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Enzymatic synthesis of phenylpyruvic acid labeled with deuterium, tritium, and carbon-14

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The synthesis of isotopomers of phenylpyruvic acid, PPA, selectively labeled with hydrogen isotopes in the 3-position of the side-chain is reported. Three deuterium or tritium labeled isotopomers of L-phenylalanine, L-Phe, i.e. [(3*S*)-^{2}H]-L-, [(3*S*)-^{3}H]-L-, and doubly labeled [(3*S*)-^{2}H/^{3}H]-L-Phe were synthesized using the enzyme phenylalanine ammonia lyase (EC 4.3.1.5). In the second step these isotopomers of L-Phe were converted into [(3*S*)-^{2}H], [(3*S*)-^{3}H]-, and [(3*S*)-^{2}H/^{3}H]-isotopomers of PPA using the enzyme L-phenylalanine dedydrogenase (EC 1.4.1.20). The isotopomer of PPA labeled with ^{14}C in carboxylic group, [1-^{14}C]-PPA, was obtained in a two-step enzymatic reaction using [1-^{14}C]-cinnamic acid as the starting substrate.

Keywords: carbon-14; deuterium; enzyme; phenylpyruvic acid; tritium

Introduction

Phenylketonuria (PKU), a human genetic disease, named in recognition of its unusual metabolic by-products, is inherited with a high average birth incidence (10^{-4}– one for 10^{4} births) in European and Asian populations.[1] The disease is accompanied by elevated levels of phenylalanine metabolites such as phenylacetate, phenyllactate, and phenylpyruvate in body fluids. Detailed knowledge of the mechanisms of enzymatic conversion of L-Phe into its derivatives is essential for understanding PKU and the proper treatment and control of dietary therapy of PKU patients. One of metabolic paths of conversion of L-Phe into phenylpyruvic acid is NAD^{+} dependent, reversible, oxidative deamination[2,3] catalyzed by the enzyme L-phenylalanine dehydrogenase (EC 1.4.1.20), PheDH, Figure 1.

The metabolism of l-Phe, a key step in PKU disease, is not clearly understood. In addition, the mechanism of the industrial manufacture of L-Phe[4] from PPA or phenyllactic acid has not been understood to date. The production of phenylalanine as a starting material for the sweetener aspartame has been a target for industrial research; [5–7] this prompted research to seek for new enzymatic methods for the synthesis of PPA.

The goal of our planned research was to investigate some details of this reversible oxidative deamination reaction presented in Figure 1 by applying the kinetic isotope effect (KIE) and solvent isotope effect methods and determining kinetic isotope values in the rate determining step.[8] We assume that in the course of this reaction some tautomerization of PPA takes place, and in the process the stereospecific abstraction of proton from the 3-position of PPA is involved.[9] The numerical values of the isotope effects allowed us to elucidate the intrinsic details of the mechanism and were useful to draw a distinction between alternative mechanisms. The aforementioned studies require the use of isotopomers of PPA labeled with deuterium and tritium in the 3-position. The ^{14}C-labeled isotopomer PPA was used as an internal radiometric standard for the KIE assays. For this purpose, PPA, labeled in the carboxylic acid group [1-^{14}C]-PPA was chosen, as the ^{14}C label is placed in a position remote from the center of reaction.

In literature there are a few papers that describe the synthesis of deuterium-,[9] tritium-,[10] and ^{14}C-labeled[11] isotopomers of PPA. Unfortunately, most of them lead to isotopomers bearing the label in positions not useful for our proposed studies.

In this paper, the synthesis of four isotopomers of PPA labeled with deuterium and tritium in the 3-position, i. e. [3-^{2}H]-PPA, [3-^{3}H]-PPA, doubly labeled [3-^{2}H/^{3}H]-PPA, and ^{14}C-labeled in the carboxylic group [1-^{14}C]-PPA was reported.

