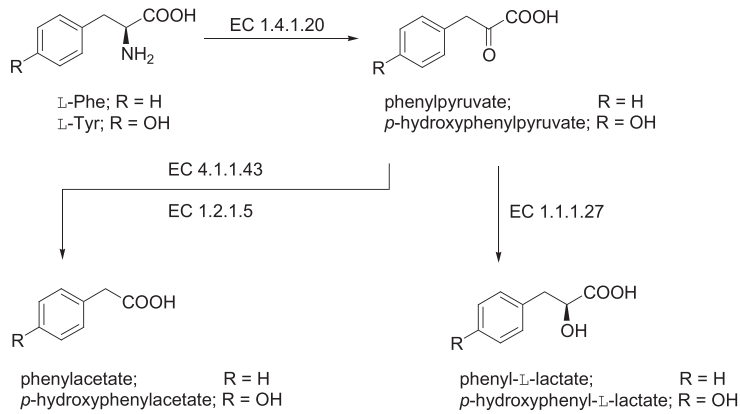
The other chemicals and solvents needed for trial synthesis, kinetics runs, and separation and purification procedures were purchased from Aldrich.

Methods

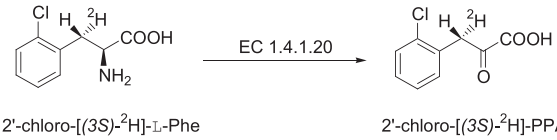
The radioactivity of all samples was determined using a liquid scintillation counter (LISA, LSC PWW470, Germany) or Tri-Carb 2910 TR, PerkinElmer (Waltham, MA, USA). In the course of synthesis and the column chromatographic separation, the presence of substrates and products was checked by thin-layer chromatography using silica gel plates (visualization by UV lamp). The concentration of substrates and products were determined using UV–VIS spectrometer (Shimadzu-UV-1202, Kyoto, Japan). The ^{1}H NMR spectra were recorded on 200 MHz Unity + spectrometer using heavy water as solvent and TMS (tetramethylsilane) as internal standard.



Scheme 3. The reversible enzymatic synthesis/decomposition of L-Trp catalyzed by the enzyme tryptophanase (EC 4.1.99.1).



Scheme 4. The enforced metabolic pathways occurring in persons suffering from PKU disease.

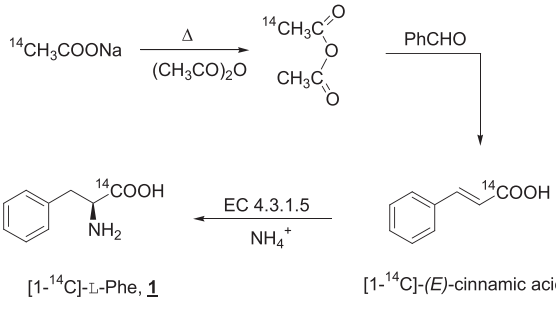


Scheme 5. The deamination of 2′-chloro-[(3*S*)-^{2}H]-L-Phe catalyzed by the enzyme phenylalanine dehydrogenase (EC 1.4.1.20).

