Enzymatic Synthesis of Carboxy-^{11}C-Labelled L-Tyrosine, L-DOPA, L-Tryptophan and 5-Hydroxy-L-tryptophan

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The multi-enzymatic syntheses of carboxy-^{11}C-labelled L-tyrosine (**3**), L-DOPA (**4**), L-tryptophan (**5**) and 5-hydroxy-L-tryptophan (**6**) via racemic [1-^{11}C]alanine (**1**) are presented. DL-[1-^{11}C]Alanine was synthesised from [^{11}C]cyanide by reaction with the bisulfite adduct of acetaldehyde and ammonia, and subsequent hydrolysis of the resulting [^{11}C]amino nitrile. The enzymatic reactions were performed using D-amino acid oxidase (D-AAO)/catalase, glutamic–pyruvic transaminase (GPT), and β-tyrosinase (for L-tyrosine and L-DOPA) or tryptophanase (for L-tryptophan and 5-hydroxy-L-tryptophan), in a *one-pot* reaction. L-[1-^{11}C]Alanine was also synthesised using the reversibility of GPT in combination with D-AAO. Total synthesis time was ca. 50 min, counted from the start of synthesis of hydrogen [^{11}C]cyanide, obtained from [^{11}C]carbon dioxide. Decay corrected radiochemical yields of the aromatic amino acids were 45–60 %, based on [^{11}C]cyanide, with radiochemical purities higher than 98 %. The specific radioactivities of the amino acids were in the order of 0.4–2.0 GBq μmol^{-1}. The enantiomeric purities were determined by HPLC, after derivatisation with *N*-(5-fluoro-2,4-dinitrophenyl)-L-alaninamide to the corresponding diastereomers, to be higher than 99 % L. In a typical run starting with 1.2 GBq hydrogen [^{11}C]cyanide, 150 MBq 5-hydroxy-L-tryptophan was obtained within 45 min.

The positron emission tomography, PET, technique has, in combination with biomolecules labelled with a positron emitting radionuclide, shown to be a valuable non-invasive tool for studying metabolic and physiological processes, e.g. energy metabolism and receptor binding, *in vivo*.[1,2] The extensive use of PET has created an increasing demand for new compounds labelled with radionuclides such as ^{11}C, ^{13}N and ^{18}F (with half-lives of 20.3, 9.97 and 109.7 min, respectively). The amino acids represent one class of compound which is of great interest as applied to PET investigations. ^{11}C- and ^{13}N-labelled amino acids, as well as ^{18}F-labelled analogues, have been used in studies of cerebral protein synthesis, neurological diseases, amino acid transport, and clinically for studying tumours in the human brain. L-Tyrosine (L-TYR), 3,4-dihydroxy-L-phenylalanine (L-DOPA), L-tryptophan (L-TRP) and 5-hydroxy-L-tryptophan (L-HTP) are of special interest, being the biological precursors for the neurotransmitters dopamine and serotonin. In previous work,[3,4] these amino acids have been labelled in the β-position with ^{11}C in order to visualise the regional distribution of the two neurotransmitters in the brain with PET.[5,6] To demonstrate that the accumulation of radioactivity was due to dopamine and serotonin formation and not only a regional concentration of L-DOPA or L-HTP, we searched for a method to synthesize the carboxy-labelled amino acids with the appropriate stereochemistry. Dopamine and serotonin are formed by an enzymatic decarboxylation of L-DOPA and L-HTP, respectively. Hence, applying the carboxy-^{11}C-labelled amino acids should result in a random distribution of the radioactivity in the brain, if we are observing neurotransmitter synthesis *in vivo*.

