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Research Article

Enzymatic synthesis of isotopomers of tyramine labeled with deuterium and tritium

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Abstract: The combined chemical and enzymatic methods of synthesis of five isotopomers of L-tyrosine, L-Tyr, and their derivatives, i.e. corresponding isotopomers of tyramine (TA), labeled with deuterium and tritium have been reported. Two-step synthesis consists with introduction of deuterium or tritium label into intermediate L-Tyr using isotope exchange followed by enzymatic decarboxylation using enzyme tyrosine decarboxylase (EC 4.1.1.25). This way five isotopomers of L-tyrosine, i.e. [2-^{2}H]-L-, [2-^{3}H]-L-,[2-^{2}H/^{3}H]-L-, [3′,5′-^{2}H$_2$]-L-,[3′,5′-^{3}H$_2$]-L-Tyr, and six isotopomers of tyramine i.e. [1S-^{2}H]-, [1S-^{3}H]-, [1S-^{2}H/^{3}H]-, [3′,5′-^{2}H$_2$]-, [3′,5′-^{2}H$_2$]-, [2′,6′-^{2}H$_2$]-TA were obtained.
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Keywords: deuterium; enzyme; labeling; tritium; tyramine

Introduction

Tyramine, TA, a biogenic amine, found in plant cells, mammalian tissues and fluids, plays an important role in many metabolic processes. It is one of the neurotransmitters in the central nervous system in humans[1–3] and a substrate for enzymatic hydroxylation to other important neurotransmitters such as dopamine. As an intermediate tyramine is responsible for generating melanin in humans and for browning fruits and vegetables.[4,5] In living cells tyramine is produced by enzymatic decarboxylation of L-tyrosine, L-Tyr, a reaction catalyzed by the enzyme tyrosine decarboxylase (EC 4.1.1.25),[6] Figure 1.

Despite much research the mechanisms of the above depicted reactions are not completely understood. It would be interesting to study the decarboxylation and hydroxylation reactions using isotope effects methods.[7–10] Determination of numerical values for solvent isotope effects, SIE, and kinetic isotope effects, KIE, could be useful for distinguishing between the alternative intrinsic details, so that the bonds involved in formation of the active complexes can be determined. For this kind of investigation the specifically labeled isotopomers of L-Tyr and TA are needed. In this paper the combined chemical and enzymatic methods for synthesizing ring and side chain labeled (^{2}H, ^{3}H) of five isotopomers of L-Tyr and six isotopomers of TA are described.

Results and discussion

For synthesis of L-Tyr with the label in the 2-position of the side chain we modified some of the methods described previously.[11–17] The synthetic pathway depicted in Figure 2 uses two enzymes; first, tryptophanase (EC. 4.1.99.1) which introduces the label into the 2-position of L-Tyr, and second, tyrosine decarboxylase (EC 4.1.1.25) which catalyses the carboxylation of the labeled L-Tyr to TA. In the course of decarboxylation of L-Tyr a solvent proton replaces the carboxyl group with retention of configuration.[18,19] Therefore, the products obtained by enzymatic decarboxylation of the isotopomers of L-Tyr (**2**, **3** and **4**), the labeled isotopomers of TA (**5**, **6** and **7)** retain the label at configuration S, Figure 3.

[3′,5′-^{2}H$_2$]-TA, **9**, was synthesized *via* two different routes. In the first H/D isotope exchange between heavy water and L-tyrosine gave [3′,5′-^{2}H$_2$]-L-Tyr · DCl, **8**. Under acid catalyzed conditions the exchange between D_2O and L-Tyr, **1**, introduces the label exclusively into the *ortho* position[16] (respectively, to ring hydroxyl group) in tyrosine yielding the isotopomer **8**, Figure 4. To avoid isotopic dilution the exchange was

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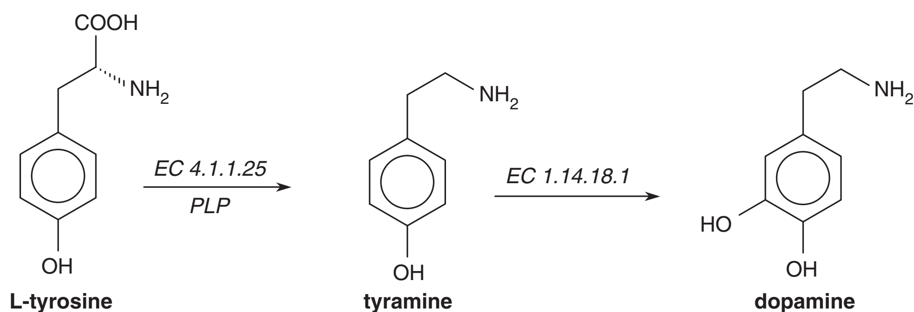

Figure 1 Enzymatic conversion of L-tyrosine into tyramine and dopamine.