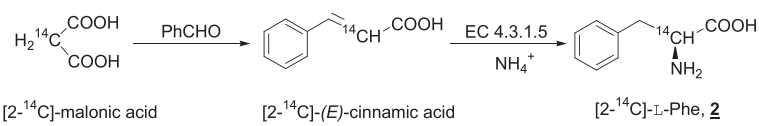
Synthesis and kinetics runs

KIE values for the elimination of ammonia from L-Phe catalyzed by the enzyme PAL

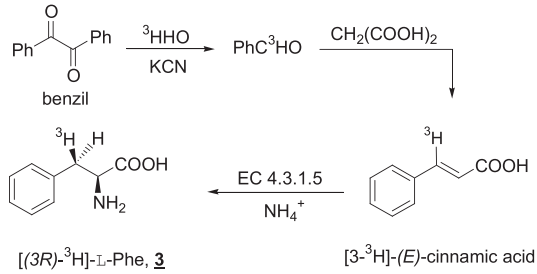
All isotopomers of L-Phe labeled with deuterium, tritium, and ^{14}C, needed for kinetics assays, were synthesized according to the methods described earlier. The isotopomer [1-^{14}C]-L-Phe, **1**, (used as internal radiometric standard) was prepared by the conversion of [1-^{14}C]-CH_3COONa in acetic anhydride, [1-^{14}C]-$(CH_3O)_2O$ and the followed condensation of the resultant product with an benzaldehyde, PhCHO, leading to [1-^{14}C]-cinnamic acid, which was enzymatically converted[13] into **1**, Scheme 6. The isotopomer [2-^{14}C]-L-Phe, **2**, was obtained by the, partly similar, combined chemical and enzymatic methods[14] using [2-^{14}C]-malonic acid as a source of label, Scheme 7. Also, the isotopomers [(3*R*)-^{3}H]-L-Phe, **3**, Scheme 8, and [(3*S*)-^{3}H]-L-Phe, **4**, Scheme 9, were synthesized by the chemical and enzymatic procedures.[15] The doubly labeled [(3*S*)-^{2}H/^{3}H]-L-Phe, **5**, was obtained according to the Scheme 9 by addition of ammonia to cinnamic acid carried out in fully deuteriated incubation medium containing tritiated water. The deuteriated [(3*R*)-^{2}H]-L-Phe, **6**, was synthesized as in Scheme 8 by cleaving benzil by the cyanide ion dissolved in heavy water and condensing the resulted deuteriated benzaldehyde, Ph^{2}HO, with malonic acid. The addition of ammonia to resulted [2-^{2}H]-cinnamic acid, catalyzed by PAL leads to **6**. [^{1}H NMR spectrum of native L-Phe: δ 3.115 (1H, β-H_S, d); 3.283 (1H, β-H_R, d); 3.983 (1H, α-H, d); 7.325 (2H, 2′and 6′-ArH, m); 7.371 (1H, 4′-ArH, m); 7.423(2H, 3′and 5′ArH). ^{1}H NMR spectrum of **6** – δ 3.117 (1H, β-H_S, d); 3.989 (1H, α-H, t); 7.327 (2H, 2′ and 6′-ArH, m); 7.374 (1H, 4′-ArH, m); 7.425 (2H, 3′ and 5′-ArH)]. The loss of the signal of the β-H_R-proton (δ 3.283) in the spectrum of **6** proves the near 100% incorporation of deuterium in this position. Also, the isotopomer [(3*S*)-^{2}H]-L-Phe, **7**, was obtained as in Scheme 9 by the addition of ammonia to cinnamic acid in deuteriated incubation medium. [^{1}H NMR for **7** – δ 3.285 (1H, β-H_R, d); 3.987 (1H, α-H, t); 7.326 (2H, 2′ and 6′-ArH, m); 7.375 (1H, 4′-ArH, m); 7.426 (2H, 3′and 5′-ArH)]. The disappearance of the signal from β-H_R-proton (δ 3.283) in



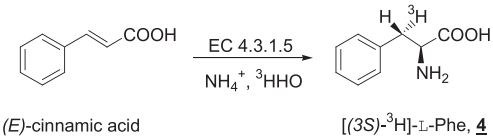
Scheme 6. Synthesis of [1-^{14}C]-L-Phe, **1** catalyzed by the enzyme phenylalanine ammonia lyase (EC 4.3.1.5).



Scheme 7. Synthesis of [2-^{14}C]-L-Phe, **2**.



Scheme 8. Synthesis of [(3*R*)-^{3}H]-L-Phe, **3**.



Scheme 9. Synthesis of [(3*S*)-^{3}H]-L-Phe, **4**, using the enzyme phenylalanine ammonia lyase (EC 4.3.1.5).

the spectrum of **7** proves the near 100% incorporation of deuterium in this position.

Procedure for ^{14}C KIE determination for the 1- and 2-positions of L-Phe. The kinetic runs of ammonia elimination from L-Phe were carried out in 25 mL encapped vials containing reaction mixture (labeled isotopomers of L-Phe, buffer, and enzyme PAL) at 25°C. The concentration and the specific activity of [1-^{14}C]-, and [2-^{14}C]-L-Phe in the mixture prepared for kinetics assays were determined as follows: The concentration of L-Phe in a precise sampled volume of reaction mixture was determined indirectly by its conversion into cinnamic acid, catalyzed by enzyme PAL. The subsequent spectroscopic measurement of absorbance of this product allows to calculate the concentration of L-Phe (c). The radioactivity (r) of exactly similar sample of reaction mixture was measured using liquid scintillation counter. Thus, the specific activity of substrate before the start of reaction – $R_0 = r/c$. In the course of kinetic assays, the samples with the exact similar volume were taken from the reaction mixture, and the reaction was quenched by the acidification to pH ~ 0. The unconverted substrate (L-Phe) and the resultant product (cinnamic acid) were separated by the extraction with 3 × 10 mL portions of diethyl ether. The concentration of cinnamic acid was determined spectroscopically while the radioactivity of L-Phe and cinnamic acid was measured on scintillation counter. This approach allows to calculate R_0, f (conversion factor), R_s (specific activity of L-Phe at the f), and R_p (specific activity of cinnamic acid at f). The ^{14}C KIE values for the 1- and 2-positions of L-Phe, calculated on the basis of formulas of Tong and Yankwich[16] are given in the Table 1.

Procedure for H/^{3}H KIE determination in the (3R)-position of L-Phe. For these kinetic runs as the internal radiometric standard **1** was used. First, for the prepared reaction mixture, containing the samples of [(3*R*)-^{3}H]-, and [1-^{14}C]-L-Phe in the boric buffer (pH 8.7), the total radioactivity of ^{14}C and the ^{3}H/^{14}C radioactivity ratio (R_0) were determined. During the reaction, the samples were taken at the specified time periods in order to achieve the degrees of conversion (f) in the range of 2–20%. The elimination reaction for each taken sample was immediately quenched by the acidification to pH ~ 0, and cinnamic acid was extracted with diethyl ether, and its ^{3}H/^{14}C ratio (R_p) was determined. The conversion factor (f) was calculated by dividing the ^{14}C-radioactivity in the starting sample by the ^{14}C-radioactivity of sample taken in course of kinetics run.

