Syntheses of β-^{11}C-Labelled L-Tryptophan and 5-Hydroxy-L-tryptophan using a Multi-enzymatic Reaction Route

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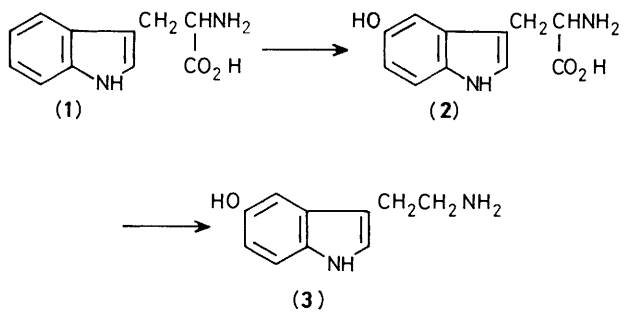
Multi-enzymatic syntheses of L-[β-^{11}C]tryptophan and 5-hydroxy-L-[β-^{11}C]tryptophan from racemic [3-^{11}C]alanine are reported. ^{11}C-Labelled alanine was prepared by an alkylation of a glycine derivative, *N*-(diphenylmethylene)glycine t-butyl ester, with [^{11}C]methyl iodide obtained from [^{11}C]carbon dioxide, and subsequent hydrolysis. The enzymatic syntheses were carried out in a one-pot reaction using D-amino acid oxidase/catalase, glutamic-pyruvic transaminase, and tryptophanase. The total synthesis time was 50 to 55 min, including h.p.l.c. purification, counted from the start of [^{11}C]methyl iodide synthesis. The yields were *ca.* 25%, decay corrected, of purified sterilized enantiomerically pure L-[β-^{11}C]tryptophan and 5-hydroxy-L-[β-^{11}C]tryptophan with radiochemical purities of >98%. The specific activities were in the order of 2.5 GBq/μmol. In a typical run starting with 4.4 GBq [^{11}C]carbon dioxide, 220 MBq of β-^{11}C-labelled L-tryptophan or 5-hydroxy-L-tryptophan was obtained ready for use in human studies.

Biomolecules labelled with short-lived positron emitting radionuclides, such as ^{11}C, ^{13}N, and ^{18}F ($t_{\frac{1}{2}}$ = 20.3, 10.0, and 110 min respectively), can be used with the positron emission tomography (p.e.t.) technique for studying a number of biologically interesting processes *in vivo*,[1] *e.g.* energy metabolism[1b] and receptor binding.[1c–f] Labelled amino acids and analogues can be used for studying protein synthesis,[1g] amino acid transport,[1h] neurological diseases,[1i–j] and, in the case of ^{11}C-labelled methionine, for the visualisation of tumours in the human brain.[1k–m] Some amino acids are of special interest because of their role as neurotransmitter precursors in the brain. Tryptophan [TRP, (**1**)] and 5-hydroxytryptophan [5-HTP, (**2**)] serve as substrates in the enzyme catalysed biosynthesis of the neurotransmitter serotonin (**3**), Scheme 1. In order to be able to study the serotoninergic neurons *in vivo* in human brain, by means of p.e.t., we therefore looked for methods to synthesize these amino acids labelled with ^{11}C.

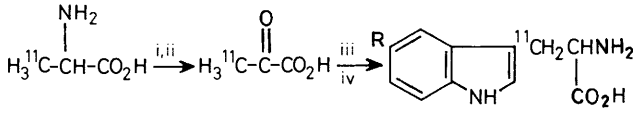
TRP has previously been labelled with ^{11}C at the carboxylic acid carbon when used in human tumour studies.[2] In these cases a modified Bücherer–Strecker synthesis, using ^{11}C-labelled cyanide, produced the racemic amino acid.[3] Since the labelling with this method is in the carboxy position, the radioactivity will be lost during the last enzymatic step in the formation of serotonin. All other positions of labelling will however result in a labelled neurotransmitter. Therefore an alternative approach to label TRP and 5-HTP in a metabolically stable position was desired.

Increasingly, enzymes are being used in organic synthesis.[4] The main advantages of using enzymes in conjunction with short-lived isotopes, are that they are often highly enantioselective and work quickly under mild conditions. Since only one of the enantiomers is usually biologically active in a living system, and optimal information from p.e.t.-investigations is achieved using the pure labelled enantiomer, this enantioselectivity can be a great advantage. Rapid resolution of racemic ^{11}C-labelled amino acids is possible, either enzymatically[5a] or chromatographically.[3d,5b–c] The drawback with these methods is, however, that half of the radioactivity is lost in the resolution.

