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Research Article

Synthesis of [^{14}C]-L-tryptophan and [^{14}C]-5′-hydroxy-L-tryptophan labeled in the carboxyl group

E. Boroda, R. Kański and M. Kańska*
Department of Chemistry, Warsaw University, Pasteur 1 Street, 02-089 Warsaw, Poland

Summary

The synthesis of selectively ^{14}C-labeled L-tryptophan and its derivative 5-hydroxy-L-tryptophan using chemical and multienzymatic methods is reported. The mixture containing [1-^{14}C[-DL-alanine, indole or 5-hydroxyindole has been converted to [1-^{14}C]-L-tryptophan or 5′-hydroxy-[1-^{14}C]-L-tryptophan, respectively, in a one-pot multienzymatic reaction using four enzymes: D-amino acid oxidase, catalase, glutamic-pyruvic transaminase and tryptophanase.

Key Words: ^{14}C−labeling; optical isotopomer; L-tryptophan; 5′-hydroxy-L-tryptophan; enzyme

Introduction

The enzyme tryptophanase, TPase, (L-tryptophan indole-lyase, EC 4.1.99.1) has been shown[1–3] to catalyze the degradation of L-tryptophan, L-Trp, and some of its analogues to indole, pyruvate and ammonia (Scheme 1).

*Correspondence to: M. Kańska, Department of Chemistry, Warsaw University, Pasteur 1 Street, 02-089 Warsaw, Poland. E-mail: mkanska@alfa.chem.uw.edu.pl

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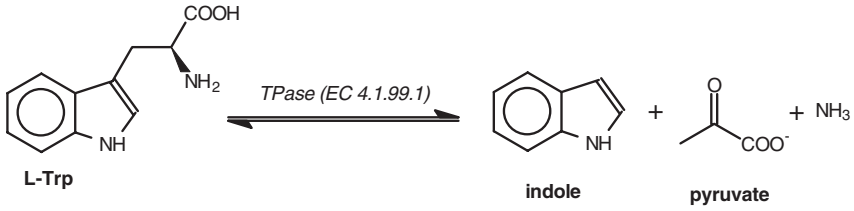


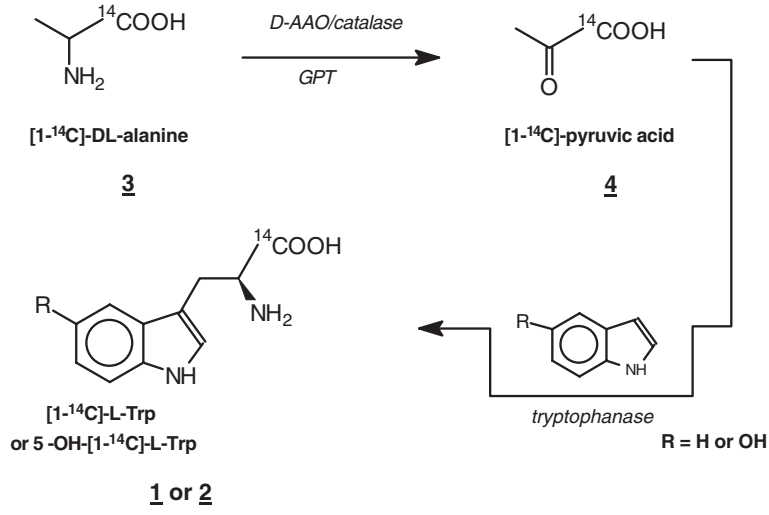
Scheme 1. The reversible reaction catalyzed by enzyme TPase