Procedure for H/^{3}H and ^{2}H/^{3}H KIE determination in the (3S)-position of L-Phe. For the kinetic runs **1**, **3**, and **5**, that is, the isotopomers of [1-^{14}C]-, [(3*S*)-^{3}H]-, and [(3*S*)-^{2}H/^{3}H]-L-Phe were used. The procedure for determining some kinetic parameters (R_0 and f) of the reaction was similar, but some modifications should be performed because of tritium label was loss by cinnamic acid (product) during the course of reaction. Thus, instead of R_p, the R_r (^{3}H/^{14}C of unreacted substrate) was determined. Therefore, after aqueous layer extraction, the remaining unreacted L-Phe was loaded onto chromatographic column (Amberlite IR-120 H^+, 10×100 mm, Aldrich, Poznan, Poland), washed with water to remove the labile tritium label. Then, L-Phe was eluted with 0.3 M NH_3(aq), concentrated under the reduced pressure, its ^{3}H/^{14}C ratio (R_r) was determined by liquid scintillation counting, and KIE was calculated.

Table 1. Tritium and ^{14}C KIE's values for deamination of L-Phe.

Substrate	KIE
[1-^{14}C]-L-Phe, **1**	1.006 ± 0.008
[2-^{14}C]-L-Phe, **2**	0.993 ± 0.006
[(3*R*)-^{3}H]-L-Phe, **3**	1.055 ± 0.005
[(3*S*)-^{3}H]-L-Phe, **4**	1.30 ± 0.02
[(3*S*)-^{2}H/^{3}H]-L-Phe, **5**	1.08 ± 001

*Spectrophotometric procedure for KIE determination on K_M (Michaelis constant) and KIE on V_{max} (maximum reaction rate) for [(3R)-^{2}H]-, **6** and [(3S)-^{2}H]-L-Phe, **7**.* The reaction of ammonia elimination was carried out in the 6 mL quartz spectrophotometric cuvette containing the reaction mixture and placed inside measuring spectrometer's chamber. The reaction was carried out in 0.2 M boric buffer (pH 8.7) at 25°C. The progress of reaction was measured on the basis of the increasing absorbance of cinnamic acid at λ = 290 nm. The results are given in the Table 2.

*Procedure for ^{14}C KIE determination in [1-^{14}C]-L-DOPA, **8**, in its decarboxylation catalyzed by tyrosine decarboxylase (EC 4.1.1.25)*

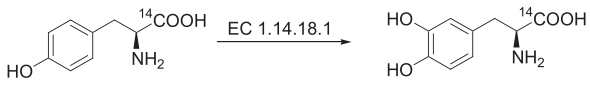
This KIE was determined using the tritium ring labeled isotopomer [2′,5′,6′-$^3H_3$]-L-DOPA as the internal radiometric standard, which was obtained by the acid-catalyzed exchange between tritiated water and authentic L-DOPA carried out at 50°C as described earlier.[17,18] The assayed substrate **8** was synthesized by the hydroxylation of commercial [1-^{14}C]-L-tyrosine catalyzed by enzyme tyrosinase according to slightly modified method,[19,20] Scheme 10. In this case of kinetic run, only the R_0, R_S, and f parameters were determined. First, the ^{3}H/^{14}C ratio (R_0) of reaction mixture used for kinetics assays (composed of [1-^{14}C]- and [2′,5′,6′-$^3H_3$]-L-DOPA) was measured. The measurements of kinetic parameters for the calculation of KIE were carried out as follows: the exact portion of two aforementioned isotopomers of L-DOPA were added to the 25 mL reaction vial containing 0.1 M phosphate buffer (pH 5.5). The reaction (carried out at 25°C) started after addition of tyrosine decarboxylase enzyme and PLP. In the 5-min intervals, the 1 mL samples were taken and immediately frozen in liquid nitrogen. In the course of each experiment, six samples were collected, and the degree of conversion (f) of L-DOPA into dopamine was in the range of 0.15–0.38. The substrate from product was separated on chromatographic column (Amberlit IRC-50(H^+), 10 × 100 mm) by elution with 0.1 M phosphate buffer (pH 6.5). The fractions containing radioactivity were collected, lyophilized, and their radioactivity measured. The R_s and f were calculated from the radioactivity measurements.

Procedure for deuterium KIE and SIE determination for the decomposition of 5′-chloro-L-Trp using a noncompetitive spectrophotometric method

5′-chloro-[2-^{2}H]-L-Trp, **9**, was synthesized by coupling 5-chloroindole with *S*-methyl-L-cysteine catalyzed by the enzyme TPase in fully deuteriated incubation medium according to the procedure described earlier[21,22]; see Scheme 11. The product was obtained with 17% chemical yield and the near 100% deuterium incorporation into the α-position of **9** as shown by ^{1}H NMR spectrum. [^{1}H NMR for authentic 2′-chloro-L-tryptophan – δ 3.355 (2H, β-H, m); 4.034 (1H, α-H, q); 7.235 (1H, 6′-ArH, dd); 7.336(1H, 2′-ArH, s); 7.473 (1H, 7′-ArH, d); 7.731(1H, 4′-ArH, d). ^{1}H

Table 2. Deuterium KIE's values in the (3*R*)- and (3*S*)-positions for deamination of L-Phe.