We now report the multi-enzymatic syntheses of L-[β-^{11}C]tryptophan (**6**) and 5-hydroxy-L-[β-^{11}C]tryptophan (**7**)



Scheme 1.



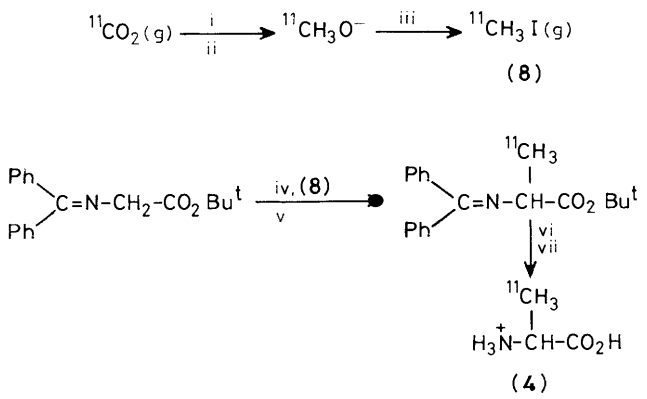
(**4**) (**5**) (**6**) R = H (**7**) R = HO

Scheme 2. *Reagents:* i, D-AAO/Catalase; ii, GPT; iii, TPase; iv, indole or 5-hydroxyindole

achieved in a one-pot reaction from racemic [3-^{11}C]alanine (**4**) using D-amino acid oxidase (D-AAO)/catalase, glutamic-pyruvic transaminase (GPT), and tryptophanase (TPase), as shown in Scheme 2.

Results and Discussion

The ^{11}C-labelled alanine was synthesized by a method described previously[6] with some modifications,[7] Scheme 3. [^{11}C]Methyl iodide was obtained after reduction of [^{11}C]carbon dioxide and subsequent treatment of the resulting methoxide anion with hydriodic acid in a one-pot procedure described in detail elsewhere.[8] The synthesis of [3-^{11}C]alanine was then achieved by alkylation in dimethylformamide/dimethyl sulphoxide[7] (DMF/DMSO) rather than by a phase-transfer



Scheme 3. *Reagents:* i, $LiAlH_4$; ii, THF; iii, HI; iv, KOH; v, DMF/DMSO; vi, HCl; vii, CH_2Cl_2

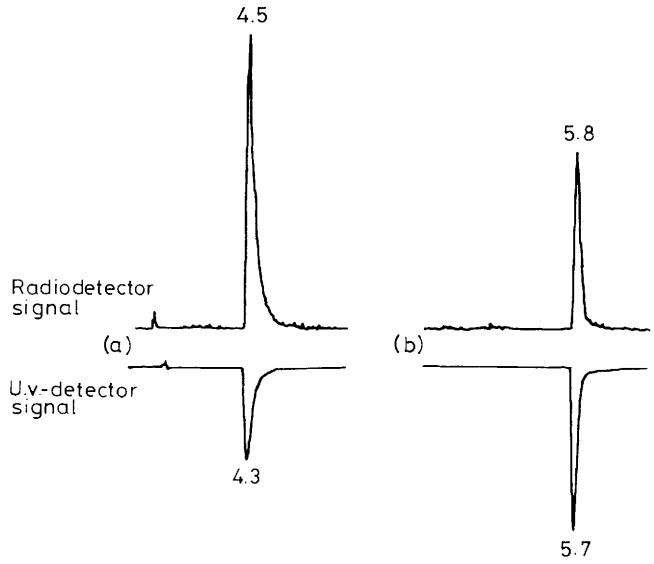


Figure 1. L.c.-analyses of the radiochemical purities of (a) L-[β-^{11}C]-tryptophan and (b) 5-hydroxy-L-[β-^{11}C]tryptophan with added references using column B and the conditions described in the Experimental section, the retention time is given in min.

procedure.[6] In this way it was easier to obtain a sufficiently pure alanine solution for use in the subsequent enzyme catalysed reactions. DL-[3-^{11}C]Alanine (**4**) was obtained in 70% radiochemical yield, decay corrected, within 18 min and with a radiochemical purity of >99%.[7]

TPase is known to catalyse the α,β-elimination of L-TRP to indole, pyruvate, and ammonia.[9] Watanabe and Snell have shown that this reaction is reversible at high concentrations of pyruvate and ammonia.[10] Furthermore, indole can be replaced by other substrates such as 5-hydroxyindole and 5-methylindole to obtain other amino acids. However, high concentrations of indole can not be used to shift the equilibrium, since TPase is inhibited at increasing indole concentration.[10] In pre-studies with ^{14}C-labelled pyruvate we found that it was also possible to synthesize TRP using TPase at low pyruvate concentrations. Since [3-^{11}C]pyruvic acid (**5**) can be obtained from [3-^{11}C]-alanine (**4**) using D-AAO/catalase and GPT,[7] the combination of these enzymes with TPase was a conceivable way to obtain ^{11}C-labelled L-TRP (**6**) and L-5-HTP (**7**), respectively, as outlined in Scheme 2.

