ENZYMATIC SYNTHESIS OF ISOTOPOMERS OF TYRAMINE LABELED WITH DEUTERIUM AND tritium

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Summary

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'-$^{3}H_2$]-, [2',6'-$^{2}H_2$]-tyramine were obtained.

Introduction

Tyramine, TA, a biogenic amine, found in plant cells and mammalian tissues and fluids, play an important role in very metabolic processes. It is one of the neurotransmitters in central nervous systems in human[1-3] and a substrate for enzymatic hydroxylation to other important neurotransmitter i.e., dopamine **[4,5]** As a intermediate tyramine is responsible for generating the melanin in human and for browning fruits and vegetables.[4,5] In the living cells tyramine is produced by enzymatic decarboxylation of L-tyrosine, L-Tyr, catalyzed by enzyme tyrosine decarboxylase (EC 4.1.1.25)[6], Fig. 1.

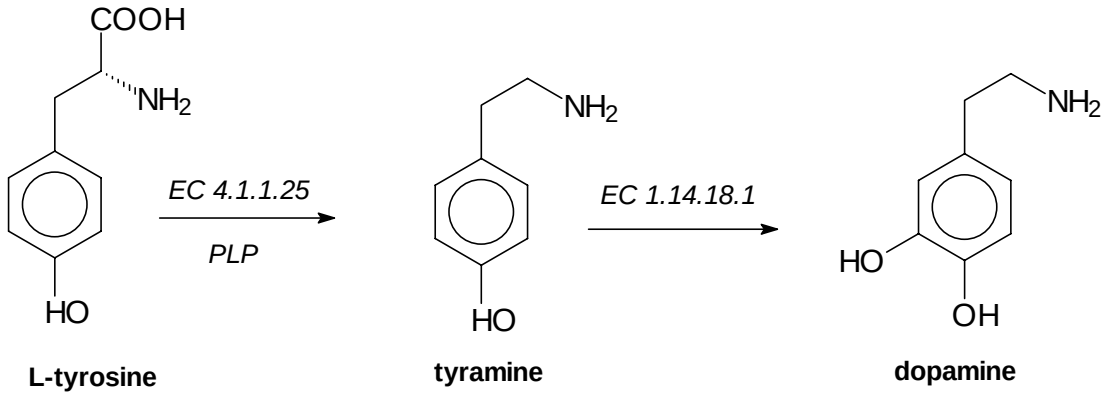


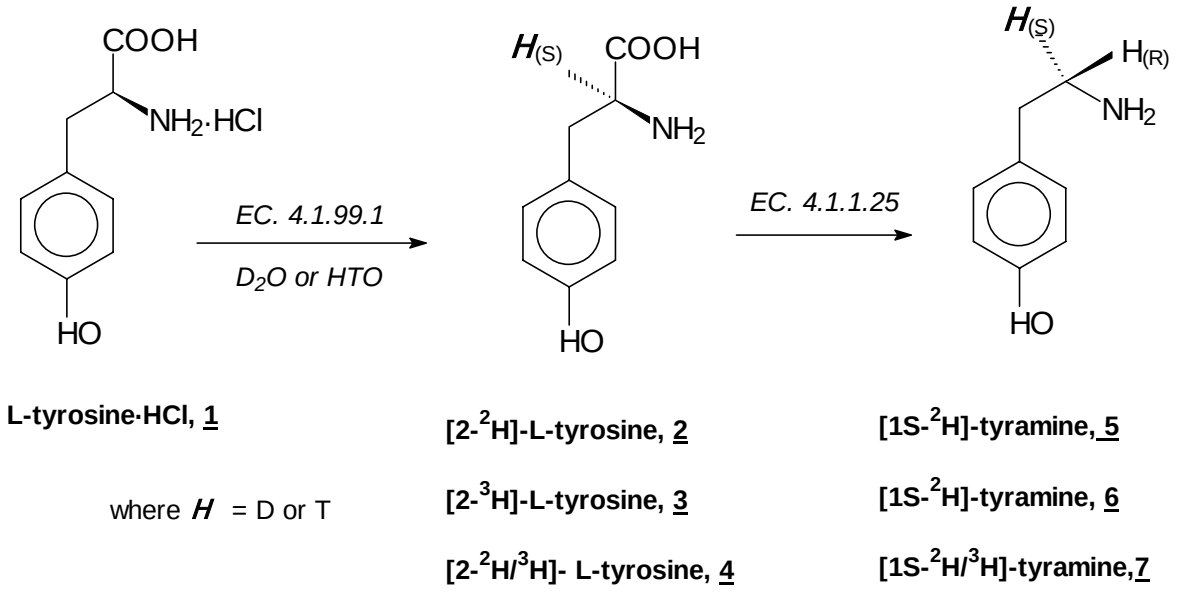
Fig.1. Enzymatic conversion of L-tyrosine into tyramine and dopamine