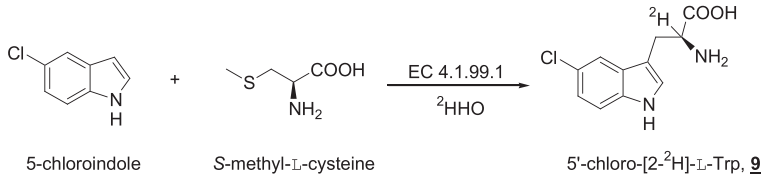
Substrate	KIE on V_{max}	KIE on V_{max}/K_M
[(3*R*)-^{2}H]-L-Phe, **6**	1.031 ± 0.009	1.032 ± 0.01
[(3*S*)-^{2}H]-L-Phe, **7**	1.143 ± 0.012	1.134 ± 0.015



[1-^{14}C]-L-Tyr

[1-^{14}C]-L-DOPA, **8**

Scheme 10. Synthesis of [1-^{14}C]-L-DOPA, **8**, catalyzed by the enzyme tyrosinase (EC 1.14.18.1).



5-chloroindole S-methyl-L-cysteine 5'-chloro-[2-^{2}H]-L-Trp, **9**

Scheme 11. Synthesis of 5′-chloro-[2-^{2}H]-L-Trp, **9**, catalyzed by the enzyme tryptophanase (EC 4.1.99.1).

NMR spectrum for **9** – δ 3.229 (2H, β-H, m); 7.239 (1H, 6′-ArH, dd); 7.339 (1H, 2′-ArH, s); 7.479 (1H, 7′-ArH, d); 7.736(1H, 4′-ArH, d)] The loss of signal from the α-proton of **9** (4.034) indicates near complete incorporation of deuterium in this position. During the kinetic runs, the progress of 5′-chloro-L-Trp decomposition was measured indirectly as the substrate and products, that is, 5-chloroindole and pyruvate, do not adsorb the photons with energy suitable for the detection with a spectrophotometer. For this purpose, to the mixture, an excess of enzyme lactate dehydrogenase (LDH – EC 1.1.1.27) and NADH were added. LDH catalyzes the conversion of pyruvate to L-lactate which oxidizes NADH to NAD^+. The formed NADH adsorbs strongly at λ = 340 nm, whereas NAD^+ is invisible. This detailed spectrometric procedure for measuring kinetic parameters, needed for the calculation of KIE and SIE, is described elsewhere.[23] The values of KIE and SIE are presented in Table 3.

Procedure for deuterium KIE and SIE determination for the deamination of 2′-chloro-L-phenylalanine to 2′-chlorophenylpyruvic acid catalyzed by PheDH

2′-Chloro-[(3*S*)-^{2}H]-L-Phe], **10**, for this oxidative-deaminative reaction was obtained by addition of ammonia to 2′-chlorocinnamic acid (Scheme 6) catalyzed by the enzyme PAL carried out in fully deuteriated incubation medium as described earlier.[15] The product, that is, **10** was obtained with 48% chemical yield and the near 100% deuterium incorporation into the (3*S*)-position of **10** , as shown by ^{1}H NMR spectra. [^{1}H NMR for authentic 2′-chloro-L-phenylalanine – δ 3.086 (1H, β-$H_{(S)}$, d); 3.096 (1H, β-$H_{(R)}$, d); 3.705 (1H, α-H, t); 6.908 (1H, 3′-ArH, m); 6.975 (1H, 5′-ArH, m); 7.153 (1H, 6′-ArH, m); 7.312 (1H, 4′-ArH, m). ^{1}H NMR spectrum for **10** δ 3.096 (1H, β-$H_{(R)}$, s); 3.705 (1H_{α}, d); 7.142 (1H, 3′-ArH, m); 7.182 (1H, 5′-ArH, m); 7.313 (1H, 6′-ArH, m); 7.363 (1H, 4′-ArH, m)]. In the region of the ^{1}H NMR spectrum, covering the signals from the protons of the side chain[24] of **10**, the signal from the (3*S*)-proton disappears (δ 3.086) indicating the 100% deuterium incorporation in this position. The kinetic runs of oxidative–deaminative conversion of **10** into 2′-chlorophenylpyruvic acid were carried out in 0.1 M glycine buffer (pH 10.7) in presence of PheDH and NAD^+. The progress of this reaction was monitored spectroscopically[25] by measuring the increase in absorbance (at λ = 340 nm) of NADH formed.