Results and discussion

The addition of ammonia to cinnamic acid catalyzed by the enzyme PAL (phenylalanine ammonia lyase, EC 4.3.5.1) carried out in fully deuteriated ammonia buffer (1.8 M NH_4Cl in D_2O, pD 9.8) led to the formation of [(3*S*)-^{2}H]-L-Phe (detailed protocol of this step was earlier described[12] by us). The similar way in which this addition was carried out in tritiated ammonia buffer gave [(3*S*)-^{3}H]-L-Phe, as well as by using ammonia buffer composed of deuteriated and tritiated water doubly labeled [(3*S*)-^{2}H/^{3}H]-L-Phe was afforded. The extent of deuterium incorporation at 3-position of the obtained products were checked by ^{1}H NMR spectra (97% enrichment).

We had to perform certain preliminary kinetic studies to find the optimum reaction conditions for obtaining a high yield of

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the desired labeled isotopomers of PPA. We selected the UV-spectrometric technique for monitoring the progress of oxidative deamination of L-Phe, Figure 2. By observing the absorbance of the NADH at 340 nm we studied the dependence of the reaction yield on time, concentration of enzyme and substrates. All the kinetic studies were carried out at room temperature using glycine buffer.[13] We have found that the yield of PPA, depends on concentration of L-Phe, and this increases to achieve a steady state after 30–40 min incubation time. The maximal yield of PPA can be reached at 8 mM concentration of L-phenylalanine in the reaction medium, Figure 3. A higher concentration of L-Phe does not improve the yield of PPA. More detailed kinetics studies allowed us to elaborate the optimal parameters for carrying out this key reaction step (pH 10.7, room temperature, reaction time ca 4 h, concentration of buffer equal to 0.1 M, and quantity of enzyme ca 2 U) to afford the highest possible chemical yield of PPA (ca 45% under these experimental conditions).

Therefore, in a subsequent step these three isotopomers of L-Phe were converted into corresponding isotopomers of PPA, i. e. [(3*S*)-^{2}H]-PPA, [(3*S*)-^{3}H]-PPA, and [(3*S*)-^{2}H/^{3}H]-PPA using the enzyme PheDH (L-phenylalanine dehydrogenase, EC 1.4.1.20).

[1-^{14}C]-cinnamic acid, the substrate for the synthesis of [1-^{14}C]-L-Phe, was obtained by the method described earlier[14,15] and subsequently ^{14}C-labeled L-Phe (43% yield), and [1-^{14}C]-PPA (40% yield) were prepared as described above.

The extent of deuterium incorporation at 3*S*-position of the products was confirmed by disappearing the signal from 3*S*-proton in ^{1}H NMR spectrum (98% D enrichment).

The degree of incorporation of label, via stereospecifically labeled L-Phe, into 3*S*- (or 3*R*-) positions of PPA can be affected by the keto-enol tautomerism observed in keto acids, Figure 4. We have confirmed by ^{1}H NMR that under our experimental conditions (synthesis, separation, and purification) the enol form of PPA is present in a negligible amount (ca 1%).

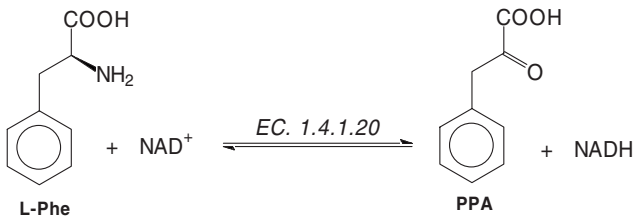

Figure 1. Enzymatic conversion of L-Phe into PPA catalyzed by enzyme L-phenylalanine dehydrogenase.

Experimental

Materials

Enzymes PAL (phenylalanine ammonia lyase, EC 4.3.1.5) from *Rhodotorula glutinis* and PheDH (L-phenylalanine dehydrogenase, EC 1.4.1.20) from *Sporosacrina* sp, cofactor NAD^{+} were purchased by Sigma.