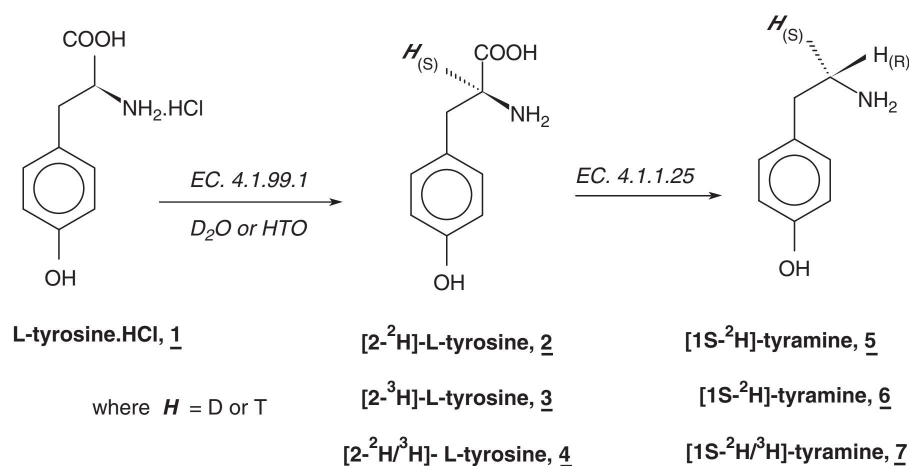

Figure 2 Enzymatic synthesis of isotopomers of L-tyrosine and tyramine labeled at the side chain with deuterium and tritium.

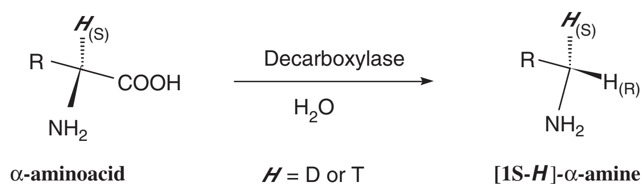

Figure 3 Retention of configuration in the course of enzymatic decarboxylation of α-amino acids.

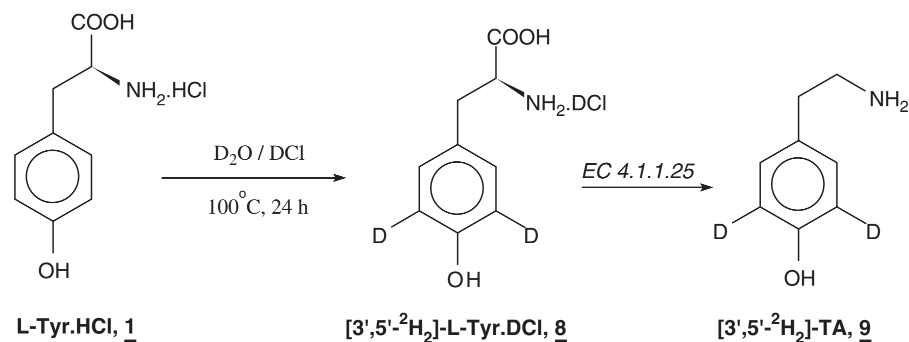

Figure 4 The combined chemical and enzymatic conversion of L-Tyr into [3′,5′-$^2H_2$]-TA.

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carried out in a fully deuteriated medium (D_2O/DCl). The second step involves enzymatic decarboxylation of deuteriated L-tyrosine, **8**, to the desired product, **9**, and this is catalyzed by the enzyme tyrosine decarboxylase (EC 4.1.1.25) from *Streptococcus faecalis*. In the same manner the commercial [2′,6′-$^2H_2$]-L-Tyr was decarboxylated to the [2′,6′-$^2H_2$]-TA, **10**, isotopomer.[13] The position and degree of incorporation of deuterium into aromatic ring of L-tyrosine and tyramine was determined using ^{1}H NMR spectroscopy.

In the second direct synthetic route deuteriated [3′,5′-$^2H_2$]-TA · DCl, **11**, as well as, tritiated [3′,5′-$^3H_2$]-TA, **12**, were obtained in the course of acid catalyzed isotopic exchange[20] carried out between deuteriated or tritiated water and unlabeled tyramine, Figure 5. In this case, as mentioned above, the deuterium is incorporated exclusively in the 3 and 5 ring positions of tyramine.