Procedure for deuterium KIE and SIE determination for the (4R)- and (4S)-positions of NADPH during reduction of DOPAL to DOPET catalyzed by ALDH

The substrates, that is, [(4*R*)-^{2}H]-, **11**, and [(4*S*)-^{2}H]-NADPH, **12**, for kinetic runs for the reduction of DOPAL to DOPET were synthesized according to known procedures.[26–29]

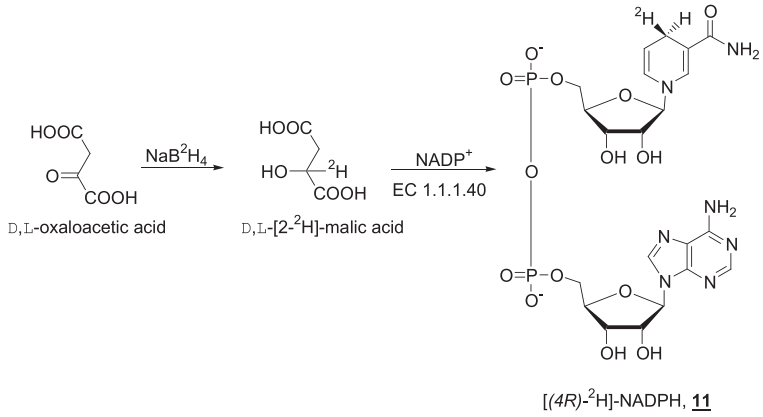
*Synthesis of [(4R)-^{2}H]-NADPH, **11**,* Scheme 12. A sample of 66 mg (0.5 mmol) of D,L-oxaloacetic acid dissolved in ^2H_2O was reduced to D,L-[2-^{2}H]-malic acid with 5 mg (0.12 mmol) of NaB^2H_4 at 0°C. The procedure of separation and purification of the obtained D,L-[2-^{2}H]-malic acid was the same as described earlier[26] giving the 53.4 mg (0.3 mmol) sample of deuteriated product obtained with 60% chem. yield. Then, 2.5 units of malic enzyme (EC 1.1.1.40) and 15 mg (0.02 mmol) of $NADP^+$ were added to 15 mg (0.08 mmol) portion of D,L-[2-^{2}H]-malic acid dissolved in TRIS · HCl buffer. The reaction was carried out for 1 h at 30°C. The separation and purification (made by the procedures given earlier[27,28]) gave 9.6 mg (0.011 mmol) of **11** (obtained with 57% chem. yield). The near 100% deuterium enrichment in the (4*R*)-position were proved by ^{1}H NMR spectra. In the authentic NADPH, the signals from the (4*S*)- and (4*R*)-protons (δ 2.757, $1H_S$, and board d), and δ (2.793, $1H_R$, and broad t) are compatible with literature data,[29] while for obtained **11,** only the signal from (4*S*)-protons (δ 2.752, $1H_{4S}$, and broad d) is observed, while the signal from the (4*R*)-protons has disappeared. The ^{1}H NMR spectrum of authentic NADHP: δ **2.757 (1H, H_{4S}, broad d), 2.793 (1H, H_{4R}, broad t)**, 4.038 (2H, d), 4.186 (2H, m), 4.264 (1H, m), 4.375 (1H, m), 5.952 (1H, d), 6.196 (1H, d), 6.918 (1H, s), 8.222 (1H, s), 8.464 (1H, s). The ^{1}H NMR spectrum of product **11** – δ **2.752 (1H, H_{4S}, broad d),** 4.049 (2H, d), 4.180 (2H, m), 4.213 (1H, m), 4.393 (1H, m), 5.952 (1H, d), 6,189 (1H, d), 6.964 (1H, s), 8.225 (1H, s), 8.496 (1H, s).

*Synthesis of [(4S)-^{2}H]-NADPH, **12**, Scheme 13.* This was carried out according to the procedure described previously.[29] A sample of 15 mg (0.02 mmol) of $NADH^+$ was reduced by 10 mg (0.055 mmol) of [1-^{2}H]-D-glucose in the presence of 136 units of enzyme glucose-L-dehydrogenase (EC 1.1.1.47). As a result, 10.5 mg (0.012 mmol) of **12** were obtained with 62.5% chem. yield and 100% deuterium enrichment at the (4*S*)-position verified by ^{1}H NMR spectrum. Only the signal from the (4*R*)-protons (δ 2.798, H_{4R}, broad t) is observed, while the signal from the (4*S*)-protons disappeared. The ^{1}H NMR spectrum of product **12**, δ **2.798 (1H, H_{4R}, broad t),** 4.031 (2H, d), 4.197 (2H, m), 4.259 (1H, m), 4.376 (1H, m), 5.947 (1H, d), 6.188 (1H, d), 6.923 (1H, s),8.218 (1H, s), 8.468 (1H, s).

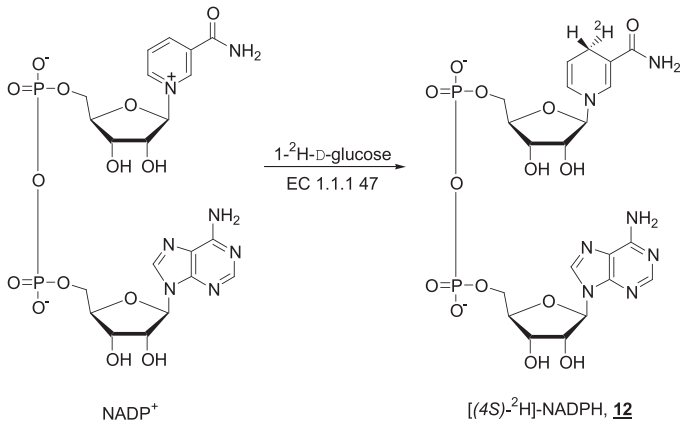
Noncompetitive spectrophotometric method. For the kinetic runs for the determination of parameters, needed to calculate the KIE and SIE values for the reduction of DOPAL to DOPET, the noncompetitive spectrophotometric method was used. The rate of this reaction, carried out in phosphate buffer (pH 7.2 or pD 7.6 for protonated or deuteriated medium, respectively) and catalyzed by enzyme ALDH in the presence of [(4*R*)-^{2}H]- **11**, or [(4*S*)-^{2}H]-NADPH **12**, (cofactor) was determined by the measuring at λ = 340 nm the increase of absorbance of $NADP^+$ formed. The calculated KIE and SIE for this reaction are given in Table 4.

