ENZYMATIC SYNTHESIS OF MULTILABELED L-TYROSINE

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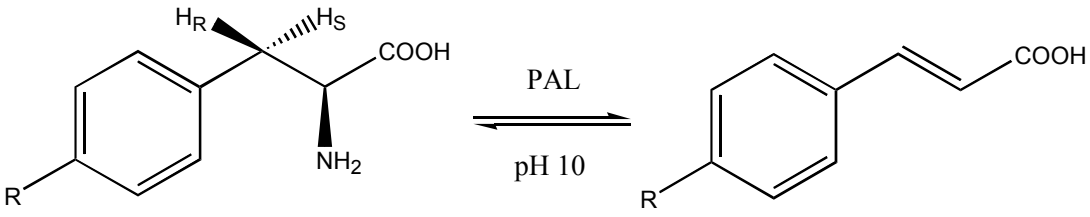
(*long abstract)*

Summary

L-Tyrosine (L-Tyr) doubly labeled with isotope of carbon-14 and with isotopes of hydrogen in 3-*R* and 3-*S* positions was synthesized using specific properties of the enzymes PAL (Phenylalanine Ammonia-Lyase, E.C. 4.3.5.1.) and L-phenylalanine hydroxylase (E.C. 1.14.16.1.) from rat liver. The synthesis selectively labeled L-Tyr via cinnamic acid and L-phenylalanine (L-Phe) applying combined chemical and enzymatic methods has been elaborated. Potassium [C-14]-cyanide, heavy and tritiated water have been used as the sources of stable or radioactive labels. In the first step, L-Phe labeled with carbon-14 was obtained by the addition of ammonia to (*E*)-[1-C-14]-cinnamic acid using enzyme PAL as catalyst. Next, L-Phe was enzymatically converted to doubly labeled L-Tyr (with deuterium or tritium in 3-*S* and 3-*R* positions and with carbon C-14 in carboxylic group) applying L-phenylalanine hydroxylase activity. Yields of obtained isotopomers of L-Tyr have been determined by enzymatic and radiochemical methods.

Phenylalanine ammonia lyase (E.C. 4.3.5.1), PAL, is an enzyme which catalyses elimination of ammonia and *pro*-3*S*-hydrogen from L-phenylalanine (L-Phe) leading to formation of (*E*)-cinnamate .(*Scheme 1).*

SCHEME 1



R = H, OH, OMe

The above mentioned reaction is a multistep process involving several intermediates and therefore it is important to determine the structure of active complex formed in the rate determining step. There are several literature reports on PAL including the structure of the active center and inhibitor studies [1-9]. There are also publications on a new scope on the proposed mechanism of action and on the some currently unclear intrinsic details [10-13]. The enzyme PAL catalyses the elimination of ammonia from L-tyrosine (L-Tyr) and *O*-methyl-L-Tyr [14]. There are no systematic kinetic studies of the elimination of ammonia from derivatives L-Phe possessing the electron-donating and electron-withdrawing groups in *para* position of the ring. The available kinetic data permitting to evaluate the structure of transition state of the rate limiting step are of qualitative nature and concern on the kinetics of elimination of ammonia from L-Phe only.

The goal of our planned research is to investigate the mechanism of this reaction by applying kinetic isotope effect (KIE) method. The ratios of reaction rates with lighter and heavier isotopes should be measured, since their numerical values will be used to find out the rate determining step [15]. Numerical values of deuterium and tritium KIEs on the bonds directly involved in chemical reaction allow to determine the presence of tunneling effect [16,17] in a given reaction according to the Saunders criterion [18,19]. Atoms taking part in the ammonia elimination reaction with L-Phe derivatives shall be labeled with radioactive or stable isotopes. It is possible to find out the number of reactive atoms and elucidate the structure of the transition state by determination of primary and secondary KIEs of deuterium and tritium. The presence of *para* substituents in the ring would also allow to investigate the effect of donor-acceptor properties of these groups on the reaction mechanism.