[1-^{14}C]-cinnamic acid was produced from K^{14}CN (supplied by Polatom - Swierk, Poland) by a multistep synthesis according to literature procedure.[15] Deuteriated water (99.9% D) was obtained from Aldrich. Tritiated water was purchased from INC Pharmaceutical Inc., Irvine, CA, USA. Scintillation cocktail was obtained from Rotiszint (Germany).

Silica gel plates were from Merck (silica gel 60 F_{254}, catalog no 105554). Aluminum oxide for column chromatography was obtained from POCh (Poland); Amberlit IR 120 was from Aldrich.

L-phenylalanine, phenylpyruvic acid, glycine, and other chemicals needed for trial synthesis and kinetic measurements were from Aldrich.

Methods

The radioactivity of all the samples was determined using a liquid scintillation counter (LISA LSC PW470,Germany). The extent of deuterium incorporation into 3*S* position of L-Phe and PPA, and the presence of enol form of PPA were determined from ^{1}H NMR spectra recorded on a Varian Unity+200 MHz spectrometer.

The concentration of PPA was determined indirectly by measuring the concentration of NADH spectrophotometrically.[16,17] The enzyme PheDH converts L-Phe into PPA in the presence of coenzyme NAD^{+}, Figure 1. The concentration change of NADH was determined by measuring the absorbance at 340 nm using a Shimadzu UV-102-CE-LV spectrometer.

The presence of phenylalanine and phenylpyruvic acid were checked qualitatively by TLC using aluminum oxide plates and developing solvents: butan-1-ol:acetic acid:water 4:1:5, v/v, and acetonitrile:water, 4:1, v/v for phenylalanine and phenylpyruvic acid, respectively (visualization by UV lamp or by exposing the plates to iodine vapors).

Synthesis

Synthesis of [(3*S*)-^{2}H]-phenylpyruvic acid, 3

Synthesis of [(3S)-^{2}H]-l-phenylalanine, ***3***

In a capped vial, to cinnamic acid (30 mg, 0.2 mmol) dissolved in 5.25 mL of fully deuteriated 1.8 M ammonium buffer (NH_4Cl

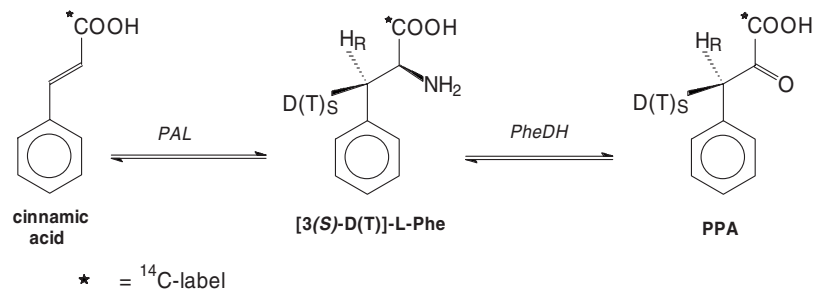

Figure 2. The enzymatic conversion of cinnamic acid into PPA.

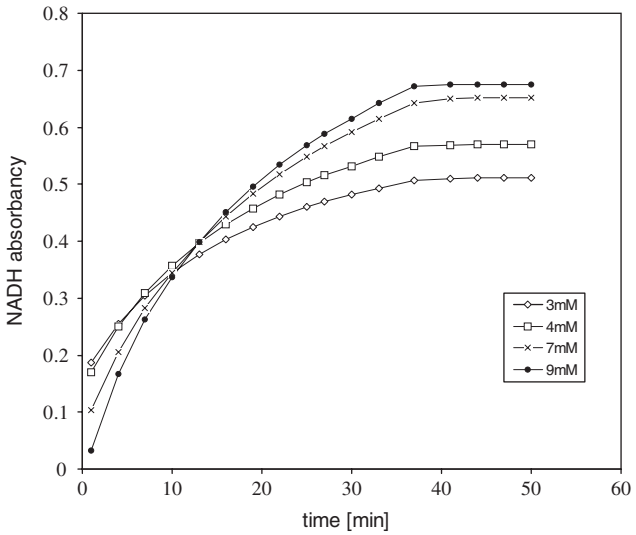

Figure 3. The dependence of conversion of L-Phe into PPA from its concentration in the incubation medium.

