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ORIGINAL PAPER

Enzymatic reductive amination of *p*-hydroxy- and phenylpyruvic acids as a method of synthesis of L-tyrosine and L-phenylalanine labelled with deuterium and tritium

Katarzyna Pałka, Marianna Kańska

Abstract. We report the synthesis of isotopomers of L-phenylalanine and L-tyrosine selectively labelled with hydrogen isotopes in the 2-position of the side chain. The deuterium or tritium label was introduced using reductive amination activity of enzyme L-phenylalanine dehydrogenase (EC 1.4.1.20). This way *p*-phenylpyruvic acid was converted into [2-^{2}H]-, [2-^{3}H]-, and doubly labelled [2-^{2}H/^{3}H]-isotopomers of L-phenylalanine, using deuteriated, tritiated, and mixed (DTO) incubation media, respectively. Similarly, *p*-hydroxyphenylpyruvic acid was converted into [2-^{2}H]-, [2-^{3}H]-, and [2-^{2}H/^{3}H]-L-tyrosine. Deuterium labelled isotopomers of L-phenylalanine and L-tyrosine can be used as markers in the investigation of abnormal metabolism of these amino acids observed in patients with inborn genetic diseases such as phenylketonuria and tyrosinemia.

Key words: deuterium • enzyme • L-phenylalanine • tritium • L-tyrosine

K. Pałka
Department of Chemistry,
University of Warsaw,
1 L. Pasteur Str., 02-093 Warsaw, Poland

M. Kańska✉
Department of Chemistry,
University of Warsaw,
1 L. Pasteur Str., 02-093 Warsaw, Poland
and 2nd Faculty of Medicine,
Department of Biochemistry,
Medical University of Warsaw,
101 Żwirki and Wigury Ave., 02-089 Warsaw, Poland,
Tel.: +48 22 822 0211 ext. 509,
Fax: +48 22 822 0211 ext. 434,
E-mail: mkanska@chem.uw.edu.pl

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Introduction

A human inborn genetic disease, type I phenylketonuria [13] (PKU) is caused by a mutation in the gene encoding the enzyme phenylalanine dehydrogenase necessary to convert L-phenylalanine (L-Phe) to L-tyrosine, (L-Tyr). The accumulated L-Phe is metabolized by enforced side reaction to phenylpyruvate, phenylacetate, and phenyl-L-lactate excreted with urine, Fig. 1. The excess of phenylalanine hinders also the transport of other amino acids in the cell, causing a reduction in the synthesis of neurotransmitters (dopamine, norepinephrine and epinephrine) and melanins. Type I PKU non cured leads to mental retardation, brain damage, seizures, and skin discoloration.

Type I tyrasinemia [10], the most severe form of tyrasinemia is inborn genetic disorder, in which L-Tyr cannot be effectively break down, is accompanied by elevated levels L-Tyr and its metabolites: *p*-hydroxyphenylpyruvate, *p*-hydroxyphenylacetate, and *p*-hydroxyphenyl-L-lactate in body fluids, Fig. 1. This disease is caused by a deficiency of enzyme fumarylacetoacetate hydrolase. The abnormal concentration of these metabolites inhibits the activity of other enzymes. This type I tyrasinemia causes liver, spleen, and kidney disturbances and mental retardation. Similarly, type II tyrosinemia is caused by a deficiency of the enzyme tyrosine aminotransferase. In this case the accumulation of L-Tyr causes the start of alternative path of its de-

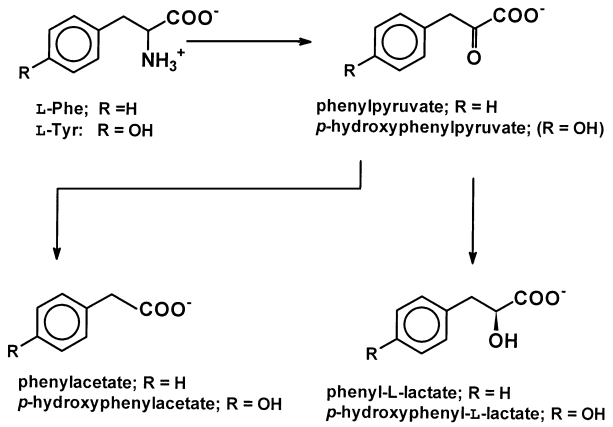

Fig. 1. Alternative metabolic pathways of accumulated L-Phe or L-Tyr in patients suffering from PKU and type I tyrasinemia diseases.