Experimental

Materials

Tritiated water was purchased from ICN Pharmaceuticals Inc, Irvine, California, USA. Deuteriated water (99.9% deuterium), solutions of 37% DCl/D_2O, 83% D_3PO_4/D_2O, and 30% KOD/D_2O, needed for preparation of fully deuteriated phosphate buffer were obtained from Polatom (Poland). Deuteriated [2′,6′-$^2H_2$]-L-tyrosine was purchased from Sigma. Scintillation cocktail was purchased from Rotiszint (Germany). TLC plates (*DC Plastikfolien Aluminiumoxid 60 F_{256}, neutral, type E*), and *Kieselgel 60* were from Merck. The enzymes: tryptophanase (EC 4.1.99.1) from *E. coli* and tyrosine decarboxylase (EC 4.1.1.25) from *Steptococcus faecalis*, and coenzyme pyridoxal 5-phosphate, PLP, were purchased from Sigma. L-Tyrosine and tyramine hydrochlorides, and other chemicals, needed for the enzymatic synthesis and control experiments, were obtained from Sigma.

Methods

The proton NMR spectra were recorded in DMSO-d_6 or D_2O using TMS as internal standard using a Varian 200 MHz Unity-Plus spectrometer. The radioactivity of all samples was determined using liquid scintillation counting on an automatic counter LISA LSC PW470 (Germany).

Synthesis

1. *Synthesis of* [2-^{2}H]-*L-tyrosine*, **2**: In an incubation vial containing 42 ml of heavy water (99.8% atom D) were dissolved in turn: 27.3 mg (0.126 mmol) of L-tyrosine hydrochloride, **1**, 371 mg (2.13 mmol) of K_2HPO_4, and 1.1 mg (4.5 μmol) of PLP. This reaction medium was adjusted to pH 8.3 with solid KH_2PO_4, and about 1 U of enzyme tryptophanase, isolated from *E. coli* (EC 4.1.99.1), was added. This reaction mixture was incubated for 7 days at room temperature. The exchange was stopped by immersing the vial in hot water for a few minutes. The precipitated proteins were separated by centrifugation, and the supernatant was loaded onto the column (Amberlite 120, H^+; 10 × 100 mm) and **2** was eluted with 0.3 M $NH_{3(aq)}$. The presence of tyrosine in each eluted fraction was checked by TLC (silica gel, eluent: methanol–25% $NH_{3(aq)}$; v/v, visualization by ninhydrine). The fractions containing **2** were combined and solvent was evaporated under reduced pressure at 50°C. The precipitated crystals were washed with ethanol and ethyl ether, and dried under vacuum. As a result 22.2 mg (0.123 mmol) of **2** was obtained (97% yield). The ^{1}H NMR spectrum showed near 100% incorporation of deuterium into the 2-position of **2**. ^{1}H NMR (200 MHz, DMSO-d_6): δ 3.03 (1 H, β-H, d, 14.4 Hz), 3.18 (1 H, β-H, d, 14.4 Hz), 6.89 (2 H, ArH, d, 8.7 Hz), 7.18 (2 H, ArH, d, 8.7 Hz). Signal from α-proton i.e. δ 3.93 (1 H, α-H, dd, 5.7 Hz) disappeared.

2. *Synthesis of* [2-^{3}H]-*L-tyrosine*, **3**: To 2.8 ml of 0.05 M potassium phosphate buffer (pH 8.3) was added

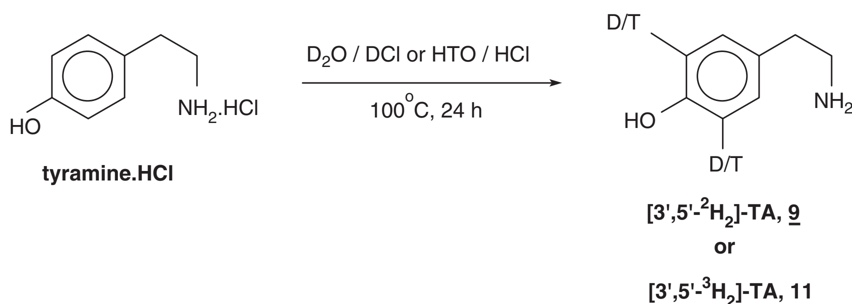

Figure 5 Acid catalyzed hydrogen exchange between tyramine and D_2O or HTO.