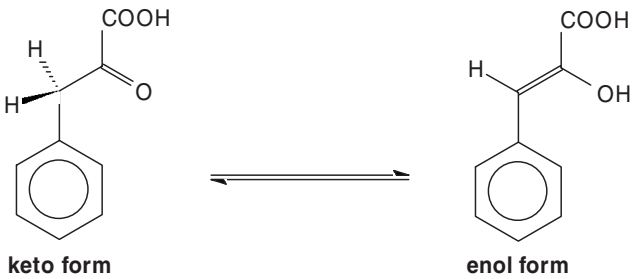

Figure 4. The equilibrium between the keto and enol forms of phenylpyruvic acid.

dissolved in D_2O and adjusted to pD 9.8 with 30% solution of KOD in D_2O) was added a solution of enzyme PAL (300 μL, 2 U) and the reaction mixture was incubated at 37°C for 7 days. The progress of reaction was monitored by TLC using butan-1-ol:acetic acid:water (4:1:5, v/v) as developing solvent. Crude deuteriated product, **2**, was extracted five times by 10 mL diethyl ether and purified by chromatography. Therefore, the excess of diethyl ether was evaporated to 2–3 mL volume, and the residue was loaded onto Amberlite IR 120 H^+ column (100 × 10 mm) and **2** was eluted with 1 M NH_3(aq) and collected as 5 mL fractions. The fractions containing **2** were combined and evaporated to dryness under reduced pressure at 40°C. As a result a 12.5 mg (0.076 mmol) sample of **2** was obtained with 38% yield. The position and the extent of deuterium incorporation were verified by ^{1}H NMR (97% D incorporation in 3-position).

*Synthesis of [3S-^{2}H]-phenylpyruvic acid, **3***

In a capped vial, 10 mg (0.06 mmol) sample of **2** was dissolved in 0.1 M of glycine buffer (5 mL, pH 10.7), and 20 mg NAD^+, and 1.2 mg of enzyme PheDH (1.6 U) was added. The reaction mixture was incubated for 4 h at room temperature. Progress of the reaction was monitored by TLC using acetonitrile:water (4:1, v/v, visualization by UV light) as the mobile phase. Next, the reaction mixture was loaded onto silica gel column (100 × 10 mm), and **3** was washed out with eluent composed with diethyl ether, methanol, and formic acid (98:1:1, v/v) and collected as 5 mL fractions. The presence of **3** in eluted fractions was checked by TLC as above. The fractions containing **3** were concentrated to a volume of about 3 mL under reduced pressure and lyophilized to dryness under vacuum. As a result about 4.8 mg (0.029 mmol) of **3** was obtained (48% yield).

Synthesis of [3-^{3}H]-phenylpyruvic acid, 4.

*Synthesis of [(3S)-^{3}H]-l-phenylalanine, **5***

In a capped vial, to cinnamic acid (8.6 mg, 0.058 mmol) dissolved in ammonium buffer (1.5 mL, pH 9.4) were added a solution of enzyme PAL (150 μL, 2 U) and 300 μL tritiated water with total radioactivity of about 11 GBq. The incubation was carried out as described in the section 'synthesis of [(3S)-^{2}H]-L-phenylalanine, **2**'. Next, the tritiated water from post-reaction mixture was removed by lyophilization. The tritiated **5** was extracted from the residue five times by diethyl ether (10 mL) to separate it from unreacted cinnamic acid and purified chromatographically as described in the section 'synthesis of [(3S)-^{2}H]-l-phenylalanine, **2**'. The remaining of HTO and tritium from labile position of L-Phe was washed out with water, and in the next **5** was eluted with 1 M NH_3(aq), and collected as 5 mL fractions. From each fraction 100 μL sample was taken for radioactivity assay. The fractions containing **5** were combined and evaporated to dryness under reduced pressure at 40°C. A 3.8 mg (0.0232 mmol) sample of **5** was obtained with total radioactivity 1.7×10^6 Bq (sp. activity 73.2 MBq/mmol, 40% yield).