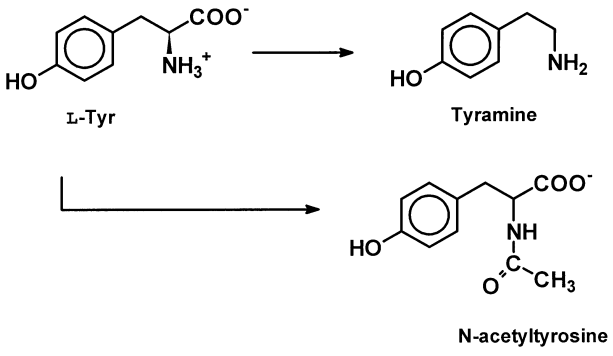

Fig. 2. Alternative decomposition pathways of L-Tyr in patients suffering from type II tyrasinemia.

composition leading to N-acetyltyrosine and tyramine, Fig. 2. This form of desease can affect the eyes, skin, and mental development.

The researches on these diseases, caused by abnormal catabolism of L-phenylalanine and L-tyrosine are widely carried out. The use for this purpose such isotopic markers as L-Phe and L-Tyr can give a new tool helpful in studies of irregular metabolism of natural aromatic amino acids.

One of metabolic path of conversion of L-Phe into phenylpyruvic acid (PPA) is the reversible oxidative deamination [4, 5] catalyzed by enzyme L-phenylalanine dehydrogenase (PheDH) (EC 1.4.1.20), Fig. 1. Under basic conditions, the enzyme PheDH catalyzes the oxidative deamination of L-α-amino acids into the corresponding 2-oxoacids [14, 15], showing a maximum activity at pH 10.7. The reversible reductive amination (conversion of L-α-amino acids into 2-oxoacids) [1, 6, 8] takes place in a milder basic incubation medium, reaching the highest rate at pH 8.5. The mechanisms of metabolism of L-Phe and L-Tyr, the key steps in PKU and tyrasinemia diseases, are not clearly understood. Consequently, this prompted our research to investigate some details of this reversible reaction presented in Fig. 1, by applying the kinetic isotope effect (KIE) [16] and solvent isotope effect (SIE) [12] methods. The determined numerical values of the isotope effects allowed us to elucidate the intrinsic details of the mechanism and were useful to make distinctions between alternative mechanisms. The aforementioned studies required the use of isotopomers of L-Phe and L-Tyr labelled with deuterium and tritium in the 2-position of side chain. Earlier, in the course of our studies of the mechanism of reactions catalyzed by enzymes phenylalanine ammonia lyase (EC 4.3.5.1), tyrosine phenol lyase (EC 4.1.99.2), and tyrosine decarboxylase (EC 4.1.1.25), we described the methods of enzymatic synthesis of isotopomers of L-Phe and L-Tyr labelled in the 2-positions [9]. Specifically, we obtained [2-^{2}H]-, and [2-^{3}H]-L-Phe as well as the corresponding isotopomers of L-Tyr ([2-^{2}H]-, [2-^{3}H]-, and [2-^{2}H/^{3}H]-L-Tyr) [2, 3, 11]. These combined chemoenzymatic synthetic methods are long lasting and final products are afforded with moderate yields. Therefore, we are seeking for new synthetic methods, which would be less time and costs consuming and more effective. In course of our study of reversible reaction presented in Fig. 1 and the literature data [1, 6, 8], it became feasible to use reductive amination activity of PheDH for synthesis of the desired isotopomers of L-Phe and L-Tyr. Our goal is to elaborate the synthesis of L-Phe and L-Tyr labelled with deuterium and tritium in the 2-position using as the substrates PPA and *p*-hydroxyphenylpyruvic acid (*p*-OH-PPA), respectively. Deuteriated and tritiated water will serve as the sources of labels, and enzyme PheDH as reductive amination catalyst.