Table 3. The deuterium KIE's and SIE's values for the 2-position of 5′-chloro-L-Trp for its decomposition catalyzed by TPase.

Substrate	KIE on V_{max}	KIE on V_{max}/K_M
5′-chloro-L-Trp in H_2O/ 5′-chloro-[2-^{2}H]-L-Trp, **8**, in H_2O	1.80 ± 0.65	1.87 ± 0.15
5′-chloro-L-Trp in H_2O/ 5′-chloro-[2-^{2}H]-L-Trp, **8**, in ^2H_2O	1.11 ± 0.08	1.59 ± 0.11
	SIE on V_{max}	SIE on V_{max}/K_M
5′-chloro-L-Trp in H_2O/ 5′-chloro-[2-^{2}H]-L-Trp, **8**, in H_2O	2.83 ± 0.19	0.68 ± 0.06
5′-chloro-L-Trp in H_2O/ 5′-chloro-[2-^{2}H]-L-Trp, **8**, in ^2H_2O	1.75 ± 0.13	0.58 ± 0.03



Scheme 12. Synthesis of [(4*R*)-^{2}H]-NADPH, **11**.



Scheme 13. Synthesis of [(4*S*)-^{2}H]-NADPH, **12**, catalyzed by the enzyme glucose 1-dehydrogenase (EC 1.1.1.47).

Table 4. KIE's values for oxidation NADPH to NADP$^+$ during the reduction of DOPAL to DOPET.

Substrate	KIE on V_{max}	KIE on V_{max}/K_M
[(4*R*)-^{2}H]-NADPH, **11**	2.52 ± 0.02	2.8 ± 0.2
[(4*S*)-^{2}H]-NADPH, **12**,	1.46 ± 0.09	2.08 ± 0.32
	SIE on V_{max}	SIE on V_{max}/K_M
DOPAL	2.31 ± 0.15	1.27 ± 0.11

KIE, kinetic isotope effect; SIE, solvent isotope effect; NADPH, nicotinamide adenine dinucleotide phosphate; DOPAL, 3′,4′-dihydroxyphenylacelaldehyde; DOPET, 3′,4′-dihydroxyphenylethanol.

$$KIE = \frac{k_1}{k_2} = \frac{\ln\left[(1-f)\times\frac{R0)}{R_s}\right]}{\ln[1-f]} \quad (1)$$

$$KIE = \frac{k_1}{k_2} = \frac{\ln\left(1-\frac{f\times R_0}{R_p}\right)}{\ln(1-f)} \quad (2)$$

$$KIE = \frac{k_1}{k_2} = \frac{\ln\frac{(R_p-R_0)}{(R_p-R_s)}}{\ln\frac{R_s\times(R_p-R_0)}{R_0\times(R_p-R_s)}} \quad (3)$$

$$KIE = \frac{k_1}{k_2} = \frac{\ln\left[\frac{1}{(1-f)}-\frac{f\times(R_p-R_s)}{(1-f)\times R_s}\right]}{\ln\left[\frac{1}{(1-f)}-\frac{f\times(R_p-R_s)}{(1-f)\times R_s\times R_p}\right]} \quad (4)$$

Results and discussion

The kinetic parameters needed to calculate the values of ^{14}C KIE for the 1- and 2-positions during the deammination of L-Phe to cinnamic acid catalyzed by enzyme PAL, and carried out at 30°, were determined using the competitive radiometric method[30]; see Scheme 1. The KIE = k_1/k_2 (reaction rates with ^{12}C and ^{14}C, respectively) was calculated using Yankwich–Tong Equations[16] (1)–(4) and the kinetic data measured in the course of five independent experimental runs.

where: R_0 – starting molar activity of substrate; R_s or R_p – molar activities of substrate or product, respectively, at given degree of conversion, f- degree of conversion.

^{14}C- KIE values were determined at low degrees of conversion (f – from 0.1 to 0.23) and using three parameters selected from the set of four (R_0, R_s, R_p, and f) and four equations to obtain the compatible results indicating that during the kinetics measurements the systematic errors were avoided. The calculated values of ^{14}C KIE values for the 1- and 2-position of L-Phe are near 1 – (1.006 ± 0.005 and 1. 008 ± 0.006). This means

that there is no KIE in this position, and so these two isotopomers can be used as internal radiometric standards to investigate the tritium KIE in the aforementioned reaction as the ^{14}C-label is distant from the center of reaction and can not affect its rate.

Tritium KIE values were determined for low conversion of substrate ($f = 0.1 - 0.25$) and using [1-^{14}C]-L-Phe as internal radiometric standard. In these cases, KIE values were calculated using only one from Equations (1) to (4) and the parameters from five independent kinetics runs.