TYR and DOPA have earlier been labelled with ^{11}C in the carboxylic position using [^{11}C]carbon dioxide in a carboxylation reaction of the appropriate isonitrile.[7,8] The Bücherer–Strecker synthesis utilizing [^{11}C]cyanide has also been used to obtain [carboxy-^{11}C]TYR,[9] as well as [carboxy-^{11}C]TRP.[10] Synthesis of carboxy-^{11}C-labelled DOPA has also been achieved, utilizing the reaction of [^{11}C]cyanide and ammonia on the bisulfite adduct of 3,4-dimethoxyphenylethanal.[11] With these methods racemic mixtures are obtained and subsequent resolution is needed. This means that the radiochemical yield will be lowered since half of the radioactivity is lost in the resolution. One way to circumvent this problem is to use asymmetric synthesis using enzymes as chiral catalysts. A number of enantiomerically pure amino acids have been labelled in different positions using enzymatic syntheses.[12,13] The main advantages of applying enzymes in labelling syntheses are that they often exhibit high stereoselectivity and high reaction rates under mild conditions.

In this paper, the syntheses of carboxy-^{11}C-labelled L-TYR (**3**), L-DOPA (**4**), L-TRP (**5**), L-HTP (**6**) and L-alanine (L-ALA) from [^{11}C]carbon dioxide, via [^{11}C]cyanide and DL-[1-^{11}C]alanine, are described. The multi-enzymatic syn-

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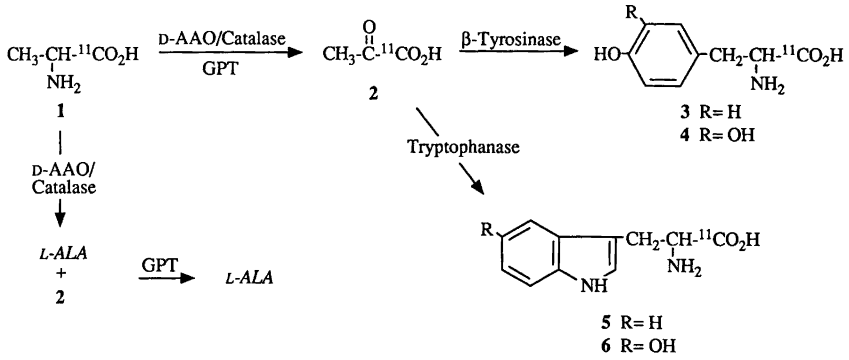

Scheme 1.

theses of the aromatic amino acids were performed using D-amino acid oxidase (D-AAO)/catalase, glutamic–pyruvic transaminase (GPT) and β-tyrosinase or tryptophanase in a *one-pot* reaction from racemic [1-^{11}C]alanine (**1**), see Scheme 1. L-[1-^{11}C]ALA was synthesised from racemic alanine using D-AAO/catalase and the reversibility of GPT. The present synthetic approach is also applicable to labelling the amino acids with ^{13}C and ^{14}C, which is here exemplified by the synthesis of L-(*carboxy*-^{13}C)tyrosine.

Results and discussion

β-Tyrosinase (tyrosine phenol-lyase, EC 4.1.99.2)[14] and tryptophanase (tryptophan indole-lyase, EC 4.1.99.1)[15] are two pyridoxal phosphate requiring enzymes which catalyse the α,β-elimination of L-TYR and L-TRP, respectively, to pyruvic acid, ammonia and phenol or indole. It has been shown that both these enzymatic reactions are reversible, and they have been used to synthesize L-TYR[16] and L-TRP.[17] Furthermore, other substrates can be used to obtain structurally related amino acids such as L-DOPA and L-HTP. Since pyruvic acid is a common precursor for both these reactions, labelling pyruvic acid was a conceivable way of producing these labelled amino acids.