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in turn: 0.2 ml of tritiated water (total radioactivity of 55.6 GBq), 11.7 mg (54 μmol) of L-tyrosine hydrochloride, **1**, 1 μmol of PLP, and 0.5 U of enzyme tryptophanase. The reaction mixture was kept at 30°C for 5 days. The reaction was quenched by adding 40 μl of conc. HCl. Tritiated water was removed by lyophilisation, and the residue dissolved in a small amount of water and loaded onto a column (Amberlite IR 120, H^+, 10 × 100 mm). The latter was washed with water to remove the buffer salts and any tritium from the labile positions of tyrosine until the radioactivity of the eluted fraction was close to bπground. Next, **3** was eluted with 0.3 M $NH_{3(aq)}$ and collected as 5 ml fractions. From each fraction 10 μl samples were taken for radioactivity assay. The fractions containing **3** were combined and treated as described in 1. As a result 9.5 mg (52.5 μmol) of **3** with a total radioactivity of 1.94 MBq was obtained (97% radiochemical yield, specific activity—3.7×10^7 Bq/mmol).

3. *Synthesis of doubly labeled* [2-^{2}H/^{3}H]-*L-tyrosine*, **4**: A 4.3 mg (19.8 μmol) sample of L-tyrosine, **1**, was dissolved in 1.5 ml of fully deuteriated phosphate buffer (pD 8.3) prepared with 83% D_3PO_4/D_2O, 30% KOD/D_2O and heavy water. To this solution were added in turn: 0.26 U of enzyme tryptophanase, 1 μmol PLP in D_2O, 10 μl of mercaptoethanol, and 0.3 ml of tritiated water with total radioactivity 58 GBq. The reaction mixture was incubated at 30°C for 7 days with constant stirring. The reaction was quenched by adding 40 μl of conc. HCl. The procedure for separation and purification of **4** was the same as described in points 1 and 2. As a result a 3.4 mg (18.8 μmol) sample of **4** with total radioactivity of 7.8×10^5 Bq was obtained (94% radiochemical yield, specific activity—4.15×10^7 Bq/mmol).

4. *Synthesis of* [1S-^{2}H]-*tyramine*, **5**: To an incubation vial containing 5 ml of 0.1 M phosphate buffer were added: 3.5 mg (19.3 μmol) sample of previously obtained **2**, 1 μmol of PLP, and 1 mg (12.5 U) of enzyme tyrosine decarboxylase (EC 4.1.1.25) from *Streptococcus faecalis*. The reaction mixture was thermostated at 37°C for 1 h. Next, the post reaction mixture was loaded onto the Kieselgel 60 (10 × 100) column and eluted with solution: methanol: 25% $NH_{3(aq)}$, (20:1, v/v), and collected as 1.5 ml fractions. The presence of tyramine in each eluted fraction was checked by TLC as described in point 1. The fractions containing tyramine were combined and evaporated under reduced pressure at 50°C. As a result a 2.1 mg (15.3 μmol) sample of **5** was obtained in 80% chemical yield.

5. *Synthesis of* [1S-^{3}H]-*tyramine*, **6**: Decarboxylation of a 3.5 mg sample of [2-^{3}H]-L-tyrosine, **3**, (obtained as described in point 2) with total radioactivity 7.1×10^5 Bq was achieved in the same manner as for the deuteriated compound (see point 4). The presence of labeled compounds in eluted fractions was checked by radioassays as described in point 2. The fractions containing **6** were combined and evaporated under reduced pressure. The purity of the product was checked by TLC as above. As a result a 1.6 mg (11.6 μmol) sample of **6** with total radioactivity of 4.25×10^5 Bq was obtained (60% radiochemical yield, specific activity of 3.66×10^7 Bq/mmol).

6. *Synthesis of doubly labeled* [1S-^{2}H/^{3}H]-*tyramine*, **7**: The synthesis of this isotopomer of tyramine was carried out as described in point 5. In the decarboxylation procedure all of the sample of **4** (3.4 mg, total radioactivity of 7.8×10^5 Bq) was used. A 1.7 mg (12.5 μmol) sample of **7** was obtained with total radioactivity of 5.15×10^5 Bq (66% radiochemical yield, specific activity 4.1×10^7 Bq/mmol).