Despite many literature data the mechanisms of two above depicted reactions are not completely understood. It would be interesting to study these decarboxylation and hydroxylation reactions using isotope effects methods. [7-10] Determination of numerical values for solvent isotope effects, SIE, and kinetic isotope effects, KIE, will be useful for distinguishing between the alternative intrinsic details, allows find the bonds involved in formation of active complex etc. 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 of synthesis of ring and side chain labeled with deuterium and tritium five isotopomers of L-Tyr and six isotopomers of TA are described.

Result and discussion

For synthesis of labeled in 2-position of side chain L-Tyr, a substrate to obtaining a corresponding isotopomers of tyramine, we modified some methods early described.[11-17] The synthetic path depicted in Fig. 2 consists of using subsequently two enzymes; first of them, i.e., tryptophanase (EC. 4.1.99.1) introduces the label (deuterium or tritium into 2-position of L-Tyr, second one, i.e., tyrosine

decarboxylase (EC 4.1.1.25) is catalyzing the carboxylation of 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]



L-tyrosine·HCl, 1

where *H* = D or T

[2-^{2}H]-L-tyrosine, 2

[2-^{3}H]-L-tyrosine, 3

[2-^{2}H/^{3}H]- L-tyrosine, 4

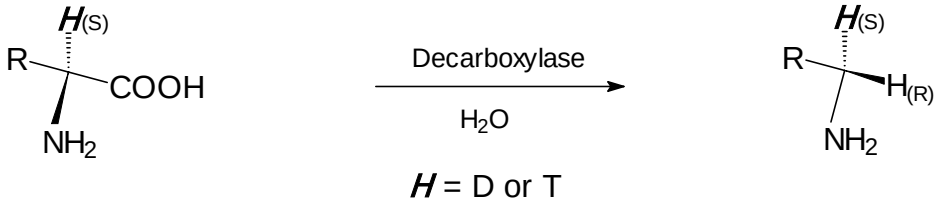
[1S-^{2}H]-tyramine, 5

[1S-^{2}H]-tyramine, 6

[1S-^{2}H/^{3}H]-tyramine, 7

Fig. 2. Enzymatic synthesis of compounds of L-tyrosine and tyramine labeled with deuterium and tritium at side chain

Therefore, the obtained by enzymatic decarboxylation of isotopomers of L-Tyr (**2**, **3** and **4**) the labeled isotopomers of TA (**5**, **6** and **7)** retain the label (deuterium or tritium) at configuration S., Fig. 3.

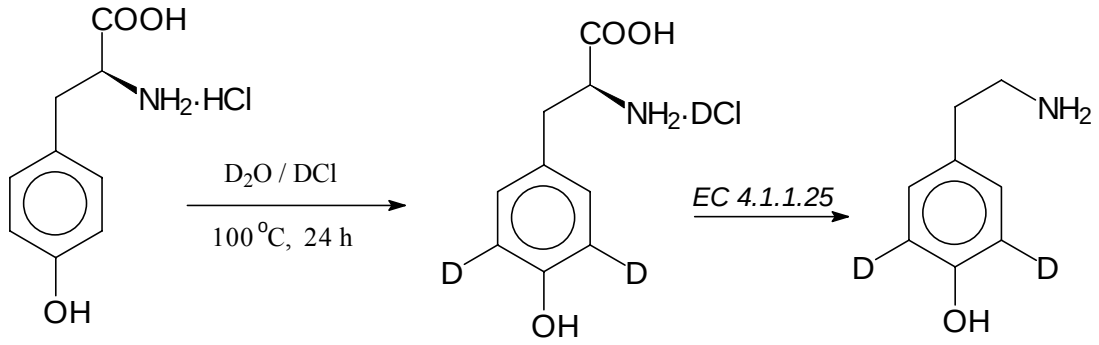


α- amino acid

[1S-*H*]-α-amine

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

The deuterium, labeled **[**3',5'-$^2H_2$]-TA, **9**, was synthesized in two different ways. The first one consists with two steps, Fig 4.