Pyruvic acid has previously been synthesised labelled with ^{11}C in the carboxy-position by enzymatic methods[18–20] and by carboxylation of a masked acyl carbanion.[21] Since we had problems with reproducing the carboxylation reaction and since [3-^{11}C]pyruvic acid was obtained in high yield from racemic alanine using D-AAO/catalase and GPT,[22] we decided to use the enzymatic method. DL-[1-^{11}C]Alanine was produced by reaction of the bisulfite adduct of acetaldehyde with [^{11}C]cyanide and ammonia, followed by hydrolysis with hydrochloric acid according to the method of Iwata *et al.*[23] with some modifications. The synthesis was carried out using a lower substrate concentration and the precursor solution was not preheated. Hydrolysis of the resulting aminonitrile was performed directly using concentrated hydrochloric acid, without isolation of the intermediate. In this way a *one-pot* synthesis was achieved and the reaction time could be shortened. The racemic [1-^{11}C]alanine was produced in almost quantitative yield, based on hydrogen cyanide, within ca. 15 min with a radiochemical purity higher than 97 %.

The enzymatic synthesis using D-AAO/catalase, GPT and β-tyrosinase or tryptophanase was then achieved in a *one-pot* procedure, as described earlier.[3,4] The optimum reaction conditions were found to be the same as for the syntheses of the β-^{11}C-labelled amino acids. After evaporation of the racemic alanine solution, large amounts of salts, mainly ammonium chloride, were obtained. However, this did not affect the yield of the enzymatic reactions. The multi-enzymatic syntheses were carried out at 45 °C, pH 9.0, for 4.5 min during which 50–70 % of the labelled alanine was converted into L-TYR, L-DOPA, L-TRP or L-HTP, respectively.

After the addition of hydrochloric acid, the precipitated proteins were removed by filtration and then the crude product was purified by HPLC. In the case of DOPA, ascorbic acid was added to the mobile phase in order to prevent oxidation of the amino acid. After adjustment of the pH (not above 5 for DOPA and HTP, since they are sensitive to high pH) the solutions were sterile-filtered before use in PET-investigations. The identity of the labelled amino acids was determined by comparison with an original sample using two different HPLC-systems. The radiochemical purities were analysed by HPLC and were found to be higher than 98 %. The carboxy-^{11}C-labelled amino acids were obtained in 45–60 % radiochemical yield, decay corrected, based on hydrogen [^{11}C]cyanide. The synthesis time was approximately 50 min, counted from release of [^{11}C]carbon dioxide.

In order to confirm the position of the label in the amino acids a ^{13}C-synthesis of L-TYR using potassium (^{13}C)cyanide was carried out. After purification by HPLC, a ^{13}C NMR spectrum was recorded and compared with a spectrum of original L-TYR. The spectrum of the ^{13}C-labelled TYR shows a very strong peak at δ 171.4 which is consistent with the carboxy carbon in L-TYR. The enantiomeric purities were determined by HPLC after derivatisation with *N*-(5-fluoro-2,4-dinitrophenyl)-L-alaninamide to the corresponding diastereomers.[24] A racemic sample of the appropriate amino acid was always added before the derivatisation of the ^{11}C-labelled amino acid took place, in order

to make sure that no kinetic resolution occurred. In the case of TYR and DOPA, more than one diastereomeric pair was obtained. This is probably due to the formation of the nitrogen and oxygen derivatives of the amino acids. However, no radioactive peaks corresponding to the D-enantiomers were detected.

The possibility of producing L-[1-^{11}C]alanine using the reversibility of GPT was also investigated. In this experiment L-glutamic acid was added, instead of α-ketoglutarate, to the reaction mixture containing D-AAO/catalase and GPT. D-AAO catalyses the conversion of D-alanine into pyruvic acid which then is converted into L-alanine by GPT. After purification using a cation exchange column the labelled alanine was derivatised and analysed as described previously. The L-[1-^{11}C]alanine obtained was found to be enantiomerically pure.

In the described no-carrier-added synthesis of carboxy-^{11}C-labelled L-TYR, L-DOPA, L-TRP or L-HTP, the amount of unlabelled amino acid varied between 0.1 and 0.2 μmol, determined by UV absorption detection at 278 nm. The specific radioactivities were 0.4–2.0 GBq μmol^{-1} at the end of synthesis, depending on the starting radioactivity. In a typical experiment 150 MBq L-HTP was obtained from 1.2 GBq hydrogen [^{11}C]cyanide within 45 min. The solutions of the ^{11}C-labelled amino acids were controlled and found to be sterile and free from pyrogens, and they have been applied in human PET investigations.

