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Research Article

Enzymatic synthesis of tritium-labelled isotopomers of histamine

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Summary

Three tritium-labelled isotopomers of histamine (HA) have been synthesized using combined chemical and enzymatic methods. In the first step the tritium-labelled isotopomers of histidine have been obtained by catalysed exchange with tritiated water. These intermediates have been converted into [1S-^{3}H]-HA, [1R-^{3}H]-HA and [2′,4′,1S,-$^3H_3$]-HA using the enzyme histidine decarboxylase (HDC, EC 4.1.1.22) from *Lactobacillus* 30a. Copyright © 2004 John Wiley & Sons, Ltd.

Key Words: enzyme; histamine; histidine; labelling; tritium

Introduction

The enzyme histidine decarboxylase, HDC, (EC 4.1.1.22) catalyses the decarboxylation of L-histidine (L-His) leading to formation of histamine[1,2] (HA) according to Scheme 1.

This biogenic amine (HA) plays an important role in various physiological functions[1–3] such as cell growth, neurotransmission, allergic response, gastric secretion. Undesirable effects of HA have led to much scientific research for the development of more selective inhibitors of the synthesis of histamine and especially for selective blocking of the activity of the enzyme HDC, the main source of histamine in living organisms.[4] The goal of our planned research is directed at investigating some details of the mechanism of the decarboxylation reaction presented in Scheme 1 by applying the kinetic isotope effect method (KIE) and determining the primary kinetic isotope effect values in the rate determining step.[5] For this kind of study the isotopomers and isotopologues of

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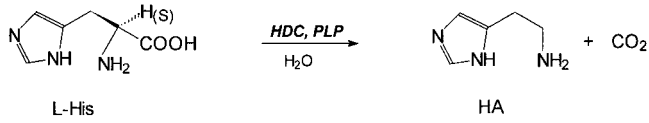


L-His HA

Scheme 1. Enzymatic decarboxylation of L-histidine

histamine (HA) specifically labelled with tritium are needed. In this paper, a synthesis of three isotopomers of HA, i.e., [1S-^{3}H]-, [1R-^{3}H]-, and [2′,4′,1S-$^3H_3$]-HA is presented.

In the literature, there are several methods describing the synthesis of selectively labelled deuterium or tritium isotopomers of HA. Generally, for this purpose the enzymatic decarboxylation[6] reaction of appropriate isotopomers of L-histidine can be used. [2-^{2}H]-, [2-^{3}H]-, and the version labelled with tritium and ^{14}C [2-^{3}H,1-^{14}C]-HA were obtained[7,8] by decarboxylation of labelled L-His catalysed by the enzyme HDC isolated from *Clostridium welchii*.

Results and discussion

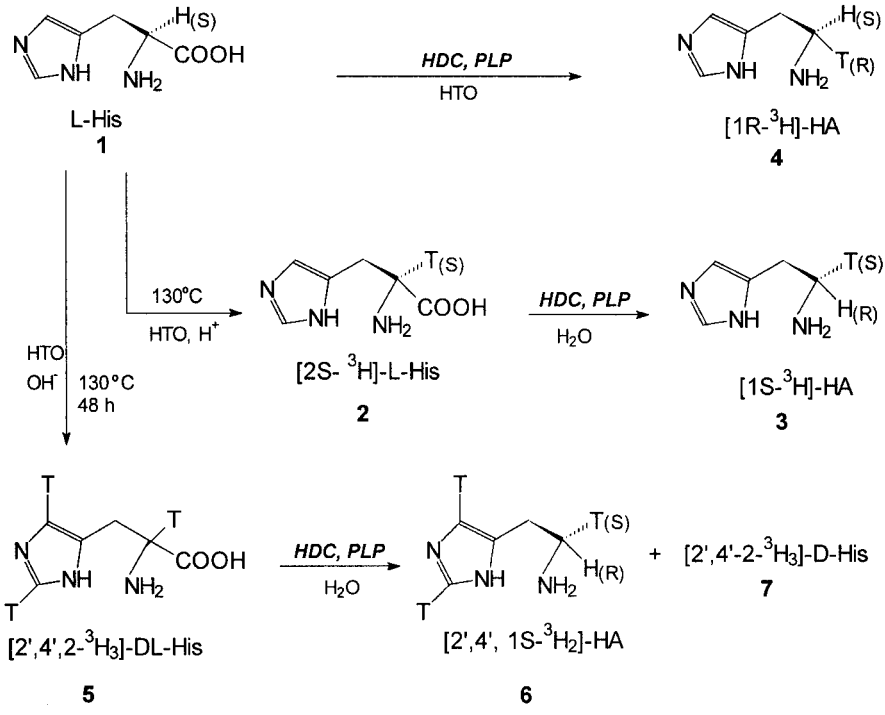
The chosen reaction scheme for the synthesis of the isotopomers of histamine (HA) labelled with tritium (Scheme 2) consists of two parts: in the first, the appropriate isotopomer of L-His labelled with tritium is obtained which is then subjected, in the second stage, to the enzymatic decarboxylation, yielding selectively labelled histamine. To label histidine with tritium, isotopic exchange between tritiated water and L-His was chosen. For this purpose the methods described in the literature[9–12] have been appropriately modified.