*Synthesis of [(3S)-^{3}H]-phenylpyruvic acid, **4***

The whole obtained sample of **5** was dissolved in a vial containing 0.1 M of glycine buffer (4.5 mL, pH 10.7), and 15 mg NAD^+, and enzyme PheDH (1,2 mg, 1.7 U) were added. The reaction mixture was incubated, separated, and purified as described in the section 'synthesis of [3S-^{2}H]-phenylpyruvic acid, **3**'. As a result about 2.1 mg (0.013 mmol) sample of **4** was obtained (56% yield) with total radioactivity 0.95 MBq (specific activity 73.2 MBq/mmol).

Synthesis of doubly labeled [(3S-^{2}H/^{3}H]-phenylpyruvic acid, 6

*Synthesis of [(3S)-^{2}H/^{3}H]-L-phenylalanine, **7***

In a capped vial, to cinnamic acid (8.6 mg, 0.058 mmol) dissolved in 1.8 M ammonium buffer (1.5 mL, pD 9.8) were added a solution of enzyme PAL (150 μl, 2 U) and 300 μL of tritiated water with total radioactivity of 5.2 GBq. The incubation and the separation protocol were the same as described in the section 'synthesis of [(3S)-^{2}H]-l-phenylalanine, **2**'. As a result 4.1 mg (0.025 mmol) sample of **7** was obtained with total radioactivity of 96 kBq (sp. activity 3.85 MBq/mmol, yield 43%)

*Synthesis of [3S-^{2}H/^{3}H]-phenylpyruvic acid, **6***

The whole obtained sample of **8** was dissolved in a vial with 0.1 M of glycine buffer (3 mL, pH 10.7) and to this 16 mg NAD^+, and enzyme PheDH (1.6 mg, 2.6 U) were added. The incubation, separation and purification of **6** were carried out as described in the section 'synthesis of [3S-^{2}H]-phenylpyruvic acid, **3**'. As a result 1.9 mg (0.012 mmol) sample of **6** was obtained with total

radioactivity of 44 kBq (sp. activity of 3.7 MBq/mmol, yield 46%).

Synthesis of [1-^{14}C-phenylpyruvic acid, 8

*Synthesis of [1-^{14}C]-L-phenylalanine, **9***

In a capped vial, to [1-^{14}C]-cinnamic acid (30 mg, 0.2 mmol, total radioactivity of 1.42 MBq) dissolved in 1.8 M ammonium buffer (5.25 mL, pH 9.4) was added a solution of enzyme PAL (300 μL, 2 U). The incubation, separation, and purification of the obtained **9** were carried out as described in the section 'synthesis of [(3*S*)-^{2}H]-l-phenylalanine, **2**'. As a result 11.6 mg (0.07 mmol) sample of **9** was obtained with total radioactivity of 0.45 MBq (yield 35%).

*Synthesis of [1-^{14}C]-phenylpyruvic acid, **8***

The whole sample of **9** was dissolved in 5 mL of 0.1 M glycine buffer and enzyme PheDH and NAD^+ were added in the same manner as described in the section 'synthesis of [3*S*-^{2}H]-phenylpyruvic acid, **3**'. The incubation and purification protocols of **8** were the same as described in the section 'synthesis of [3*S*-^{2}H]-phenylpyruvic acid, **3**'. As a result a 4.6 mg (0.028 mmol) sample of **8** was obtained with total radioactivity 0.2 MBq (sp. activity 7.1 MBq/mmol, yield 40%).

Acknowledgement

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