The aforementioned studies require the synthesis of one of the derivatives of L-Phe, i.e. L-tyrosine (L-Tyr) doubly labeled with deuterium tritium in 3*S* and 3*R* position of side chain and with carbon-14 in the carboxylic group. The ^{14}C

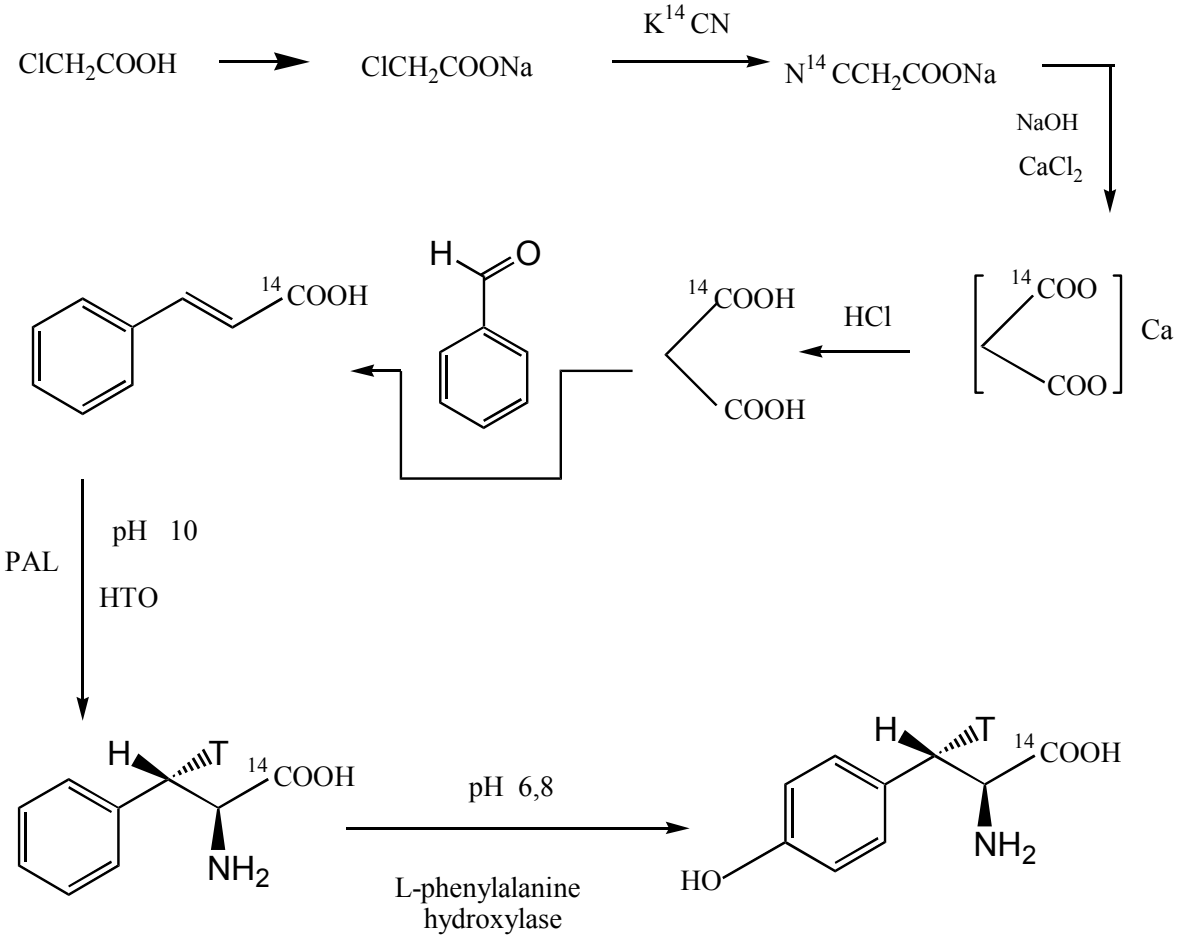
label in carboxylic group will serve as an internal radiometric standard during hydrogen KIE determination. There is no literature data for synthesis of doubly and specifically labeled isotopomers of L-Tyr. Some papers deal with synthesis of ring-non-specifically, uniformly or single labeled tyrosine only [20-24]. Therefore we have elaborated the routes of synthesis leading to the desired products.

L-Tyr specifically labeled with deuterium, tritium and carbon-14 have been prepared with combined chemical and enzymatic methods. We used the enzyme PAL, which converts cinnamic acid into L-Phe and the enzyme L-phenylalanine hydroxylase (E.C. 1.14.16.1.) from rat liver which oxidized L-Phe to L-tyrosine. PAL was a commercial product from SIGMA and L-phenylalanine hydroxylase was isolated from rat liver according to the described procedure [25]. Heavy or tritiated water and sodium [^{14}C]-cyanide were used as sources of deuterium, tritium and ^{14}C label for syntheses of all isotopomers of L-Tyr.