In this paper the synthesis of carboxy-^{13}C-labelled L-TYR is described. The synthesis, which is also applicable to the other amino acids mentioned in this paper, was carried out on a milligram scale. It is presumably possible to scale up the synthesis, work which is now in progress.

Experimental

General. The ^{11}C was produced by the ^{14}N(p,α)^{11}C reaction using a tandem Van der Graaf accelerator at Uppsala University. The [^{11}C]carbon dioxide obtained in the nuclear reaction was trapped in lead-shielded 4 Å molecular sieves, and transported to the chemistry laboratory. Hydrogen [^{11}C]cyanide was prepared by reduction of [^{11}C]carbon dioxide to [^{11}C]methane with hydrogen gas and a nickel catalyst at 400 °C and subsequent reaction with ammonia gas at 1000 °C, catalysed by platinum.[23,25]

Analytical HPLC was carried out using a Hewlett–Packard 1090 liquid chromatograph equipped with a UV-diode array detector in series with a β$^+$-flow detector.[26] The columns used were: (A) 250 × 4.6 mm LC-NH_2, Nucleosil, 10 μm and (B) 250 × 4.6 mm C-18, Nucleosil, 10 μm. Mobile phases used were: (C) 0.01 M potassium dihydrogen phosphate, pH 4.6; (D) 500:70 (v:v) acetonitrile–water; (E) 17 mM acetic acid; (F) methanol; (G) 0.05 M ammonium formate, pH 3.5. Preparative HPLC was carried out using a Waters pump, M-6000A, and a 250 × 10 mm C-18, Nucleosil, 10 μm column in series with a Waters UV-detector, M-441, and a tubing coiled around a GM tube. 17 mM Acetic acid–ethanol 95:5 (v:v) for TYR, 80:20 for TRP, 93:7 for HTP, and 17 mM acetic acid containing 1 mM ascorbic acid for DOPA were used as mobile phases. Flow 4.0 ml min^{-1}, for TYR and DOPA, and 6.0 ml min^{-1}, for TRP and HTP. ^{13}C NMR spectra were recorded on a Varian XL-300 NMR spectrometer.

All the enzymes except β-tyrosinase and tryptophanase were purchased from Sigma. D-Amino acid oxidase, D-AAO (EC 1.4.3.3), from porcine kidney, crystalline suspension in 3.6 M ammonium sulfate, pH 6.5. Glutamic–pyruvic transaminase, GPT (EC 2.6.1.2), from porcine heart, lyophilized powder, was dissolved in 50 mM potassium phosphate buffer, pH 7.5, containing 0.2 mM pyridoxal 5-phosphate (PLP). Catalase (EC 1.11.1.6) from bovine liver, crystalline suspension in water containing 0.1 % thymol, was dialysed against 50 mM potassium phosphate buffer, pH 7.5. β-Tyrosinase, tyrosine phenol-lyase (EC 4.1.99.2), was purified from *Citrobacter intermedius*.[4] Tryptophanase, tryptophan indole-lyase (EC 4.1.99.1), was obtained from *Escherichia coli* according to a literature procedure.[27] All the enzymes except D-AAO were frozen in small portions in order to preserve their catalytic activity.

The bisulfite adduct of acetaldehyde was prepared by adding an excess amount of acetaldehyde to a saturated aqueous solution of sodium hydrogen sulfite. After being cooled in the refrigerator, the precipated crystalline solid was filtered off and washed with cold ethanol.