L-Tyr·HCl, 1

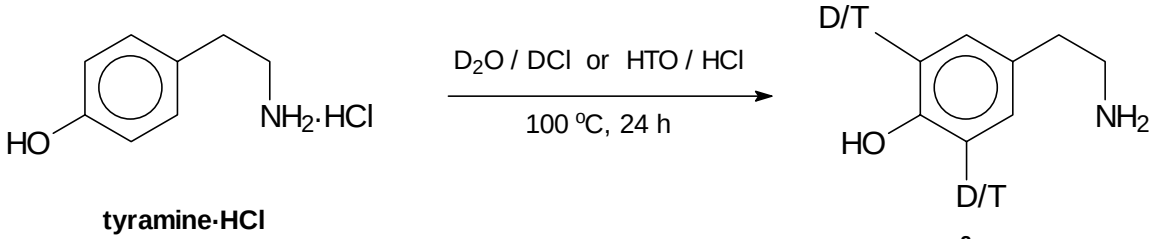
(3,,5'-$^2H_2$]-L-Tyr·DCl, 8

(3',5'- $^2H_2$]-TA, 9

Fig. 4. The combined chemical and enzymatic conversion of L-Try into [3',5'-$^2H_2$]-TA.

In the first one using H/D isotope exchange method between heavy water and L-tyrosine a intermediate [3',5'-$^2H_2$]-L-Tyr·DCl, **8**, was achieved. In the acid catalyzed conditions the exchange between D_2O an L-Tyr, **1**, introduces deuterium exclusively into *orto* position[16] (respectively to ring hydroxyl group) in tyrosine yielding above mentioned isotopomer **8** . To avoid isotopic dilution the exchange was carried out in fully deuteriated medium (D_2O/DCl). The second step consists with enzymatic decarboxylation of deuteriated L-tyrosine, **8**, to desired product, **9**, catalyzed by enzyme tyrosine decarboxylase (EC 4.1.1.25) from *Streptococcus faecalis.* The same manner the commercial [2',6'-$^2H_2$]-L-Tyr was decarboxylized to [2',6'-$^2H_2$]-TA, **10** isotopomer.[13] The position and degree of incorporation of deuterium into aromatic ring of L-tyrosine and tyramine were determined using ^1H NMR spectra.

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, Fig. 5. In this case, as mentioned above, the deuterium is incorporated exclusively in 3 and 5 ring position of tyramine.



tyramine·HCl

(3',5'- $^2H_2$]-TA, 9
or
(3',5'- $^3H_2$]-TA, 11

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

Experimental

Materials

Tritiated water was purchased from ICN Pharmaceutical Inc, Irvine Ca, 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 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 on Varian 200 MHz Unity-Plus spectrometer. The radioactivity of all samples was determined using liquid scintillation technique on automatic counter LISA LSC PW470 (Germany).

Synthesis

1. *Synthesis of [2-^2H]-L-tyrosine*, **2**. In 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 in room temperature. The exchange was quenched 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 was 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 ^1H NMR