Time is an essential factor in work with radionuclides with short half-lives such as ^{11}C, $t_{\frac{1}{2}}$ = 20.3 min.[11] In order to minimize synthesis time, technical handling, and exposure of the chemist, a one-pot method utilizing free enzymes was developed. Hence, optimization of the reaction conditions with respect to all the enzymes was required. For example: the maximum rate of conversion of alanine into TRP was obtained at pH 9.0. On the other hand, a pH of 8.5 was found to be the best choice if only pyruvic acid was to be produced.[7] However, since D-AAO and GPT have a rather broad pH maximum, the overall multi-enzymatic reaction is favoured by a higher pH. One problem which arises using free enzymes is their rather low stability over longer periods. This was overcome by freezing the enzymes (GPT, catalase, and TPase) in small portions and then thawing them one at a time, as the need arose.

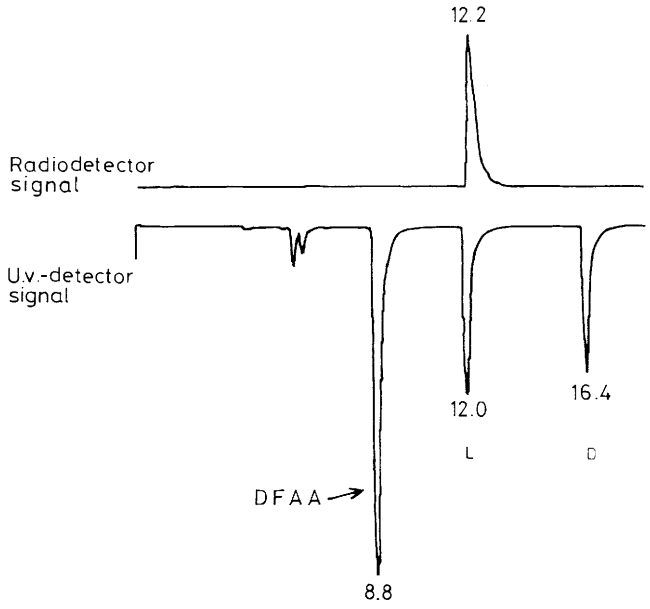


Figure 2. L.c.-analysis of the enantiomeric purity of L-[β-^{11}C]tryptophan with added racemic reference, after derivatization with DFAA using column C and the conditions described in the Experimental section, the retention time is given in min

The maximum yield of ^{11}C-labelled amino acids was obtained within 3 min. After denaturation of the enzymes with hydrochloric acid the crude product was purified using semi-preparative h.p.l.c. The total synthesis time was 50–55 min counted from release of [^{11}C]carbon dioxide. The yields were *ca.* 25%, decay corrected, of purified, sterilized L-amino acids with radiochemical purities of >98% (Figure 1).

The enantiomeric purities of the ^{11}C-labelled amino acids were analyzed by h.p.l.c. using the corresponding diastereo-isomers obtained after derivatization with *N*-(2,4-dinitro-5-fluorophenyl)-L-alaninamide[12] (DFAA), exemplified by the analysis of TRP in Figure 2, a method used earlier to determine the enantiomeric excess of ^{11}C-labelled amino acids.[13]

The solutions containing the ^{11}C-labelled amino acids, when sterile and free of pyrogens, were found to be suitable for use in animal and human applications. The specific activities were determined by comparison with standards and were found to be *ca.* 2.5 GBq/μmol. In a typical run starting with 4.4 GBq [^{11}C]carbon dioxide, 220 MBq of purified, sterilized L-[β-^{11}C]-tryptophan or 5-hydroxy-L-[β-^{11}C]tryptophan was obtained.