At sufficiently high concentration of pyruvate and ammonia the TPase also catalyzes the reverse reaction leading to formation[3] of L-Trp. This compound and its derivative 5′-hydroxy-L-tryptophan, 5-OH-L-Trp, are of special interest as the biological precursors for the synthesis of dopamine and serotonin. It is therefore important to know the exact mechanism behind the biological synthesis. Moreover, the above multistep reaction involving hydrogen transfer is still not clear. A number of mechanistic questions can be resolved[4–6] by determining kinetic isotope effects, KIE, using tritium attached to selected positions of L-Trp and 5-OH-L-Trp. The numerical values of the KIE may be useful in distinguishing between alternative mechanisms. Such studies require L-Trp and 5-OH-Trp selectively ^{14}C-labeled in the carboxyl group. This label will serve as an internal radiometric standard during the determination of the KIE for hydrogen isotopes.[7–8] Such studies require the use of only one biologically active enantiomer. Therefore, enzymes are being widely used for introducing the label as they exhibit high stereoselectivity and operate under mild conditions. This paper reports on the multienzymatic one-pot synthesis of L-Trp and 5-OH-L-Trp labeled with ^{14}C in the carboxyl group. As a source of ^{14}C $^{14}CO_2$ has used, which was converted to the starting intermediate i.e., [1-^{14}C]-DL-alanine on the chemical route.[9,10] In the literature there are several methods describing the synthesis of the L-enantiomers of Trp and 5-OH-Trp selectively labeled with isotopic carbon. The ^{13}C-labeled indoles have been converted to the corresponding isotopomers of L-Trp using transformed *Esterechia coli* cells containing large amounts of enzyme tryptophan synthetase.[11–13] The ^{11}C-labeling of some isotopomers of L-Trp and its derivatives have been reported; they are required for tumor diagnosis using positron emission tomography.[14–16] In addition several tedious chemical routes for obtaining the different isotopomers of ^{14}C-labeled DL-Trp have also been described[17].

Results and discussion

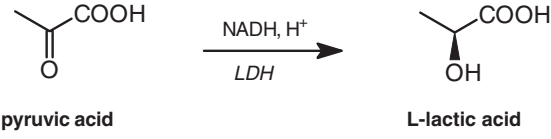
[1-^{14}C]-L-Trp, **1**, and 5-OH-[1-^{14}C]-L-Trp, **2**, specifically labeled with ^{14}C in the carboxyl group, have been prepared using a combination of chemical and multienzymatic methods. For this synthesis we applied, as an intermediate, a racemic mixture of [1-^{14}C]-DL-alanine, **3**, which was obtained in a multistep synthesis that has been previously reported.[9,10] As a source of ^{14}C-label $^{14}CO_2$ (obtained from $Ba^{14}CO_3$) was used. It has been converted in turn into [1-^{14}C]-DL-alanine, **3**, *via* [1-^{14}C]propionic acid (carbonation of the Grignard reagent with $^{14}CO_2$, C_2H_5MgJ, and decomposition of the complex formed), 2-bromo[1-^{14}C]-propionic acid, followed by ammonolysis. In a one-pot multienzymatic synthesis **3** was converted into [1-^{14}C]-pyruvic acid, **4**, using the enzymes D-AAO/catalase and GPT.[18,19] In turn **4** is coupled with indole or 5-hydroxyindole by the enzyme TPase giving **1** or **2**, respectively (Scheme 2). Enzyme D-AAO rapidly transforms D-alanine to pyruvic acid only, therefore, to avoid the loss of half of radioactivity from the L-enantiomer we also used the second enzyme, GTP, converting L-alanine into pyruvic acid only. The medium also contained the enzyme catalase (removing the H_2O_2 formed) and coenzymes for D-AAO and GPT, i.e., flavine adenine dinucleotide, FAD, and pyridoxal 5-phosphate, PLP, respectively.

For this synthesis a reliable spectrophotometric method of determining the concentration of tryptophans was used. The enzyme TPase



Scheme 2. Enzymatic synthesis of [1-^{14}C]-L-Trp or 5-OH-[1-^{14}C]-L-Trp

decomposes L-Trp and its derivatives to pyruvic acid (Scheme 1). The pyruvic acid is converted into lactic acid in the presence of the enzyme lactic acid dehydrogenase, LDH, and nicotine amide dinucleotide, NADH (Scheme 3). NADH gives a strong absorbance at 340 nm that is easily registered spectrophotometrically. The concentration of NADH enables the concentration of tryptophan in the measured sample to be calculated.[6,20]