[3*S*-^{3}H]-[1-^{14}C]-L-Tyrosine: doubly labeled with tritium in 3-*S* position and ^{14}C in the carboxylic group

Sodium [^{14}C]-cyanide via [1-^{14}C]-malonic acid was condensed with benzaldehyde to [1-^{14}C]-cinnamic acid [26]. Addition of ammonia to [1-^{14}C]-cinnamic acid [27] catalyzed by the enzyme PAL in the buffer containing tritiated water leads to [3*S*-^{3}H]-[1-^{14}C]-L-Phe. Obtained L-Phe was converted into doubly labeled L-Tyr by hydroxylation in the presence of L-phenylalanine hydroxylase (*Scheme 2).*

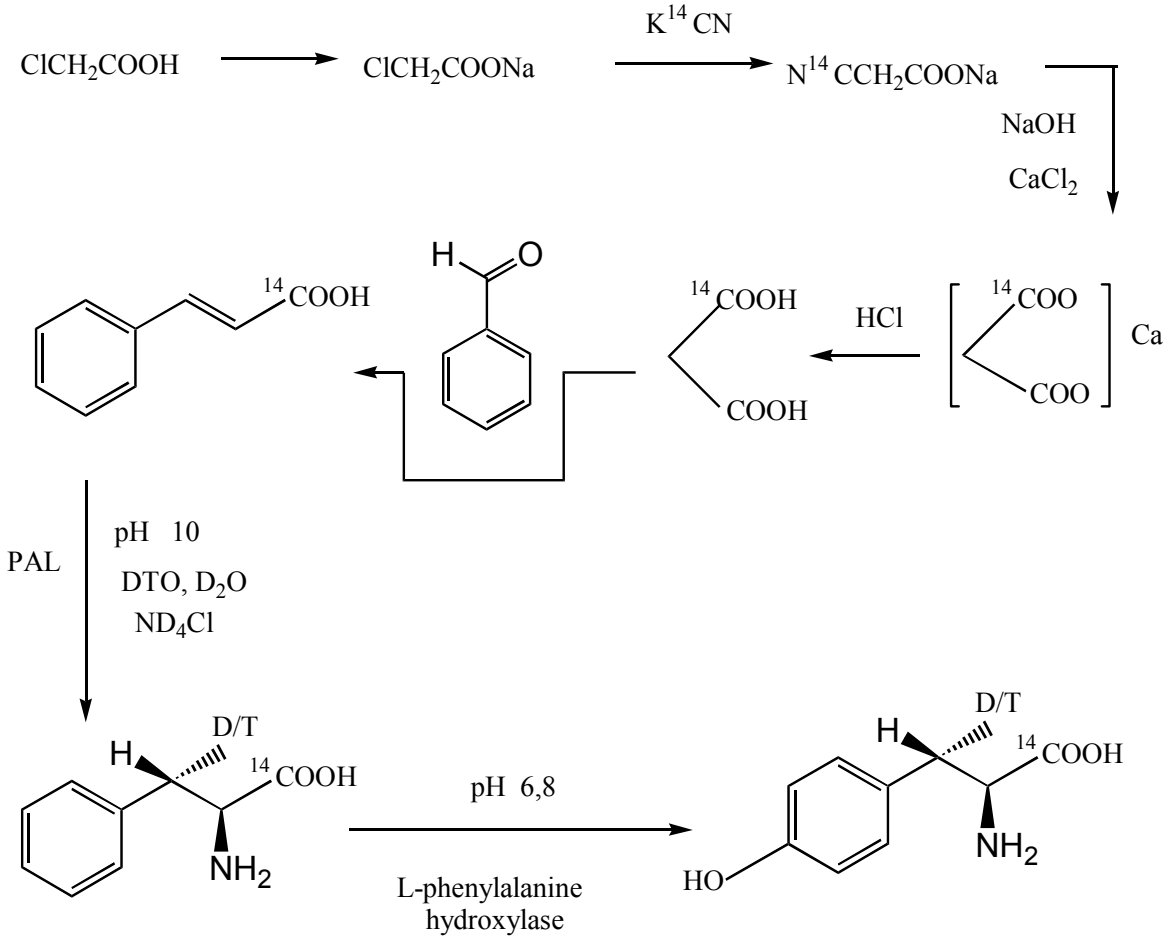
SCHEME 2



[3*S*-^{3}H/^{2}H]-[1-^{14}C]-L-Tyr: mutlilabeled with tritium and deuterium in 3-*S* position and ^{14}C in carboxylic group