For the (3*R*)-position of [(3*R*)-^{3}H]-L-Phe, **3**, the secondary H/T KIE was calculated using the isotopic ratios $^{14}C/^{3}H$ of substrate (R_0) and cinnamic acid (R_p) isolated after reaction and Equation (2).

For the (3*S*)-position of [(3*S*)-^{3}H]-L-Phe, **4**, the primary H/T KIE was determined using isotopic ratios $^{14}C/^{3}H$ of substrate before (R_0) and isolated after reaction (R_s), and Equation (1).

For the (3*S*)-position of [(3*S*)-^{2}H/^{3}H]-L-Phe, **5**, the D/T KIE was determined using isotopic ratios $^{14}C/^{3}H$ of substrate before (R_0) and cinnamic acid isolated after reaction (R_p), and Equation (2). The summarized KIE, values are listed in Table 1.

The kinetic parameters for calculation of deuterium KIE's, that is, KIE on V_{max} and KIE on V_{max}/K_M, for the reaction presented in Scheme 1, were determined using the noncompetitive spectrophotometric method.[30] The absorbance measurements of cinnamic acid allow to determine V_{max} and calculation K_M according to Michaelis Equation (5).

$$K_M = S\left(\frac{V_{max}}{v} - 1\right) \quad (5)$$

where ***v*** is the reaction rate at substrate concentration S, V_{max} is a maximum reaction rate, and K_M is a Michaelis constant. K_M and V_{max} are interrelated, and approximately, K_M is a measure of strength of enzyme-substrate binding, and V_{max} is a measure of the rate of reaction under conditions given.

The deuterium KIE's for (3*R*)- and (3*S*)-positions of L-Phe are given in Table 2.

Despite of the numerous literature data, the mechanism of deamination of L-Phe catalyzed by the enzyme PAL is still unclear and has gained much attention for long time. The mechanism proposed by Hanson and Havier[5] assumes that dehydroalanine (DHA) – the rare prosthetic group forming essential part of PAL and the important factor for catalysis – acts as the electrophile forming a covalently bonded 'enzyme-intermediate' complex with quaternary nitrogen of L-Phe. Therefore, this complex enhances the leaving ability of the amino group and withdraws negative charge from the hydrogen at β-carbon, so it becomes sufficiently acidic to be abstracted by a base. The alternative mechanism postulated by Schuster and Retey[6] assumes that in the first step of this reaction, a Friedel–Crafts type of electrophilic attack of DHA on the aromatic ring occurs, followed by hydrogen abstraction. To carry out ammonia elimination from L-Phe (Scheme 1), two events are necessary: firstly, the activation of hydrogen at β-carbon making it capable to leave, and secondly, the quarternization of Phe-nitrogen and thus facilitating the C-N-rupture. The Hanson and Havir's mechanism[5] assumes that DHA plays both roles simultaneously, in contrary to the Shuster and Retey's mechanism,[6] where DHA activates a hydrogen, while protonation of Phe is a separate step occurring upon. Also the data published[7] of H/T KIE at ortho position of ring of L-Phe provides a strong argument for the Friedel–Crafts reaction type in the early steps of elimination of ammonia, which was confirmed by semi empiric calculation. For the confirmation of these hypotheses, the investigation of carbon KIE at the ortho position of L-Phe is needed.

The lack of large isotope effects in ammonia elimination from L-Phe catalyzed by PAL proves that the mechanism is complicated and it is difficult to identify the rate determining the step of this reaction. Perhaps, more than one stage has a decisive influence on the overall rate of reaction. The attention must be paid to the following facts: the size of tritium KIE in the (3*S*)- and (3*R*)-position,(1.3 and 1.08, respectively), however, both being small, differ from each other. The (3*S*)-position (where hydrogen is abstracted in the process of elimination) is more isotopically sensitive. KIE in the (3*S*)-position may be the result of specific interaction of (3*S*)-proton with the enzyme, while KIE in the (3*R*)-position may be associated with a change of hybridizing with sp^3 to sp^2. It is likely that the internal isotope effect at this position is substantially greater than that observed and may be suppressed by other processes of a similar rate.

The determined ^{14}C KIE for the 2-position of L-Phe (to which amino group is attached) is about 1 (no effect), which indicates that the leaving amino group does not decide of the reaction rate. The lack of KIE excludes the two-step (E2) and carbocation (E1) mechanisms. It is most likely the carboanion (equivalent E1cB) mechanism; that is, first, a proton is abstracted, and later, the amino group is leaving. Having considered that, a DHA (cofactor) electrophilic attack on the ring and formation a positive charge on C_1-ring carbon, proposed by Shuster and Retey,[6] seem likely.

^{14}C KIE for [1-^{14}C]-L-DOPA in its decarboxylation catalyzed by tyrosine decarboxylase (EC 4.1.1.25) (Scheme 2) was determined using the tritium ring labeled isotopomer [2′,5′,6′-$^{3}H_3$]-L-DOPA as the internal radiometric standard. In this case, only $^{14}C/^{3}H$ ratios of starting substrate (R_0), substrate, isolated after conversion (R_s) and f parameter were determined. KIE equal to 1.18 ± 0.01 was calculated using Equation (1). Here, the rarely found high value of ^{14}C KIE proves that C_α–C_{COOH} bond breakage occurs in the rate determining step of this enzymatic reaction.