Experimental

Materials

Enzyme PheDH (L-phenylalanine dehydrogenase, EC 1.4.1.20) from *Sporosacrina* sp. and cofactor – NADH disodium salt – were purchased by Sigma. Tritiated water was from INC Pharmaceutical Inc., Irvine, CA, USA. Deuteriated water (99.9% D), Amberlit IR 120 were obtained from Aldrich. Deuteriated 30% KOD/D_2O (99.9% D) was purchased from POLATOM, Poland. Silica gel plates were from Merck (silica gel 60 F_{254}; catalog no. 105554). Scintillation cocktail was from Rotiszint (Germany). L-Phenylalanine, L-tyrosine, sodium phenylpyruvate, *p*-hydroxyphenylpyruvic acid, and other chemicals needed for trial synthesis 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 the 2-position of L-Phe and L-Tyr was determined from ^{1}H NMR spectra recorded on a Varian Unity+ 200 MHz spectrometer. The progress of enzymatic reductive amination reaction was determined indirectly by measuring the concentration of NADH (nicotine amide dinucleotide) spectrophotometrically at 340 nm using a Shimadzu UV-102-CE-LV spectrometer. Additionally, in the course of reaction the presence of phenylalanine, tyrosine, *p*-hydroxy- and phenylpyruvic acids were checked qualitatively by TLC (thin-layer chromatography) using silica gel plates and developing solvent: acetonitrile:water, 4:1; v/v (visualization by UV lamp).

Synthesis

Synthesis of [2-^{2}H]-L-Phe, ***1***

In a capped vial, to phenylpyruvic sodium salt (10 mg, 0.054 mmol) dissolved in 2 mL of fully deuteriated 0.7 M ammonium buffer (NH_4Cl dissolved in D_2O and adjusted to pD 8.9 with 30% solution of KOD in D_2O) were added 50 mg (0.07 mmol) of NADH disodium salt and 1 mg (2.2 U) of enzyme PheDH (EC 1.4.1.20). The reaction mixture was incubated at room temperature for 24 h. The progress of reaction was monitored by TLC using developing solvent: acetonitrile:water, 4:1, v/v. The enzyme was removed by centrifugation and supernatant was acidized with 2 mL of 2 M HCl. Next, unreacted PPA was extracted with 3 × 2 mL portions of diethyl ether. The water layer containing **1** was loaded onto Amberlite IR 120 H^+ column (100 × 10 mm) and inorganic salts were washed out with water (150 mL). Deuteriated product **1** was eluted with 1 M NH_3(aq) and collected as 5 mL fractions. The presence of phenylalanine in each eluted fraction was checked by TLC. The fractions containing **1** were combined and evaporated under reduced pressure at 45°C, and finally dried under vacuum. As a result, a 6.2 mg (0.037 mmol) sample of **1** was obtained with 62% yield. The ^{1}H NMR spectrum is shown near 100% incorporation of deuterium into 2-position of **1**. ^{1}H NMR (200 MHz, DMSO-d_6): δ 3.13 (1H, β-H, d), 3.27 (1H, β-H, d), 7.29 (2H, ArH, d), 7.34 (2H, ArH, d), 7.84 (1H, ArH, d). Signal from α-proton, i.e., δ 3.99 (1H, α-H, dd) disappeared.

Synthesis of [2-^{3}H]-L-Phe, ***2***

In a capped vial, to phenylpyruvic sodium salt (10 mg, 0.054 mmol) dissolved in 2 mL of 0.7 M ammonium buffer (pD 8.5) were added 50 mg (0.07 mmol) of NADH disodium salt, 1 mg (2.2 U) of enzyme PheDH (EC 1.4.1.20), and 0.5 mL of tritiated water with total radioactivity of about 19 GBq. The reaction mixture was incubated at room temperature for 24 h. Next, the tritiated water was removed by lyophilization, the residue was dissolved in 1 mL of ammonium buffer, and the enzyme was centrifuged off. The supernatant was acidified with 2 mL of 2 M HCl and unreacted PPA was extracted with 3 × 2 mL of diethyl ether. Water layer was loaded onto an Amberlite IR 120 H^+ column (100 × 10 mm) and washed with water to remove the buffer salts and any tritium from labile position of L-Phe until the radioactivity of eluted fractions was close to steady background. Next, **2** was eluted with 1 M NH_3(aq) and collected as 5 mL fractions. From each fraction 50 μL sample was taken for radioactivity assay. The fractions containing **2** were treated as described in the section "Synthesis of [2-^{2}H]-L-Phe, **1**". As a result, a 5.2 mg (0.031 mmol) sample of **2** was obtained (52% yield) with total radioactivity of 2.28 MBq (sp. activity 73.5 MBq/mmol).

