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Research Article

Enzymatic synthesis of L-tryptophan and 5′-hydroxy-L-tryptophan labeled with deuterium and tritium at the α-carbon position

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Summary

The enzymatic synthesis of L-tryptophan and its derivative 5′-hydroxy-L-tryptophan labeled with deuterium and tritium at the α-carbon position is reported. The mixture containing *S*-methyl-L-cysteine, indole or 5-hydroxyindole dissolved in deuteriated or tritiated water has been converted to [2-^{2}H]-L-tryptophan, [2-^{3}H]-L-tryptophan, 5′-hydroxy-[2-^{2}H]-L-tryptophan, and 5′-hydroxy-[2-^{3}H]-L-tryptophan, respectively, in a one-pot reaction using the enzyme tryptophanase. The same reaction carried out in heavy water with THO added yielded either doubly labeled [2-^{2}H/^{3}H]-L-tryptophan or 5′-hydroxy-[2-^{2}H/^{3}H]-L-tryptophan. Copyright © 2003 John Wiley & Sons, Ltd.

Key Words: deuterium; tritium; labeling; L-tryptophan; 5′-hydroxy-L-tryptophan; enzyme

Introduction

The enzyme tryptophanase, TPase, (L-Tryptophan Indole-Lyase, EC 4.1.99.1) has been shown[1–4] to catalyze the reductive cleavage of L-tryptophan, L-Trp, and some of its analogues to yield indole (substituted indoles), pyruvate and ammonia (Scheme 1).

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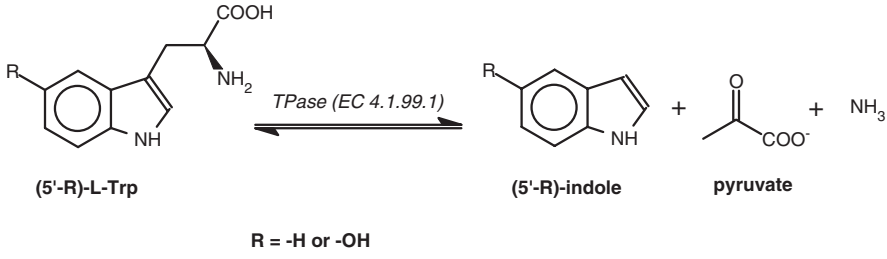


Scheme 1. The reversible reaction catalyzed by the enzyme Tpase

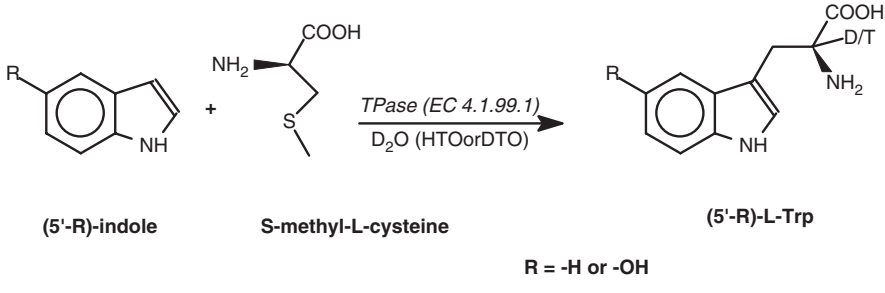
L-Tryptophan and its metabolite 5′-hydroxy-L-tryptophan, 5-OH-L-Trp, play the role of primary intermediates in the biosynthesis of some hormones and some of their derivatives exhibit pharmaceutical activity.[5,6] These reactions (Scheme 1) require multiple proton transfers and the mechanisms are not fully established. For such investigations, multiple deuterium and tritium kinetic isotope effects are excellent tools.[7–10] For some of these studies it is useful to have the label located at the α-carbon position. Such kind of studies involving enzymatic activity requires the use of L-enantiomers specifically labeled in desired position. Specific labeling with deuterium or tritium by chemical methods is inefficient and very tedious; sometimes it is even impossible yielding a DL mixture of the compound. Therefore, enzymatic reactions have been developed to produce the product with high stereochemical purity and the label specifically in the desired position. This paper reports on such an example namely the enzymatic one-pot synthesis of three isotopomers of both L-Trp and 5-OH-L-Trp labeled with deuterium and tritium at the α-carbon position, i.e., [2-^{2}H]-L-Trp, **1**, [2-^{3}H]-L-Trp, **2**, doubly labeled [2-^{2}H/^{3}H]-L-Trp, **3**, 5-OH-[2-^{2}H]-L-Trp, **4**, 5-OH-[2-^{3}H]-L-Trp, **5**, and doubly labeled 5-OH-[2-^{2}H/^{3}H]-L-Trp, **6**. These compounds will be useful for determining kinetic isotope effect values for deuterium and tritium in the reaction depicted in Scheme 1.