7. *Synthesis of deuteriated* [3′,5′-$^2H_2$]-*L-tyrosine · DCl*, **8**: A sample of 200 mg of L-tyrosine hydrochloride, **1**, was dissolved in 6 ml of 2 M deuteriochloric acid (5 ml of D_2O+1 ml conc. DCl/D_2O) and placed in a glass ampoule which was frozen in liquid nitrogen, degassed, and sealed. The ampoule was heated at 100°C for 24 h. Next, the ampoule was cooled to room temperature, opened, and lyophilized under vacuum. As a result 197 mg of **8** was obtained (97% yield). The analysis of the ^{1}H NMR spectrum (DMSO-d_6, TMS, 200 MHz Unity plus spectrometer) shows near 100% deuterium incorporation into the 3′ and 5′ positions of **8**. ^{1}H NMR: δ 3.03 (1 H, β-H, d, 14.4 Hz), 3.18 (1 H, β-H, d, 14.4 Hz), 3,93 (1 H, α-H, dd, 5.7 Hz), 7.18 (2 H, ArH, d, 8.7 Hz). Signal from 3′ and 5′ of ring protons, i.e., δ 6.89 (2 H, ArH, d, 8.7 Hz) disappeared. The purity of the product was checked by TLC (silica gel, methanol:25%$NH_{3(aq)}$, 20:1, v/v; visualization by ninhydrine) and shown to be close to 100%. So, the product was taken for further enzymatic decarboxylation without purification.

8. *Synthesis of* [3′,5′-$^2H_2$]-*tyramine*, **9**: To an incubation vial containing 6 ml of 0.1 M phosphate buffer were added: 4.7 mg (19.3 μmol) of deuteriated L-Tyr · DCl, **8**, 1 μmol of PLP and 12.5 U of enzyme tyrosine decarboxylase (EC 4.1.1.25) from *Steptococcus feacalis*. The incubation mixture was thermostated at 37°C for 1 h. Next, the reaction mixture was loaded onto the Kiesegel (E. Merck) column (10 × 100 mm) and eluted with a solution of methanol:25% $NH_{3(aq)}$, 20:1, v/v. The eluent was collected as 1.5 ml fractions. The presence of tyrosine and tyramine in each fraction was tested as above by TLC. The fractions containing **9** were combined, evaporated under reduced pressure at 50°C, and finally under vacuum. As a result 2.1 mg (15.3 μmol) of **9** was obtained in 80% yield. ^{1}H NMR

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(200 MHz, D_2O): δ 2.91 (2 H, β-2 H, t, 37 Hz), 3.23 (2 H, α-2 H, t, 37 Hz), 7.20 (2 H, ArH, d, 20 Hz). Signal from 3′ and 5′ of ring-protons, i.e. δ 6.89 (2 H, ArH, d, 21 Hz) disappeared.

9. *Synthesis of* [2′,6′-$^2H_2$]-*tyramine*, **10**: This deuteriated isotopomer of tyramine was obtained by decarboxylation of commercial [2′,6′-$^2H_2$]-L-tyrosine in the same manner as described in point 8. For decarboxylation 3.5 mg (19.3 μmol) substrate were taken and as a result 2.1 mg (15.3 μmol) of **10** was obtained in 80% yield.

10. *Synthesis of* [3′,5′-$^2H_2$]-*tyramine deuteriochloride*, **11**: The isotopic H/D exchange between heavy water and tyramine was carried out as described for the synthesis of **8**. A sample of 201 mg of tyramine hydrochloride dissolved in 6 ml of 2 M deuteriochloric acid was heated at 130°C for 24 h. The subsequent procedure was the same as in the case of **8** (point 7). As a result 198 mg of **11** was isolated (near 99% yield). The ^{1}H NMR spectrum showed, as in the of decarboxylation described in point 8, that incorporation of deuterium takes also place exclusively in the 3′ and 5′ ring positions of tyramine.

11. *Synthesis of* [3′,5′-$^3H_2$]-*tyramine*, **12**: Tritiated isotopomer **12** was obtained in a similar manner to that used for **11**. To a glass ampoule were added in turn: 200 mg (1.15 mmol) tyramine hydrochloride, 0.9 ml of water, 0.2 ml of conc. HCl, and 100 μl of tritiated water with total radioactivity 11.1 GBq. The ampoule was sealed under vacuum and heated at 130°C for 24 h. After opening the vial tritiated water was removed by lyophilisation, and the residue dissolved in a small amout of water (1–2 ml) and loaded onto column (10 × 100 mm) filled with Dowex WX-50 (H^+). The residual tritiated water and tritium from the labile -NH_2 group were washed out with distilled water until the radioactivity of eluent was close to background. Tritiated product, **12**, was eluted with 0.5 M $NH_{3(aq)}$ and collected as 6 ml fractions. From each fraction a 100 μl sample was taken for radioactivity assay. Fractions contained **12** were combined, evaporated under reduced pressure, and finally under vacuum. As a result 160 mg (0.92 mmol) of **12** was obtained (yield 74%) with total radioactivity 6.4×10^7 Bq (specific activity—6.9×10^7 Bq/mmol). The purity of product was verified by TLC.

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