Synthesis of [2-^{2}H/^{3}H]-L-Phe, ***3***

In a capped vial, to phenylpyruvic sodium salt (10 mg, 0.054 mmol) dissolved in 1 mL of fully deuteriated 0.7 M ammonium buffer (pD 8.9) were added 50 mg (0.07 mmol) of NADH disodium salt, 1 mg (2.2 U) of enzyme PheDH (EC 1.4.1.20), and 0.3 mL of tritiated water with total radioactivity of about 11 GBq. The procedure of for incubation, separation and purification of **3** was the same as described in the section "Synthesis of [2-^{3}H]-L-Phe, **2**". As a result a 5.3 mg (0.032 mmol) sample of **3** was obtained (53% yield) with total radioactivity of 2.19 MBq (sp. activity 68.4 MBq/mmol).

Synthesis [2-^{2}H]-L-Tyr, ***4***

In a capped vial, to *p*-hydroxyphenylpyruvic acid (10 mg, 0.046 mmol) dissolved in 2 mL of fully deuteriated 0.7 M ammonium buffer (pD 8.9) were added 50 mg (0.07 mmol) of disodium NADH salt and 1 mg (2.2 U) of enzyme PheDH (EC 1.4.1.20). The reaction mixture was incubated for 24 h and treated similarly as described in the section "Synthesis of [2-^{2}H]-L-Phe, **1**". A 4.6 mg (0.026 mmol) sample of **4** was obtained with 46% yield. The ^{1}H NMR spectrum shown near 100% incorporation of deuterium into 2-position of **4**. ^{1}H NMR (200 MHz, DMSO-d_6): δ 3.03 (1H, β-H, d), 3.18 (1H, β-H, d), 6.89 (2H, ArH, d), 7.18 (2H, ArH, d). Signal from α-proton i.e., δ 3.93 (1H, α-H, dd) disappeared.

Synthesis of [2-^{3}H]-L-Tyr, ***5***

In a capped vial, to *p*-hydroxyphenylpyruvic acid (10 mg, 0.046 mmol) dissolved in 2 mL of 0.7 M ammonium buffer (pH 8.5) were added 50 mg (0.07 mmol) of NADH disodium salt, 1 mg (2.2 U) of enzyme PheDH (EC 1.4.1.20), and 0.5 mL of tritiated water with total radioactivity of about 19 GBq. The followed experimental protocol was the same as described in the section "Synthesis of [2-^{3}H]-L-Phe, **2**". A 4.2 mg (0.023 mmol) sample of **4** was obtained (42% yield) with a total radioactivity of 1.94 MBq (sp. activity 84.3 MBq/mmol).

Synthesis of [2-^{2}H/^{3}H]-L-Tyr, ***6***

In a capped vial, to *p*-hydroxyphenylpyruvic acid (10 mg, 0.046 mmol) dissolved in 1 mL of fully deuteriated 0.7 M ammonium buffer (pD 8.9) were added 50 mg (0.07 mmol) of NADH disodium salt, 1 mg (2.2 U) of enzyme PheDH (EC 1.4.1.20), and 0.3 mL of tritiated water with total radioactivity of about 11 GBq. The procedure for incubation, separation and purification of **3** was the same as described in the section "Synthesis of [2-^{3}H]-L-Phe, **2**". As a result, a 4 mg (0.032 mmol) sample of **6** was obtained (40% yield) with total radioactivity of 1.9 MBq (sp. activity 86.4 MBq/mmol).