In the literature there are several synthetic routes leading to the preparation of different isotopomers of tryptophan and its 5-hydroxy derivative labeled with deuterium and tritium specifically or non-specifically. Perdeuteriated in the indonyl moiety, [$^2H_5$]-L-tryptophan was obtained[11,12] by H/D exchange between tryptophan dissolved in CF_3COOD. The (2R*S*)-[2-^{2}H]-Trp was obtained by exchange with D_2O using a racemization/acylation procedure. This intermediate was resolved with acylase yielding pure (2*S*)-[2-^{2}H]-Trp.[13] Four isotopomers of L-Trp labeled with deuterium specifically in the indole ring (i.e., 4, 5,

6 and 7) have been synthesized via coupling of the labeled indoles with L-serine. This reaction was catalyzed by extracts of *E. coli* cells containing a high concentration of enzyme tryptophan synthetase.[14] Various isotopomers of tryptophan labeled with tritium and deuterium at the 2- and 3-positions of the side chain were synthesized[15] using methods described earlier for inactive compounds.[16,17] Uniformly labeled [U-^{3}H]-5′-hydroxy-DL-tryptophan was obtained by platinum catalyzed exchange with tritiated water.[18] Also [5′-^{3}H]-DL-Trp and [5′- ^{2}H]-DL-Trp were prepared[19] by reduction of 5-bromo-DL-Trp with tritium or deuterium gas. 5′-Hydroxy-[4′-^{3}H]-DL-Trp was obtained[19] by isotope exchange between 5′-hydroxy-DL-Trp and tritiated water in the presence of DMSO and triethylamine. 5′-Hydroxy-[4′-^{3}H]-Trp has also been prepared by enzymatic hydroxylation of [5′-^{3}H]- Trp; this reaction proceeded with over 90% retention of the label.[20] The isotopomers of tryptophan labeled with tritium and deuterium at the 3-position of the side chain have been synthesized using the oxazoline method.[21] [2-^{3}H]-DL-Trp was obtained[22] as a result of the racemization of its inactive acetyl derivative in a mixture of acetic anhydride and tritiated water.

Results and discussion

Under some experimental conditions the enzyme TPase also catalyses the condensation of the indonyl moiety with *S*-methyl-L-cysteine leading[2,3,23,24] to the synthesis of L-tryptophans (Scheme 2). The enzymatic labilization of hydrogen attached to the α-carbon facilitates the H/D or H/T exchange with the solvent (deuteriated or tritiated water in this case).



Scheme 2. Enzymatic synthesis of L-Trp and 5′-OH-L-Trp labeled with deuterium and tritium at the α-carbon position

This reaction was used by us to obtain L-Trp and 5′-OH-L-Trp labeled with the isotopes of hydrogen at the α-carbon position. For the synthesis of [2-^{2}H]-L-Trp, **1**, and 5′-OH-[2-^{2}H]-L-Trp, **4**, all reagents were dissolved in fully deuteriated water. For the synthesis of [2-^{3}H]-L-Trp, **2**, and 5′,-OH-[2-^{3}H]-L-Trp, **5**, the reaction was carried out in tritiated water (HTO). Doubly labeled [2-^{2}H/^{3}H]-L-Trp, **3**, and 5′-OH-[2-^{2}H/^{3}H]-L-Trp, **6**, have been obtained using a medium containing heavy water to which DTO is added. To prevent the growth of bacteria and fungi during incubation 2-mercaptoethanol was also added.

To determine the concentration of the tryptophans we have used a previously described spectrophotometric method.[23–25] The enzyme TPase decomposes L-Trp and its derivatives to pyruvic acid (Scheme 1). In turn this acid is converted into lactic acid in the presence of the enzyme lactic acid dehydrogenase, LDH, and nicotinamide dinucleotide, NADH. This reaction is carried out in the spectrometer cuvette. The concentration of NADH registered spectrophotometrically enables the concentration of tryptophan in the sample to be calculated.

Experimental

Materials

Tritiated water (5 Ci/ml) was purchased from ICN Pharmaceutical Inc, Irvine Ca, USA. Deuteriated water (99.9% deuterium) was obtained from Polatom (Poland). Scintillation cocktail was purchased from Rotiszint (Germany). Silica gel TLC plates, 60 F_{256}, were from Merck. The enzymes:TPase (EC 4.1.99.1) from *E. coli*, and lactic acid dehydrogenase (1.1.1.27), LDH, from rabbit muscle were purchased from Sigma. Coenzymes, i.e., pyridoxal 5-phosphate, PLP, and the reduced form of nicotinamide adenine dinucleotide, NADH, were also from Sigma. Other chemicals, needed for the enzymatic synthesis and control experiments, i.e., 2-mercaptoethanol, *S*-methyl-L-cysteine, L-tryptophan, 5-hydroxy-L-tryptophan, indole and 5-hydoxyindole were obtained from 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 the tryptophans by using 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. The measured sample of L-Try or 5-OH-L-Trp was added to the cuvette with 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 extent of deuterium incorporation at the α-carbon in **1** and **2** was checked by measuring the proton NMR spectra on a Varian 500 MHz Unity-Plus spectrometer. The radioactivity of all samples was determined using an automatic liquid scintillation counter, LSC, (LISA LSC PW470 – Raytest, Germany).