Experimental

General.—The ^{11}C was prepared by the ^{14}N(p,α)^{11}C nuclear reaction using a nitrogen gas target and 10 MeV protons produced by a tandem Van der Graaff accelerator at The Svedberg Laboratory, University of Uppsala. The [^{11}C]carbon

dioxide obtained was trapped in lead shielded 4 Å molecular sieves and transported to the chemistry laboratory. The [^{11}C]methyl iodide used in the alkylation reaction was synthesized by a general method described previously.[8] *N*-(Diphenylmethylene)glycine t-butyl ester was prepared according to a literature procedure.[6]

All enzymes except tryptophanase were purchased from Sigma. D-Amino acid oxidase, D-AAO (EC 1.4.3.3), from porcine kidney, crystalline suspension in 3.6M ammonium sulphate pH 6.5, was used without further treatment. 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. 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. Tryptophanase, TPase (EC 4.1.99.1), was obtained from *Escherichia coli* prepared according to Kagamiyama *et al*[14] with some modifications.[15] The specific activity of the TPase was determined spectrophotometrically using *S*-*o*-nitrophenyl-L-cysteine (SOPC) as substrate,[16] and was found to be 48 μmol/min/mg protein. The protein concentration was 42.4 mg/ml. In order to maintain the activity of the enzymes the prepared enzyme solutions of GPT, catalase, and TPase were stored in a freezer in small portions. After thawing they can be kept in the refrigerator for *ca.* 1 month before the enzyme activity starts to decrease.

Analytical l.c. was performed on a Hewlett-Packard 1090 liquid chromatograph equipped with a u.v.-diode array detector in series with a β^+-flow detector.[17] The following columns were used: (A) 250 × 4.6 mm LC-NH_2 (Nucleosil) 10 μm column, (B) 250 × 4.6 mm C-18 (Spherisorb ODS2) 5 μm column, and (C) 150 × 4.6 mm C-18 (Supelco) 3 μm column. (D) 0.01M potassium dihydrogen phosphate pH 4.6, (E) acetonitrile–water (500:70, v:v), (F) 17 mM acetic acid, (G) 0.05M ammonium formate pH 3.5, and (H) methanol were used as mobile phases. Preparative l.c. was carried out using a Waters M-45 pump and a 250 × 10 mm C-18 (Nucleosil) 10 μm semi-preparative column in series with a Pharmacia dual path monitor UV-2 and a β^+-flow detector.[17]

Solid phase extraction C-18 (Supelco) columns were conditioned with dichloromethane (3 ml), ethanol (10 ml), and distilled water (20 ml) prior to use. Sep-Pak C-18 (Waters) columns were pre-conditioned with ethanol (10 ml) and distilled water (20 ml). Cation Exchange Resin (Bio-Rad Laboratories) AG 50W-X4, 200—400 mesh, was conditioned with distilled water.

Tetrahydrofuran (THF) was dried by distillation from sodium–benzophenone. All other chemicals were used without further purification.

[^{11}C]*Methyl Iodide.*—The [^{11}C]carbon dioxide was released from the lead shielded molecular sieves upon heating and transferred to a solution of lithium aluminium hydride in THF (0.8M; 0.5 ml) in a specially designed one-pot reaction flask [8] by a stream of nitrogen gas. After evaporation of the THF, hydriodic acid (57%; 2 ml) was added and the reaction mixture was refluxed during which the [^{11}C]methyl iodide formed was distilled off and transferred to the reaction vessel.

DL-[3-^{11}C]*Alanine.*—*N*-(Diphenylmethylene)glycine t-butyl ester (3 mg, 10 μmol) was dissolved in DMF (450 μl) and DMSO (50 μl) in a 1.5 ml vial equipped with a septum. Potassium hydroxide (5M; 2 μl) was added to the solution and then the [^{11}C]methyl iodide was transferred to the reaction vessel in a stream of nitrogen gas. The reaction mixture was heated at 80 °C for 2 min and then transferred to a syringe, containing distilled water (20 ml), connected to a C-18 solid phase extraction column. The aqueous solution was passed through the column which was washed with water (3 ml) before the radioactive components trapped in the column were eluted with dichloromethane (2 ml) into a 7 ml glass vessel containing hydrochloric acid (6M; 0.8 ml). The mixture was heated and shaken at 130 °C for 5 min, after which the racemic [3-^{11}C]-alanine solution was diluted with water (1 ml) and purified by passage through a Sep-Pak C-18 column. The radiochemical purity of the eluate was controlled by analytical l.c. using column A and the following conditions: flow 2 ml/min, solvents D/E, gradient 0—8 min 95—60% E, column temperature 40 °C, wavelength 230 nm. Retention time: 6.1 min for [3-^{11}C]alanine.