spectrum shown near 100% incorporation of deuterium into 2-position of **2**. ^{1}H NMR (200 MHz, DMSO-d_6): δ 3.03 (1H, β-H, d, 14.4 Hz), 3.18 (1H, β-H, d, 14.4 Hz), 6.89 (2H, ArH, d, 8.7 Hz), 7.18 (2H, ArH, d, 8.7 Hz). Signal from α-proton i.e., δ 3.93 (1H, 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 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 μl of PLP, and 0.5 U of enzyme tryptophanase. The reaction mixture was incubated for 5 days at 30°C. The reaction was quenched by adding 40 μl of conc. HCl. Tritiated water was removed by lyophilisation, and a residue dissolved in small amount of water was loaded onto a column (Amberlite IR 120, H^+, 10×100 mm). The column was washed with water to remove the buffer salts and a rest of tritium from labile positions of tyrosine up to the moment when radioactivity of eluted fraction was close to background. Next, **3** was eluted with 0.3 M $NH_{3(aq)}$ and collected as 5 ml fractions. From each fraction the 10 μl sample was taken for radioactivity assay. The fractions containing **3** were combined and treated as described in point 1. As a result a 9.5 mg (52.5 μmol) of **3** with 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 of 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 incubation vial containing 5 ml of 0.1 M phosphate buffer were added: 3.5 mg (19.3 μmol) sample of early 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 for 1 hour at 37°C. 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 fractions 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 2.1 mg (15.3 μmol) sample of **5** was obtained with 80% chemical yield.
5. *Synthesis of [1S-^{3}H]-tyramine,* **6**. Decarboxylation of 3.5 mg sample of [1-^{3}H]-L-tyrosine, **3**, (obtained as described in point 2) with total radioactivity 7.1×10^5 Bq was preceded the same manner as in the case of deuteriated one (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 product was checked by TLC as above. As a result 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 $3{,}66\times10^7$ 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 decarboxylation procedure all 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 glass ampoule. The ampoule was connected to vacuum apparatus, its contains frozen with liquid nitrogen, degassed under vacuum, and sealed. The ampoule was heated at 100°C for 24 h. Next, ampoule was cooled to room temperature, opened, and water with deuteriochloric acid were removed by lyophilisation under vacuum. As a result 197 mg of **8** was obtained (97% yield). The analysis of ^{1}H NMR spectrum (DMSO-d_6, TMS, 200 MHz Unity plus spectrometer) shown near 100% deuterium incorporation into 3' and 5' position of **8**. ^{1}H NMR -: δ 3.03 (1H, β-H, d, 14.4 Hz), 3.18 (1H, β-H, d, 14.4 Hz), 3,93 (1H, α-H, dd, 5,7 Hz), 7.18 (2H, ArH,

d, 8.7 Hz). Signal from 3' and 5' of ring protons, i.e., δ 6.89 (2H, ArH, d, 8.7 Hz) disappeared. Purity of product checked by TLC shown negligible amount of impurities (silica gel, methanol:25%$NH_{3(aq)}$, 20:1, v/v; visualization by ninhydrine). So, this crude product was taken for further enzymatic decarboxylation without purification.

8. *Synthesis of [3',5'-$^2H_2$]-tyramine,* **9.** To 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** , early obtained, 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 for 1 h at 37°C. Next, reaction mixture was loaded onto the Kiesegel (E. Merck) column (10x100 mm) and eluted with solution methanol:25% $NH_{3(aq)}$, 20:1, v/v. The eluent was collected as 1.5 fractions. The presence of tyrosine and tyramine in each fraction was tested as above by TLC. The fractions contained **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 with 80% yield. ^{1}H NMR (200 MHz, D_2O): δ 2.91 (2H, β-2H, t, 37 Hz), 3.23 (2H, α-2H, t, 37 Hz), 7.20 (2H, ArH, d, 20 Hz). Signal from 3' and 5' of ring-protons, i.e., δ 6.89 (2H, 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 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 with 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 similarly as described for 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 further procedure was the same as in case of **8** (point 7*)*. As a result 198 mg of **11** was isolated (near 99% yield). The ^{1}H NMR spectrum prove as in the of decarboxylation described in point 8 that incorporation of deuterium take also place exclusively in 3' and 5' ring position of tyramine.
11. *Synthesis of [3',5'-$^3H_2$]-tyramine,***12**. Tritiated isotopomer **12** was obtained similarly as deuteriated one, i.e., **11**. To 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 as in point 1a and heated at 130°C for 24 h. After opening the tritiated water was removed by lyophilisation, and residue was dissolved in small amout of water (1-2 ml) and loaded on column (10x100 mm) filled with Dowex WX-50 (H^+). The residual tritiated water and tritium with labile position of $–NH_2$ group were washed out with distilled water up to moment where 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 100 μl sample was taken for radioactivity assay on liquid scintillation counter. 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 (sp. activity – 6.9×10^7 Bq/mmol). The purity of product was verified by TLC.

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