Scheme 3. Conversion of pyruvic acid into L-latic acid by enzyme LDH

Experimental

Materials

$Ba^{14}CO_3$ was obtained from Polatom (Poland). Scintillation cocktail was purchased from Rotiszint (Germany). Silica gel TLC plates, 60 F_{256} were from Merck. The enzymes: D-amino acid oxidase (EC. 1.4.3.3), D-AAO, from PORCINE KIDNEY; catalase (EC 1.11.1.6) from BOVINE LIVER; glutamic-pyruvic tramsaminase (EC 2.6.1.2), GPT, from PORCINE HEART; tryptophanase, Tpase (EC 4.1.99.1) from *E. coli*, and lacic acid dehydrogenase (1.1.1.27), LDH, from RABBIT MUSCLE were purchased from Sigma. Coenzymes, i.e., the oxidized form of flavine adenine dinucleotide, FAD, pyridoxal 5-phosphate, PLP, and the reduced form of nicotinamide adenine dinucleotide, NADH, were also from Sigma. Other chemicals, needed for enzymatic synthesis and control experiments, i.e., α-ketoglutarate, α-KG, TRIS/HCl, L-tryptophan, DL-alanine, indole and 5-hydoxyindole were products of Sigma.

Methods

The presence of L-Trp and 5-OH-L-Trp was checked qualitatively by TLC using silica gel plates and developing solvent: acetic acid—water—butanol (1 : 1 : 4, v/v). The concentration of the above-mentioned compounds was determined indirectly by measuring the concentration of NADH spectophotometrically. This procedure

consists of eliminating pyruvic acid from tryptophans by the enzyme Tpase. In the next step, pyruvic acid is converted into L-lactic acid in the presence of the enzyme LDH and coenzyme NADH (Scheme 3). The measured sample of L-Try or 5-OH-L-Trp was added to a medium containing 0.2 M phosphate buffer (pH 8), enzymes TPase and LDH and coenzyme NADH and incubated at room temperature for 45 min.The concentration change of NADH was determined by measuring the absorbance at 340 nm using a Shimadzu-UV-102 CE-LV spectrophotometer. In the control studies with inactive compounds the optical rotation of the L-Trp and 5-OH-L-Trp was checked on the polarimeter (P 3002- A. Krüs Optronic—Germany). The radioactivity of all samples (except of $^{14}CO_2$, which was measured by an internally filled Geiger–Müller counter) was determined using an automatic liquid scintillation counter (LISA LSC PW470—Raytest, Germany).

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

Synthesis of DL-*[1-^{14}C]-alanine,* ***3***. This compound was synthesized as described elsewhere.[9,10] From 100 mg of $Ba^{14}CO_3$ with a total radioactivity of 3.7×10^7 Bq (specfic activity of 7.4×10^7 Bq/mmol) 1.66 g of DL-[1-^{14}C]alanine of 1.37×10^7 Bq radioactivity was obtained (specific activity of 1.04×10^6 Bq/mmol).

Synthesis of ***1***. To the incubation vial containing 3 ml of 0.384 M Tris/HCl buffer at pH 9, the following quantities of reactants were added in turn:

(i) 50 µl of 0.2 M α-KG;
(ii) 10 µl of 0.1 M PLP;
(iii) 10 µl of 0.001 FAD;
(iv) 100 µl of 0.3 M aqueous solution of [1-^{14}C]-[D,L]-alanine of 3.1×10^6 Bq radioactivity (sp. act. 1.04×10^6 Bq/mmol);
(v) 3 ml of catalase (1960 U/ml);
(vi) 50 µl of D-AAO (72 U/ml);
(vii) 50 µl of GPT (1400 U/ml);
(viii) 0.2 ml of 1,5 M $(NH_4)_2SO_4$;
(ix) 100 µl of 0.4 M indole.

The reaction mixture was incubated at room temperature for 2 days. The reaction was stopped by adjusting the pH to 5 with glacial acetic

acid. Non-reacted indole was extracted with toluene (2 × 6 ml) and next the aqueous layer was loaded on to a silica gel column (10 × 100 mm). The **1** formed was eluted with a mixture of acetonitrile/water (4:1 by volume). Fractions containing **1** were combined, concentrated under vacuum at 40–45°C and checked as described in *Methods*. As a result a 1.38 mg sample of **1** was obtained with total radioactivity of 6.8×10^3 Bq (sp. activity 1.03×10^6 Bq/mmol).

Enzymatic synthesis of 5-OH-[1-^{14}C]-L-Trp, 2. This derivative of L-Trp was prepared in the same manner as described in (3b) with the difference that instead of indole the 5-hydroxyindole was used. A 1.92 mg sample of **2** was obtained with total radioactivity of 8.9×10^3 Bq (sp. activity 1.02×10^6 Bq/mmole).

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