Results and discussion

For the synthesis of isotopomers L-Phe and L-Tyr labelled in the 2-position we used the reaction presented in Fig. 3. In the course of NADH dependent reductive amination of 2-oxoacids (PPA or *p*-OH-PPA) catalyzed by enzyme PheDH, the amine group replaces oxygen atom. Simultaneously, a solvent proton is introduced into the 2-position, and α-amino acids are formed. When this reaction is carried out in incubation media,

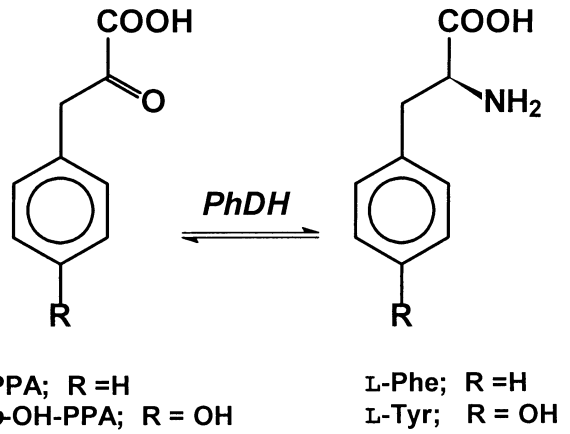

Fig. 3. Reversible conversion of L-Phe and L-Tyr into corresponding 2-oxoacids.

composed of NH_4Cl/D_2O, NH_4Cl/HTO, and NH_4Cl/DTO, PPA is converted into [2-^{2}H]-, [2-^{3}H]-, and doubly labelled [2-^{2}H/^{3}H]-L-Phe, respectively. Under the same conditions, *p*-OH-PPA is biotransformed into the corresponding isotopomers of L-Tyr.

We had to perform certain preliminary studies to elaborate the optimal experimental conditions for reductive amination of PPA and *p*-OH-PPA. The progress of reaction was determined indirectly by spectroscopic [1] measurement of absorbance of NADH at 340 nm. Additionally, for qualitative assays TLC was used (silica gel plates, acetonitrile:water, 4:1; v/v). In this case the spots corresponding to unreacted 2-oxoacids and L-Phe or L-Tyr were observed under a UV-light lamp. The near 100% deuterium enrichment at the 2-position of L-Phe and L-Tyr was determined by spectroscopy (disappearing the signals from 2-proton in ^{1}H NMR spectra). In the course of trial synthesis carried out at room temperature we found that the maximal yield of labelled amino acids (ranging from 40 to 60%) can be reached after 24 h of incubation time in 0.7 M ammonium buffer at pH 8.5, the quantity of enzyme is equal to ca. 40 U (unit) for 1 mmol of 2-oxoacid, and under 1.3–1.5 molar excess of NADH over 2-oxoacid. In the case of reactions carried out in fully deuteriated incubation media the pD was corrected to 8.9 due to higher pK(D_2O) constant [7].

The new elaborated and presented here routes of synthesis of isotopomers of L-Phe and L-Tyr labelled with deuterium and tritium in the 2-position are more efficient and less time consuming. Previously, we presented combined enzymatic/chemical methods that consisted of three steps. In the first, [2-^{2}H]- and [2-^{3}H]-malonic acid was synthesized by the acid catalyzed isotope exchange. In the second step, labelled isotopomers [2-^{2}H]- and [2-^{3}H]- cinnamic acid were obtained by Konvenagel condensation of labelled malonic acids with benzaldehyde. And finally, in the third step, ammonia was added to the labelled cinnamic acid to yield [2-^{2}H]-, and [2-^{3}H]-L-Phe via phenylalanine ammonia lyase (PAL) enzymatic catalysis [9]. The duration of the last incubation step was seven days with the reaction yield of 30% only. Also, the labelled isotopomers [2-^{2}H]-, and [2-^{3}H]-L-Tyr were obtained by enzymatic hydroxylation of appropriate isotopomer of L-Phe labelled in the 2-position by enzyme L-phenylalanine 4'-monooxidase [2, 9]. In this case, the incubation lasted 3–5 days. In another method, synthesis of hydrogen labelled L-Tyr was achieved by an exchange of the labile hydrogen at the α-carbon in the side chain of L-Tyr. This reaction was catalyzed by enzyme tryptophanase (EC 4.1.99.1) with the duration of 8 days [3, 11]. Such a long incubation time required extra care to prevent fungi growth in the reaction medium. In this study, one-step enzymatic synthesis requires 24 h reaction time only, also offering much higher reaction yield.

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