[3-^{11}C]*Pyruvic Acid.*—The DL-[3-^{11}C]alanine solution was evaporated to dryness and the residue was dissolved in tris-(hydroxymethyl)aminomethane–hydrochloric acid (TRIS/HCl) buffer pH 8.5 (0.1M; 0.8 ml). The solution was then transferred to a 5 ml glass tube containing α-ketoglutarate (α-KG) (0.2M; 50 μl), pyridoxal 5-phosphate (PLP) (10 mM; 10 μl), and flavin adenine dinucleotide (FAD) (1.7 mM; 10 μl). After the pH had been adjusted to 8.5, D-AAO (1.9 units), GPT (12 units), and catalase (3 600 units) were added and the reaction mixture was kept at 45 °C for 2.5 min. The reaction was quenched by addition of hydrochloric acid (6M; 0.2 ml). After filtration through a 0.22 μm pore filter the radiochemical yield was determined by analytical l.c. using the same system as described for DL-[3-^{11}C]alanine. Retention time: 3.7 min for [3-^{11}C]pyruvic acid.

L-[β-^{11}C]*Tryptophan and* 5-*Hydroxy*-L-[β-^{11}C]*tryptophan.*—The eluate containing the purified racemic [3-^{11}C]alanine was evaporated to dryness, and the residue was redissolved in TRIS/HCl buffer pH 9.0 (0.1M; 0.6 ml). The solution was introduced to a mixture of ammonium sulphate, TRIS/HCl pH 9.0, α-KG, FAD, and PLP, the pH was adjusted to 9.0 with 1M potassium hydroxide and then the enzymes were added. The final solution (1 ml) contained: D-AAO (1.9 units), GPT (12 units), catalase (3 600 units), TPase (100 units; 1 unit = 1 μmol SOPC/min), TRIS/HCl pH 9.0 (0.1M), ammonium sulphate (0.15M), α-KG (10 mM), PLP (0.1 mM), and FAD (17 μM). The reaction mixture was thermostatted at 45 °C for 15 s after which an ethanolic solution of indole for TRP or 5-hydroxyindole for 5-HTP (0.5M; 10 μl) was added. After another 3.0 min at 45 °C the reaction was quenched by adding hydrochloric acid (6 M; 0.2 ml). The denatured enzymes were removed by filtration through a 0.22 μm pore filter and the resulting clear solution was purified using the previously described preparative l.c. system. 17 mM Acetic acid–ethanol (80:20, v:v) for TRP or (92:8, v:v) for 5-HTP, respectively was used as mobile phase, flow 8.0 ml/min. The radioactive fractions containing the appropriate compound were collected and evaporated. The residue was dissolved in saline (5 ml) and phosphate buffer pH 7.4 (1 ml) after which the pH of the solution was adjusted to *ca.* 6. The solution was sterilized by passage through a 0.22 μm pore filter into a sterile vial, and was then used in human and animal applications. The radiochemical purity was analysed by h.p.l.c.: (i), Column A, flow 2.0 ml/min, solvents D/E, gradient 0—8 min 95—60% E, column temperature 40 °C, wavelength 230 nm. Retention times: 4.1 min for L-[β-^{11}C]tryptophan and 5-hydroxy-L-[β-^{11}C]tryptophan. (ii), Column B, flow 2.0 ml/min, solvents F/H, isocratic 40% H (for TRP) or 10% H (for 5-HTP), column temperature 60 °C, wavelength 278 nm. Retention time: 4.5 min for L-[β-^{11}C]tryptophan and 5.1 min for 5-hydroxy-L-[β-^{11}C]-tryptophan.

Enantiomeric Purity.—The enantiomeric purity was assessed by the following procedure. On completion of the enzyme reaction the mixture was directly applied to a cation exchange column, AG 50W-X4, containing *ca.* 2 ml of resin. The column

was washed with distilled water (4 ml) and the ^{11}C-labelled amino acid was eluted with ammonia (2M). The eluate was evaporated to dryness and the residue was dissolved in an aqueous solution of DL-TRP or DL-5-HTP (0.05M; 0.1 ml), respectively. The solution was transferred to a 1.5 ml glass vial and subsequently a solution of *N*-(2,4-dinitro-5-fluorophenyl)-L-alaninamide (DFAA) in acetone (1%; 0.2 ml) and sodium hydrogen carbonate (1M; 40 μl) was added. The vial was sealed with a septum and the reaction was left to proceed at 60 °C for 15 min after which hydrochloric acid (2M; 20 μl) was added. The resulting solution was analysed by h.p.l.c. using column C and the following conditions: flow 1.0 ml/min, solvents G/H, gradient 0–15 min 25—50% H, column temperature 50 °C, wavelength 340 nm. Retention times for the DFAA-derivatives of the amino acids were: 12.0, 16.4, 7.0, and 8.3 min for L-TRP, D-TRP, L-5-HTP, and D-5-HTP, respectively and 8.8 min for hydrolysed DFAA.

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