Synthesis of the isotopomers of L-*Trp labeled with deuterium and tritium*

Synthesis of [2-^{2}H]-L-Trp, **1**. To the incubation vial containing 20 ml of 0.1 M phosphate buffer at pD 8 (the proper weights of phosphate salts were dissolved in deuteriated water and pD was adjusted with KOD) the following quantities of reactants were added in turn:

(i) 133 mg (1 mmol) of *S*-methyl-L-cysteine;
(ii) 46 mg (0.4 mmol) of indole;
(iii) 10 µl of 0.1 M PLP;
(iv) 5 µl of 2-mercaptoethanol ($HSCH_2CH_2OH$);
(v) 10 mg of enzyme TPase (0.3 U)

The above mixture was incubated at room temperature for 2 days. The reaction was quenched by adjusting the pH to 5 with glacial acetic acid. The enzyme was removed by centrifugation. Non-reacted indole was extracted with toluene (2 × 10 ml). The aqueous layer containing the product, *S*-methyl-L-cysteine and salts was loaded on to a silica gel column (10 × 100 mm). The **1** formed was eluted with the 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 an 8.5 mg sample of **1** was obtained.

The NMR spectrum showed that incorporation of deuterium at the α-carbon position of **1** exceeded 97%.

Synthesis of [2-^{3}H]-L-Trp, **2**. This isotopomer was obtained similarly as described in synthesis of [2-^{2}H]-L-Trp, **1**. In this case the amounts of reactants (except TPase) were reduced about four times. (5 ml of 0.1 M phosphate buffer at pH 8, 33 mg of methyl-*S*-cysteine,12 mg of indole, 5 µl of $HSCH_2CH_2OH$, 4 mg of TPase), To this incubation medium 100 µl of tritiated water was introduced (total activity of 2.7×10^7 Bq). After 2 days of incubation the obtained **2** was separated chromatographically. The post-reaction mixture was loaded onto an ion-exchange column (Amberlit IR-120 H^+, 10×100 mm) and HTO was washed with water up to the moment when the radioactivity of the eluted fractions was steady and close to background. Next **2** was eluted with 0.3 M of ammonia and the radioactivity of the eluted fractions was checked with LSC. To complete the removal of the rest of the tritiated water the fractions containing **2** were combined, evaporated under reduced pressure to 2 ml volume and once more loaded onto the column, washed with water and next **2** was eluted with 0.3 M NH_3(aq). The fractions containing **2** were combined and evaporated. As a result a sample of 4 mg (0.02 mmol) of **2** (as determined spectrophotometrically) dissolved in 2 ml of water with a total activity of 9×10^4 Bq was obtained (specific activity of 4.5×10^6 Bq/mmol).

Synthesis of the doubly labeled [2-^{2}H/^{3}H]-L-Trp, **3**. This product, doubly labeled with deuterium and tritium was obtained in the same manner as described in synthesis of [2-^{3}H]-L-Trp, **2** with the difference that D_2O served as the solvent. The amounts of all reagents, enzyme and HTO were approximately the same. The isolation procedure and purification of **3** was also similar, as given in synthesis of [2-^{3}H]-L-Trp, **2**. As a result a sample of 3.8 mg (0.018 mmol) of **3** dissolved in 2 ml of water with a total activity of 7.9×10^4 Bq was obtained (specific activity of 4.4×10^6 Bq/mmol).

Synthesis of the isotopomers of 5′-OH-L-Trp labeled with deuterium and tritium

Synthesis of 5′-OH-[2-^{3}H]-L-Trp, **4**. This compound was prepared in the same way as described in synthesis of [2-^{2}H]-L-Trp, **1** with the difference that instead of indole the 5-hydroxyindole (52 mg, 0.4 mmol) was used. In this case the incubation time was prolonged to 3 days. A

9.5 mg sample of **4** was obtained with 97% incorporation of deuterium at the α-carbon position as determined by ^{1}H NMR.

Synthesis of 5′-OH-[2-^{3}H]-L-Trp, **5**. This tritium labeled derivative of L-Trp was prepared as described in synthesis of [2-^{3}H]-L-Trp, **2**. Instead of indole 0.1 mmole of 5-hydroxyindole was added and the incubation lasted 3 days. The resulting 2.5 mg sample of **5** contained 4.55×10^4 Bq total activity (specific activity 4.38×10^6 Bq/mmol)

Synthesis of the doubly labeled 5′-OH-[2-^{2}H/^{3}H]-L-Trp, **6**. This isotopomer was obtained in the same manner as described in synthesis of doubly labeled [2-^{2}H/^{3}H]-L-Trp, **3**. To the incubation mixture instead of indole its 5-hydroxyderivative (0.1 mmol) was introduced and reaction was carried out for 3 days. A 2.7 g sample of **6** contained 4.72×10^4 Bq total activity (specific activity 4.2×10^6 Bq/mmol).

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