This isotopomer of L-Tyr was prepared similarly as above. The difference is that ammonia addition to L-Phe was performed in deuteriated and tritiated buffer (*Scheme 3).*

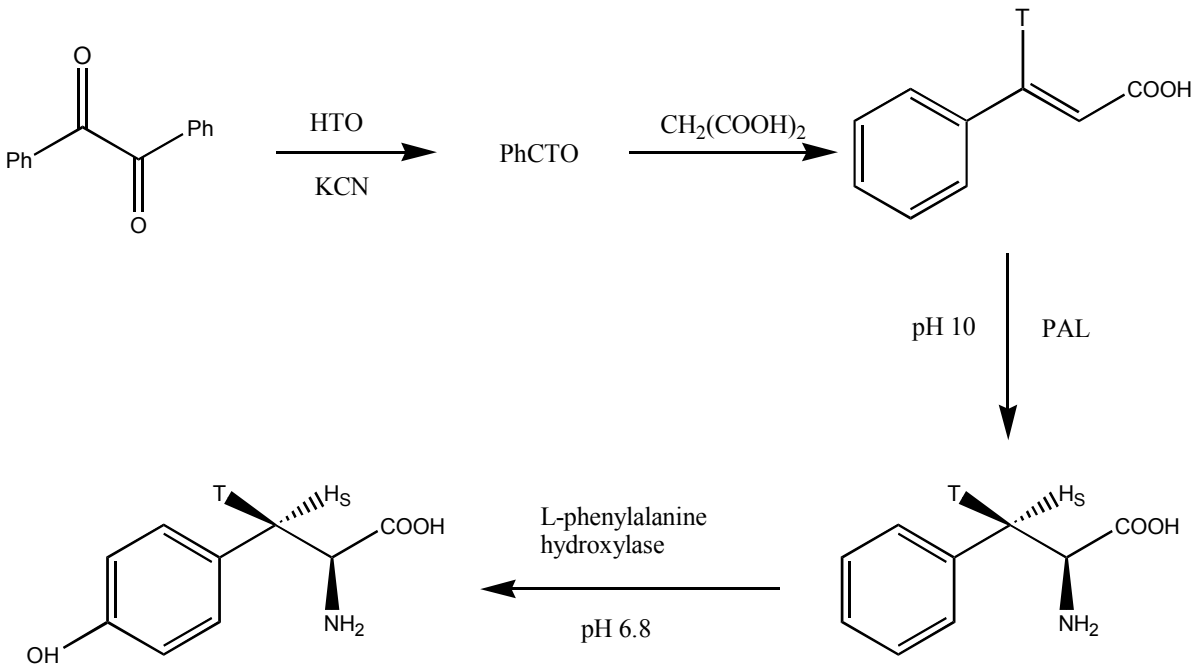
SCHEME 3



[3*R*-^{3}H]-[1-^{14}C]-L-Tyr: doubly labeled with tritium in 3-*R* position and ^{14}C in carboxylic group

Benzoil, (PhCOCOPh), was converted to benzaldehyde labeled with tritium in –CHO group in the presence of KCN and HTO. The Perkin condensation of the aldehyde with malonic acid yielded (*E*)-[3-^{3}H]-cinnamic acid. Enzymatic addition of ammonia gave [3-^{3}H]-L-Phe. Next, this amino acid was transformed into [3-^{3}H]-L-Tyr using the activity of L-phenylalanine hydroxylase (*Scheme 4).*

SCHEME 4



All the final products were purified by column chromatography and preparative TLC. Purity of the obtained amino acids was proven by TLC and enzymatic methods. Radiochemical yields of reactions were determined by liquid scintillation counting. Chemical yields were found by enzymatic and spectral methods.

Currently, we continue the work to elaborate the synthesis of the other multilabeled isotopomers of L-Tyr. We are preparing the deuteriated substrate ([3-^{2}H]cinnamic acid) and also cinnamic acid labeled with carbon ^{14}C in 2 and 3-positions of the side chain for synthesis of different isotopomers of L-Tyr.

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