Previous research has shown[6–8] that decarboxylation of L-His in heavy water, using the enzyme decarboxylase HDC from *Lactobacillus* 30a (*EC 4.1.1.22*), proceeds with the introduction of one deuterium atom to the newly generated histamine and preserving the configuration at the carbon atom in position α. This observation has been used to obtain isotopologues of histamine in configuration R or S.

The entire synthetic pathway has been tested by means of deuterium as the label with ^{1}H NMR being used to identify the deuteriation site.

As a result of isotopic exchange between tritiated water and L-His, **1**, the tritium-labelled isotopomer [2S-^{3}H]-L-His, **2**, was obtained, from which histamine [1S-^{3}H]-HA, **3**, in configuration **S** was afforded by decarboxylation carried out in the aqueous medium in the presence of enzyme HDC. In the test synthesis carried out in heavy water at a temperature of 130°C, ^{1}H NMR analysis shows that in 48 h the introduction of deuterium at the α-C of histidine proceeds with a 70% yield.

Enzymatic decarboxylation of L-His, **1**, in the tritiated water medium leads to the formation of isotopomer [1R-^{3}H]-HA, **4**, in configuration **R**.



Scheme 2. The combined chemical and enzymatic synthesis of tritium-labelled isotopomers of histamine

As a result of isotopic exchange between tritiated water and **1** carried out under basic conditions, a racemic mixture [2′,4′,2-$^{3}H_3$]-DL-His, **5**, was obtained, labelled with tritium in the ring and in position 2 of the side chain. This mixture **5** was subjected to enzymatic decarboxylation in water yielding the isotopomer of histamine [2′,4′,1S-$^{3}H_3$]-HA, **6**, of configuration **S**, leaving the D-enantiomer form, [2′,4′-2-$^{3}H_3$]-D-His, **7**, intact. Enzyme HDC works only with the L-form of histidine, so that the D-form of **5** remains unreactive, contributing to the loss of about half the radioactivity.

The base catalysed isotopic exchange test reaction between **1** and heavy water carried out at 130°C for 24 h showed 60% incorporation of deuterium in positions 2′ and 4′ of the imidazole ring, and close to 100% H/D exchange in position 2 of the side chain.

The concentration of histidine and histamine in the reaction mixtures was determined spectrophotometrically. As histidine and histamine absorb UV light in this same region of the spectrum an indirect method, developed for the assay of histamine in biological media, was used.[13] Under appropriate conditions histidine and histamine react with *o*-phthaldialdehyde yielding coloured condensation products whose concentration can be determined by

measuring the absorbance at 380 and 556 nm for histidine and histamine, respectively.

Experimental

Materials

Tritiated water 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 enzyme L-Histidine Decarboxylase (EC 4.1.1.22) isolated from *Lactobacillus* 30a, coenzyme, i.e., pyridoxal 5-phosphate, PLP, and other chemicals, needed for the enzymatic synthesis and control experiments, i.e., L-histidine free base, histamine, 2-mercaptoethanol, *o*-phtalicdialdehyde (OPD) were also obtained from Sigma.

Methods

The presence of histidine and histamine was checked qualitatively by TLC using silica gel plates and developing solvent: methanol – 25% NH_3(aq) – (20:1 v/v). The concentration of the above mentioned compounds was determined indirectly by measuring the absorptions at 380 and 560 nm using a SHIMADZU-UV-102 CE-LV spectrophotometer. The extent of deuterium incorporation at the α-carbon and at 2′ and 4′ of imidazole ring 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 histidine labelled with tritium

Synthesis of [2-^{3}H]-L-His, **2**. To the reaction ampoule were added in turn: 204 mg (1.3 mmol) of L-histidine base form, 0.4 ml of concentrated HCl, and 1.6 ml of tritiated water with total radioactivity 5.2 GBq. The ampoule was frozen in liquid nitrogen, evacuated, sealed, and thermostated at 130°C for 48 h. After cooling the ampoule was opened and tritiated water was removed by lyophylization. The residue was dissolved in a small volume of water and loaded onto a column (Dowex 50WX-4-50 H^+ form). To remove the remaining HTO and tritium incorporated into the labile positions of histidine (=NH, -NH_2, -COOH) the column was washed with water until the radioactivity of the eluted fractions was steady and close to background. Next **2** was eluted with 0.5 M NH_3(aq) and collected as 3 ml fractions. For each fraction 10 µl samples were taken for radioactivity assay. The fractions containing **2** were combined and evaporated under vacuum at 50°C and the purity checked by TLC. As a result a sample of 162 mg (1.03 mmol) of **2** was

obtained with a total radioactivity 2.45×10^7 Bq (2.38×10^7 Bq/mmol specific activity) and chemical yield 79.5%.

