ENZYMATIC SYNTHESES OF AROMATIC AMINO ACIDS LABELED WITH CARBON AND HYDROGEN ISOTOPES

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Summary

The isotopomers of L-phenylalanine, L-tyrosine, L-tryptophan, and its derivative 5'-hydroxy-L-tryptophan labeled with isotopes of carbon and hydrogen have been obtained using combined chemical and enzymatic routes. The $Ba^{14}CO_3$, ^{14}C-malonic acid, $K^{14}CN$, heavy and tritiated water have been used as a sources of stable and radioactive label.

Keywords: enzyme, isotope, labeling, L-fenylalanine, L-tryptophans, L-tyrosine

Contract/grant number: Committee for Scientific Research: KBN 4 T09 A 063 24

Introduction

The labeled L-amino acids play important role in many research of areas especially in metabolic and mechanistic investigations. Specifically labeled L-amino acids are valuable tools for probing the mechanism of enzymatic reactions using kinetic isotope effect (KIE) method. Such studies required the use different L-enantiomers of amino acids specifically labeled in desired positions. Many questions can be answered by determining the KIE for atoms involved in the postulated rate determining step[1]. We have used KIE methods for studying the mechanism of three enzymatic reactions, i. e., amination/desamination of L-phenylalanine catalyzed by enzyme PAL, reversible conversion of L-tyrosine to phenol, pyruvate and ammonia in the presence of enzyme β-tyrosinase, and decomposition of L-tryptophan catalyzed by enzyme TPase. For measuring reliable of tritium KIE values we used internal radioactive standard method. It assumes using doubly labeled substrate with probed label (^{3}H) bonded to postulated center of reaction and second one (^{14}C) placed in position non-involved in course of reaction. In our studies L-amino acids with ^{14}C- labeled carboxylic group serve as a internal standard substrates.

Results and discussion

1. Synthesis o isotopomers of L-phenylalanine, L-Phe. Enzyme phenylalanine ammonia lyase, PAL, EC 4.3.5.1, catalyses the reversible elimination of ammonia and *pro-3S*-hydrogen from L-Phe yielding (E)-cinnamic acid [2], Scheme 1. Under proper condition enzyme PAL catalyses addition of ammonia to (E)-cinnamic acid leading to formation of L-Phe. This enzymatic reaction was used to obtain the different isotopomers of L-Phe labeled with isotopes of hydrogen and carbon. The key intermediate for this reaction, i. e.,

Synthesis and Applications of Isotopically Labelled Compounds, Volume 8.
Edited by D. C. Dean, C. N. Filer and K. E. McCarthy.
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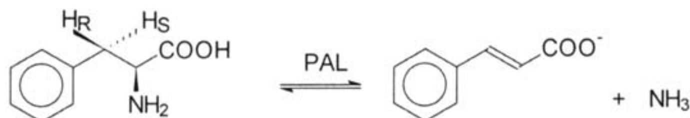
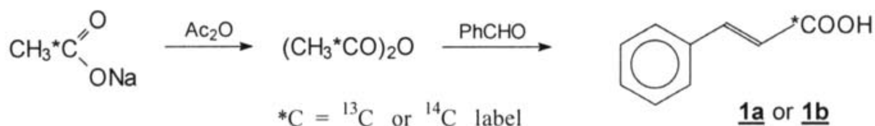
different isotopomers of cinnamic acid labeled with isotopes of hydrogen or carbon have been synthesized using chemical and enzymatic routes[3, 4].



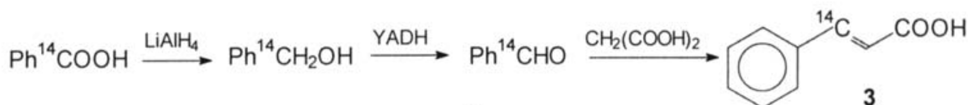
Scheme 1. The reversible deamination/amination catalyzed by enzyme PAL

a) Synthesis of [1-^{13}C]-, **1a**, *and [1-^{14}C]cinnamic acid,* **1b**, has been carried out according to Scheme 2 using sodium [1-^{13}C]- or [1-^{14}C]acetate, as a source of carbon label. Alternative route of obtaining of **1b** and [2-^{14}C]cinnamic acid, **2**, consists of the condensation of benzaldehyde with [1-^{14}C]-, or [1-^{14}C]-malonic acid respectively.