The deuterium KIE's and SIE's to the 2-position of side chain for 5′-chloro-[2-^{2}H]-L-Trp, **8**, during its decomposition to 5-indole and pyruvate (similar as shown in Scheme 3) were calculated using Equation (5) and kinetic parameters determined as stated in the Experimental Section are listed in Table 3.

The earlier reported[31] isotope effects for the 2-position for decomposition of [2-^{2}H]-L-Trp (listed in the succeeding section) carried out in the same conditions differ insignificantly from those given in Table 3.

- KIE on $V_{max} = 2.1 \pm 0.1$ and 2.1 ± 0.13 (in H_2O and $^{2}H_2O$, respectively)
- KIE on $V_{max}/K_M = 2.10 \pm 014$ and 1.45 ± 0.13 (in H_2O and $^{2}H_2O$, respectively)
- SIE on $V_{max} = 1.87 \pm 0.17$ and 1.25 ± 0.17 (in H_2O and $^{2}H_2O$, respectively)
- SIE on $V_{max}/K_M = 1.07 \pm 013$ and 1.14 ± 0.05 (in H_2O and $^{2}H_2O$, respectively)

This means that substitution of hydrogen atom by the chlorine one does not affect the reaction of degradation of chloroderivative of L-Trp in comparison to [2-^{2}H]-L-Trp.

In the course of oxidative deamination of 2′-chloro-[(3*S*)-^{2}H]-L-Phe, **8**, Scheme 5, the deuterium KIE's and SIE's for the (3*S*)-position were determined as described in Experimental Section. The kinetic data needed for isotope effects calculation were obtained using Equation (5). The numerical values of deuterium in this position are: For the KIE's on V_{max} and the KIE's on V_{max}/K_M – 1.20 ± 0.09 and 1.35 ± 0.12, respectively; for the SIE's on V_{max} and the SIE's on V_{max}/K_M – 1.46 ± 0.12 and 2.28 ± 0.13, respectively.

Earlier published[32] or not published yet isotope effects (listed in the succeeding sectionn) for the 3-position for oxidative deamination of [(3*S*)-^{2}H]-L-Phe carried out in the same conditions are

- For the KIE's on V_{max} and the KIE's on V_{max}/K_M – 1.16 ± 0.12 and 1.01 ± 0.09, respectively
- For the SIE's on V_{max} and the SIE's on V_{max}/K_M – 1.67 ± 0.11 and 1.91 ± 0.13, respectively.

As can be seen from earlier data, the values of isotope effects are almost identical (with limits of experimental error), which means that ring substitution of hydrogen atom by chlorine does not affect the oxidative deamination of 2′-chloro-[(3*S*)-^{2}H]-L-Phe in comparison to [(3*S*)-^{2}H]-L-Phe.

Small or close to 1 (no effect) the deuterium KIE and SIE values, observed in the decomposition of 5′-chloro-[2-^{2}H]-L-Trp and 2′-chloro-[(3*S*)-^{2}H]-L-Phe for the α- and β-protons, strongly suggest that a proton abstraction from these positions in not the rate determining step of the reactions mentioned.

The kinetic parameters for KIE and SIE values for the enzymatic reduction of DOPAL to DOPET in the presence of NADPH and catalyzed by enzyme ALDH (Scheme 2) were determined as described in Experimental Section and calculated using Equation (5). The SIE on V_{max} = 2.31 ± 0.15 and the SIE on V_{max}/K_M = 1.27 ± 0.11 are obtained for the reduction of DOPAL to DOPET. The KIE values for the oxidation of NADPH (reducing species) to $NADP^+$ for this reaction are listed in Table 4.

The higher value of SIE on V_{max} than SIE on V_{max}/K_M (Table 4) is affiliated with the conformational changes in the structure of ALDH occurring during the cofactor binding with the enzyme (V_{max} is very sensitive to this changes). The published crystallographic data[33] show that ALDH's conformational changes occur as a result of the side amino acids group rotation, participating in the binding of NADPH.

Moreover, the higher values of deuterium KIE on V_{max} and KIE on V_{max}/K_M for [(4*R*)-^{2}H]- than [(4*S*)-^{2}H]-NADPH (Table 4) suggest that during the DOPAL to DOPET reduction, the ALDH enzyme catalyzes the proton removal from the (4*R*)-position.[34]

Conclusion

This research presents some new data on the isotopic effects in enzymatic reactions. The numerical values of deuterium, tritium, and ^{14}C KIE and SIE values have been determined for the following reactions, catalyzed by the enzymes from lyases group: the deamination of L-Phe by PAL, the decarboxylation of L-DOPA by tyrosine decarboxylase, and the decomposition of 5′-chloro-L-Trp by TPase, as wells as the biotransformations catalyzed by dehydrogenases, that is, oxidative deamination of 2′-chloro-L-Phe by PhDH and the conversion of DOPAL to DOPET by ALDH using deuteriated in the (4*R*)- or (4*S*)-positions NADPH as cofactor. These KIE and SIE values may be useful to elucidate some mechanism details of the investigated enzymatic reactions.

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