*Synthesis of [2′,4′,2-$^{3}H_3$]-*DL*-His,* **5**. To the reaction ampoule were added 208 mg (1.32 mmol) of L-histidine base form dissolved in 1.8 ml 2 M NaOH, and 0.2 ml of tritiated water with total radioactivity 5.2 GBq. The ampoule was sealed (similarly as in point 3*a*) and heated at 130°C for 48 h. The product **5** was separated and purified in the same manner as described in point 3*a*. A sample of 158 mg (about 1 mmol) of **5** obtained with a total radioactivity 2.62×10^7 Bq (2.62×10^7 Bq/mmol specific activity) and chemical yield 78%.

Synthesis of [1S-^{3}H]-HA, **3**

The 10.2 mg (65 μmol) sample of **2** was placed in an incubation vial and dissolved in 4 ml of 0.1 M phosphate buffer at pH 6.8. To this 50 μl of 1 mM PLP, 5 μl of 2-mercaptoethanol, and 1 mg (0.23 U) of enzyme HDC were added. The reaction mixture was incubated at 37°C for 48 h with constant shaking. After reaction the enzyme was removed by centrifugation, and the post reaction-mixture was loaded onto the Amberlit IR 120 H^+ column (100×10) and washed with 100 ml of water. The residual quantities of unreacted **2** were washed out with 250 ml of 0.3 M NH_3(aq). The product, i.e., [1S-^{3}H]-histamine, **3**, was eluted with 2 M NH_3(aq). Fractions containing **3** were combined, evaporated under reduced pressure and checked as described in *Methods*. As a result a 6 mg (59 μmol) sample of **3** with total radioactivity of 1.5×10^6 Bq was obtained (specific activity of 2.54×10^7 Bq/mmol). Chemical yield was about 90%.

Synthesis of [1R-^{3}H]-HA, **4**

The 14.4 mg (90 μmol) sample of **1** was placed in an incubation vial and dissolved in 1.3 ml of 0.1 M phosphate buffer at pH 6.8. To this 0.2 ml of tritiated water with total radioactivity 5.2 GBq, 50 μl of 1 mM PLP, 5 μl of 2-mercaptoethanol, and 1 mg (0.23 U) of enzyme HDC were added. The reaction mixture was incubated at 37°C for 48 h with constant shaking. The post reaction mixture was lyophylized to remove most of the tritiated water. The residue was dissolved in water and loaded onto the Amberlit 120 IR H^+ column (10×100) and the residual HTO and labile tritium from -NH_2 and -NH- groups was washed out with water until the radioactivity of the eluted fractions was close to background. Next, product **4** was separated as described in point *4*. As a result 8 mg (79 μmol) of sample **4** was obtained with total radioactivity 1.75 MBq (specific activity 2.2×10^7 Bq/mmol) and chemical yield of 88%.

Synthesis of [2′,4′,1S-$^3H_3$]-HA, **6**

The 31 mg (197 μmol) sample of **5** with total radioactivity 5.2 MBq (specific activity 2.62×10^7 Bq/mmol) was added to an incubation vial containing 15 ml of 0.1 M phosphate buffer at pH 6.8, 60 μl of 1 mM PLP, 10 μl of 2-mercaptoethanol, and 1 mg (0.23 U) of enzyme HDC. The reaction mixture was incubated at 37°C for 48 h with constant shaking. After reaction the enzyme was removed by centrifugation and the post reaction mixture was loaded onto the Amberlit IR 120 H^+ column (100×10) and washed with 100 ml of water. The unreacted [2′,4′,2-$^3H_3$]-D-His, **7**, was washed out with 250 ml of 0.3 M NH_3(aq). The product, i.e., [2′,4′,1S-$^3H_3$]-histamine, **6**, was eluted with 2 M NH_3(aq). Fractions containing **6** were combined, evaporated under reduced pressure and checked as described in 'Methods' section. As a result a 11.5 mg (114 μmol) sample of **7** with total radioactivity of 2.97×10^6 Bq was obtained (specific activity of 2.6×10^7 Bq/mmol) and chemical yield of about 57%.

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