Enzyme assay. The activity of β-tyrosinase was assayed by measuring the amount of pyruvic acid formed from L-TYR in potassium phosphate buffer, pH 8.0, at 30 °C.[14] One unit was defined as the amount of enzyme which catalysed the formation of 1 μmol pyruvate min^{-1} under the conditions described. The specific activity of the β-tyrosinase was 1.2 μmol min^{-1} mg^{-1} protein and the protein concentration was 56.7 mg ml^{-1}. Tryptophanase activity was determined spectrophotometrically using *S*-(*o*-nitrophenyl)-L-cysteine (SOPC) as the substrate.[28] One unit was defined as the amount of enzyme which catalysed the conversion of 1 μmol SOPC min^{-1}. The specific activity was determined to be 48 μmol min^{-1} mg^{-1} protein and the protein concentration was 43.4 mg ml^{-1}.

DL-[1-^{11}C]Alanine. In a septum-equipped vial were dissolved 20 mg of the bisulfite adduct of acetaldehyde in 0.4 ml 5 M ammonia. H[^{11}C]CN was then introduced into the reaction mixture cooled in an ice bath. The solution was heated at 70 °C for 5 min after which the reaction vessel was unsealed and 1 ml conc. hydrochloric acid was added. The reaction mixture was agitated and heated at 175 °C for 5 min. The racemic [1-^{11}C]alanine formed was diluted with 2 ml water and then analysed by HPLC using column A and the following conditions: flow 2 ml min^{-1}, solvents C/D, gradient 0–8 min 95–60 % D, column temperature 40 °C, wavelength 230 nm. The retention time was 6.1 min for [1-^{11}C]alanine.

L-[1-^{11}C]Alanine. The racemic alanine solution was evaporated to dryness and the residue was redissolved in 0.8 ml

0.1 M tris(hydroxymethyl)aminomethane–hydrochloric acid (TRIS/HCl) buffer, pH 9.0. The solution was transferred to a glass tube containing 50 µl 0.5 M TRIS/HCl, pH 9.0, 50 µl 0.2 M L-glutamic acid, 10 µl 1.7 mM flavin adenine dinucleotide (FAD), and 10 µl 10 mM PLP, after which the pH was adjusted to 8.6 with 5 M potassium hydroxide. To the reaction mixture were then added 4.8 units D-AAO, 30 units GPT and 3600 units catalase and then the solution was thermostatted at 45 °C for 5 min. The crude product was purified on a cation exchange column, Bio-Rad Laboratories AG 50W-X4 200-400 mesh, containing ca. 6 ml resin. The column was washed with water and then the labelled alanine was eluted with 5 M ammonia. The L-[1-^{11}C]alanine was analysed by HPLC using the same system as described above.

Carboxy-^{11}C-labelled L-tyrosine, L-DOPA, L-tryptophan and 5-hydroxy-L-tryptophan. The racemic [1-^{11}C]alanine solution was evaporated to dryness, and the residue was dissolved in 0.8 ml 0.1 M TRIS/HCl buffer, pH 9.0, and then added to a mixture of 50 µl 0.5 M TRIS/HCl, pH 9.0, 50 µl 0.2 M α-ketoglutarate, 10 µl 1.7 mM FAD, and 10 µl 10 mM PLP. The pH was adjusted to 9.0 with 5 M potassium hydroxide after which 3.2 units D-AAO, 20 units GPT, 3600 units catalase, and 3.4 units β-tyrosinase or 100 units tryptophanase were added. The solution was thermostatted at 45 °C for 15 s after which 10 µl of an ethanolic solution of 2.5 M phenol (TYR), 2.5 M catechol (DOPA), 0.5 M indole (TRP) or 0.5 M 5-hydroxyindole (HTP) were added. After a further 4.5 min at 45 °C, the reaction was stopped by adding 200 µl conc. HCl. The precipitated proteins were removed by filtration through a 0.2 µm pore filter and the resulting clear solution was purified using the previously described semi-preparative HPLC system. The correct fractions were combined and evaporated, to remove the ethanol (TYR, TRP and HTP), after which saline was added to a final volume of ca. 6 ml. The pH was adjusted to ca. 6 for TYR and TRP, and 4.5 for DOPA and HTP before the solution was sterilized by passage through a 0.2 µm pore filter into a sterile vial. The radiochemical purity was analysed by HPLC using: (1) column A, solvents C/D, flow 2.0 ml min^{-1}, gradient 0–8 min 95–60 % D, column temperature 40 °C, wavelength 230 nm. The retention times were 4.9, 4.7, 4.1 and 4.1 min for the carboxy-^{11}C-labelled L-TYR, L-DOPA, L-TRP, and L-HTP, respectively; (2) column B, solvents E/F, flow 2.0 ml min^{-1}, isocratic 0, 0, 15 and 5 % F for TYR, DOPA, TRP and HTP, respectively, column temperature 50 °C, wavelength 278 nm. Retention times were 3.1, 2.4, 3,8 and 3.5 min for carboxy-^{11}C-labelled L-TYR, L-DOPA, L-TRP and L-HTP, respectively.