Scheme 2. Synthesis of [1-^{13}C]-, and [1-^{14}C]-cinnamic acid, **1a**, **1b**

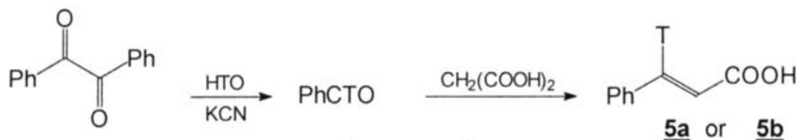
b) Synthesis of [3-^{14}C]-cinnamic acid, **3,** has been carried out starting from $^{14}CO_2$ which was converted into ^{14}C-benzoic acid. In this synthetic route presented in Scheme 3 the intermediate ^{14}C-benzyl alcohol was oxidized to ^{14}C-benzaldehyde using enzyme yeast alcohol dehydrogenase, YADH, EC.1.1.1.1, activity.



Scheme 3. Synthesis of [3-^{14}]-cinnamic acid, **3**

c) Synthesis of [2-^{2}H]- , **4a**, *and [2-^{3}H]-cinnamic acid,* **4b**. These isotopomers have been obtained by condensation of benzaldehyde with deuteriated or tritiated [2-*H]-malonic acid respectively.

d) Synthesis of [3-^{2}H]-, **5a**, *and [3-^{3}H]-*cinnamic *acid,* **5b**, has been carried out as shown in Scheme 4. For obtaining of **5a** instead of tritiated water (HTO) the heavy water was used.



Scheme 4. Synthesis of [3-$^{+2}$H]- and [3-^{3}H]-cinnamic acid

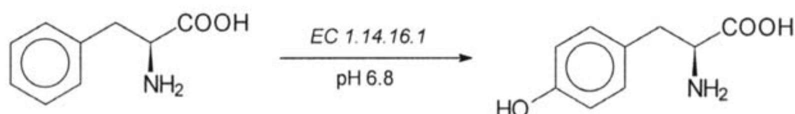
e) Synthesis of isotopomers of L-Phe labeled with isotopes of hydrogen and carbon. Specifically labeled isotopomers of L-Phe was obtained according to reverse reaction[3-5] presented in Scheme 1. Addition of ammonia to compounds of cinnamic acid was

carried out at pH 10 at 30°C (in this case deuterium or tritium label the substrates was dissolved in D_2O or HTO)[3]. The following isotopomers of L-Phe have been obtained:

(i) [1-^{13}C]-L-Phe, **6a**, and [1-^{14}C]-L-Phe, **6b**, from **1a** or **1b** respectively;
(ii) [2-^{14}C]-L-Phe, **7**, from **2**;
(iii) [3-^{14}C]-L-Phe, **8**, from **3**;
(iv) [2-^{2}H]-L-Phe, **9a** [2-^{3}H]-L-Phe, **9b** from **4a** or **4b** respectively;
(v) [3R-^{2}H]-L-Phe, **10a**, and [3R-^{3}H]-L-Phe, **10b**, from **5a** and **5b** respectively;
(vi) [3S-^{2}H]-L-Phe, **11a**, addition of ammonia to unlabeled cinnamic acid was carried out in fully deuteriated medium;
(vii) [3S-^{3}H]-L-Phe, **11b**, all substrates were dissolved in tritiated water;
(viii) Doubly labeled [3S-^{2}H/^{3}H]-L-Phe, **12**, addition of ammonia to **5b** was carried out in fully deuteriated medium;

Confirmation of tritium label in **10b**, **11b** and **12** was done using deamination reaction catalyzed by PAL (Scheme 1) and determining the radioactivity of cinnamic acid obtained from sample of given isotopomer of Phe.

2. Synthesis of isotopomers of L-tyrosine. The isotopomers of L-tyrosine, L-Tyr, have been obtained by hydroxylation (Scheme 5) of given isotopomer of L-Phe in the presence of enzyme L-phenylalanine 4'-monooxygenase (EC 1.14.16.1) [6, 7]



Scheme 5. Enzymatic hydroxylation of L-Phe

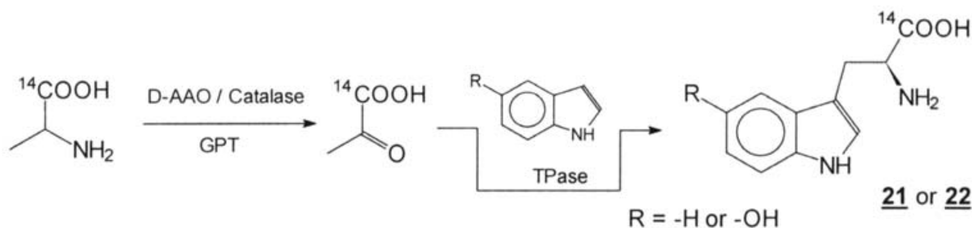
Using above enzymatic route the following isotopomers of L-Tyr have been obtained:

(i) [1-^{14}C]-L-Tyr, **13**,from **6b**;
(ii) [2-^{14}C]-L-Tyr, **14**, from **7**;
(iii) [3-^{14} C]-L-Tyr, **15**, from **8**;
(iv) [2-^{3}H]-L-Tyr, **16**, from **9b**;
(v) [3R-^{3}H]-L-Tyr, **17**, from **10b**;
(vi) [3S-^{3}H]-L-Tyr, **18**, from **11b**;
(vii) Doubly labeled [3S-^{2}H/^{3}H]-L-Tyr, **19**, from **12**;
(viii) [2',6'-$^{3}H_2$]-L-Tyr, **20**, from commercial [2',6'-$^{3}H_2$]-L-Phe.

Some isotopomers of L-Tyr will serve as a intermediates in the enzymatic synthesis of the specifically labeled L-dopa.

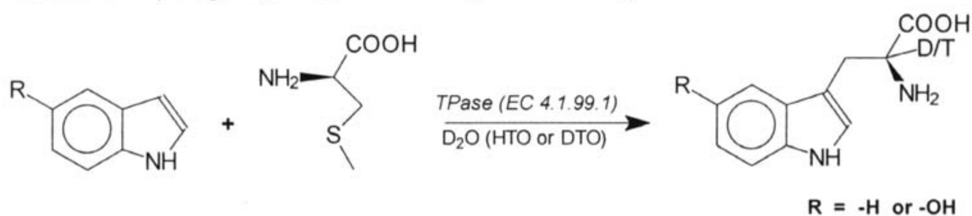
3. Synthesis of L-tryptophan, L-Trp, and 5'-hydroxy-L-tryptophan, 5-OH-L-Trp labeled with carbon-14 and isotopes of hydrogen.

a) Two ^{14}C- isotopomers of L-Trp, i.e., [1-^{14}C]-L-Trp, **21**, and 5-OH-[1-^{14}C]-L-Trp, **22**, have been synthesized using multienzymatic one-pot method[8] as presented in Scheme 6. [1-^{14}C]-DL-alanine was converted into [1-^{14}C]-pyruvic acid using enzymes: D-AAO (D-amino acid oxidase, EC 1.4.3.3), catalase (EC.1.11.6) and GPT (glutamic-pyruvic transaminase, EC 2.6.1.2). In turn [1-^{14}C]-pyruvic acid is coupled with indoles by enzyme TPase (tryptophanase, EC 4.1.99.1) giving **21** and **22**.



Scheme 6. Enzymatic synthesis of L-tryptophans labeled with ^{14}C

b) Synthesis of isotopomers of L-Trp and 5-OH-L-Trp labeled with isotopes of hydrogen. Under some experimental condition the enzyme TPase catalyses coupling of indoles with S-methyl-L-cysteine leading to formation of L-tryptophans[9]. The presence of enzyme TPase facilitates also labilization of hydrogen bonded to α-carbon and causing exchange with solvent hydrogens (HTO, DTO and D_2O in our case), Scheme 7.



Scheme 7. Synthesis of isotopomers of L-Trp and 5-OH-L-Trp

Using enzyme TPase and applied as a reaction medium water with different isotopic content the following isotopomers of L-Trp and 5'-hydroxy-L-Trp were obtained:

(i) [2-^{2}H]-L-Trp, **23**, (heavy water as a solvent);
(ii) 5-OH-[2-^{2}H]-L-Trp, **24**, (heavy water as a solvent);
(iii) [2-^{3}H]-L-Trp, **25**, (tritiated water, HTO, as a solvent);
(iv) 5-OH-[2-^{3}H]-L-Trp, **26**, (tritiated water, HTO, as a solvent);
(v) Doubly labeled [2-^{2}H/ ^{3}H]-L-Trp, **27**, (D_2O with HTO added as a solvent);
(vi) Doubly labeled 5-OH-[2-^{2}H/ ^{3}H]-L-Trp, **28**, (D_2O with HTO added as a solvent);

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