L-(Carboxy-^{13}C)tyrosine. In a septum-equipped vial were placed 19.4 mg of the bisulfite adduct of acetaldehyde, 4.0 mg potassium (^{13}C)cyanide, and 0.4 ml 5 M ammonia. After being heated at 70 °C for 15 min the vial was opened and 2 ml conc. hydrochloric acid were added. The reaction mixture was agitated and heated at 175 °C for 10 min and then evaporated to dryness. The residue was dissolved in 8 ml 0.1 M TRIS/HCl, pH 9.0, and then 0.2 ml 0.5 M TRIS/HCl, pH 9.0, 0.5 ml 2 M α-ketoglutarate, 0.1 ml 1.7 mM FAD and 0.1 ml 10 mM PLP were added. The pH was adjusted to 9.0 with 5 M potassium hydroxide and then 9.6 units D-AAO, 60 units GPT, 7200 units catalase, 3.4 units β-tyrosinase and 0.1 ml 2.5 M phenol were added. The reaction mixture was agitated and thermostatted at 40 °C for 90 min and then stopped by the addition of 0.4 ml conc. hydrochloric acid. After centrifugation, the supernatant was filtered and then purified using the previously described HPLC system. The collected fractions were evaporated to dryness and redissolved in 0.75 ml D_2O–CD_3CO_2D (2:1) with 3 drops of D_2SO_4. ^{13}C NMR (75.4 MHz, $\underline{C}D_3CO_2D$ used as the reference, δ 20.0): 171.4 (*C*-carboxy), 54.5 (C-α, d, *J* 60 Hz), 35.1 (C-β), 125.6 (C-1), 131.2 (C-2), 116.3 (C-3), 155.7 (C-4).

Enantiomeric purity. The collected fractions containing the ^{11}C-labelled amino acid were evaporated to dryness and the residue was redissolved in 100 µl water. The solution was transferred to a 1.5 ml glass vial containing 5 µmol of the corresponding unlabelled racemic amino acid and then 40 µl 1 M sodium hydrogen carbonate and 200 µl 1 % (w:v) *N*-(5-fluoro-2,4-dinitrophenyl)-L-alaninamide in acetone were added. The vial was sealed with a septum and the reaction was left to proceed at 60 °C for 20 min. After the addition of 20 µl 2 M HCl, the sample was analysed by HPLC using column B, solvents G/F, flow 2.0 ml min^{-1}, column temperature 50 °C, wavelength 340 nm, gradient 0–15 min 30–50 % F. Retention times for the *N*-derivatives of the amino acids were 6.1 (L-ALA), 9.5 D-ALA), 7.5 (L-TYR), 11.4 (D-TYR), 6.0 (L-DOPA), 8.7 (D-DOPA), 11.5 (L-TRP), 15.7 (D-TRP), 6.7 (L-HTP) and 8.5 min D-HTP).

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