Synthesis of 1- and 3-^{11}C-Labelled L-Lactic Acid using Multi-enzyme Catalysis.

Peter Bjurling and Bengt Långström.*

Department of Organic Chemistry, Institute of Chemistry, Uppsala University, P.O. Box 531, S-751 21 Uppsala, Sweden.

*Author for correspondence.

Summary

The synthesis of 1- and 3-^{11}C-labelled L-lactic acid from the corresponding racemic 1- or 3-^{11}C-labelled alanine using a multi-enzymatic reaction route, is presented. DL-[1-^{11}C]Alanine was synthesised by reacting sodium 1-hydroxy-ethyl sulfite with hydrogen [^{11}C]cyanide, obtained from [^{11}C]carbon dioxide, and ammonia followed by acid hydrolysis. DL-[3-^{11}C]-Alanine was synthesised by a methylation of a glycine derivative, *N*-(diphenylmethylene)-glycine *tert*-butyl ester, with [^{11}C]methyl iodide, obtained from [^{11}C]carbon dioxide, and subsequent hydrolysis. The racemic 1- or 3-^{11}C-labelled alanine was then converted to pyruvic acid, by D-amino acid oxidase/catalase and glutamic-pyruvic transaminase, which was directly reduced to L-lactic acid by L-lactic dehydrogenase in a *one-pot* procedure. The total synthesis time was 40 minutes, counted from release of [^{11}C]carbon dioxide. The decay corrected radiochemical yields were *ca.* 80% for L-[1-^{11}C]lactic acid, based on hydrogen cyanide, and *ca.* 60% for L-[3-^{11}C]lactic acid, based on carbon dioxide. The radiochemical purities were higher than 99% analysed by HPLC.

Key words: Multi-enzyme catalysis, Carbon-11, L-[1-^{11}C]Lactic acid, L-[3-^{11}C]Lactic acid.

Introduction

Positron emission tomography (PET) in combination with molecules labelled with a positron emitting radionuclide, has become a versatile tool for studying biochemical processes *in vivo*. In our continuing research for fast and reliable syntheses of biomolecules labelled with short-lived positron emitters such as ^{11}C (half-life 20.3 min), we have investigated the use of ^{11}C-labelled pyruvic acid as a reactive intermediate.[1] Several enzymes utilize pyruvic acid as a substrate. Using carbon-carbon lyases in combination with enzymatically produced

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pyruvic acid, the amino acids L-tyrosine, L-DOPA, L-tryptophan, and 5-hydroxy-L-tryptophan have been labelled with ^{11}C in *one-pot* procedures.[2-4] In these methods the syntheses were achieved from labelled alanine using D-amino acid oxidase (D-AAO) and glutamic-pyruvic transaminase (GPT) together with the lyases. The possibility of using a reductive enzyme such as L-lactic dehydrogenase (L-LDH) in a *one-pot* approach simultaneously with D-AAO and GPT have now been investigated.

^{11}C-Labelled L-lactic acid has been proposed as a tracer for measuring cardiac metabolism under normal and pathophysiological conditions.[5] Lactic acid has earlier been labelled with ^{11}C in its physiologically active form by enzymatic synthesis,[5-7] and in its racemic form by chemical procedures.[8,9] We now report the synthesis of 1- and 3-^{11}C-labelled L-lactic acid in excellent yields from the corresponding racemic alanine applying D-AAO/catalase, GPT, and L-LDH simultaneously in a *one-pot* procedure.

Experimental

General.- [^{11}C]Carbon dioxide was obtained by the $^{14}N(p,\alpha)^{11}C$ nuclear reaction achieved by bombardment of a nitrogen gas target with a 10 MeV proton beam produced by a tandem van der Graaff accelerator at the University of Uppsala. The radioactive carbon dioxide was trapped in a lead shielded oven containing 4-Å molecular sieves and transported to the chemistry laboratory. Hydrogen [^{11}C]cyanide was produced according to literature procedures.[10,11] The reaction was carried out by reducing the released [^{11}C]carbon dioxide to [^{11}C]methane using hydrogen gas and a nickel catalyst at 400°C and subsequent reaction with ammonia at 1000°C catalysed by platinum. The [^{11}C]methyl iodide was synthesised by reducing the carbon dioxide with lithium aluminium hydride in tetrahydrofuran followed by hydrolysis with hydriodic acid in a *one-pot* system.[12] *N*-(Diphenylmethylene)glycine *tert*-butyl ester was prepared according to a literature procedure.[13] Sodium 1-hydroxy-ethyl sulfite was prepared by adding an excess amount of acetaldehyde to a saturated aqueous solution of sodium hydrogen sulfite. After cooling in the refrigerator, the crystalline solid was filtered and washed with cold ethanol.

Analytical HPLC was performed on a Hewlett-Packard 1090 liquid chromatograph equipped with a UV-diode array detector in series with a β^+-flow detector.[14] The columns used were: (A) 250x4.6 mm LC-NH_2, Nucleosil, 10 µm and (B) 100x7.8 mm Fast Acid Analysis Column, Bio-Rad. The following mobile phases were used: (C) 10 mM potassium

dihydrogen phosphate, pH 4.6, (D) acetonitrile/water (500:70), and (E) 13 mM sulfuric acid/acetonitrile (9:1). Solid phase extraction (SPE), Supelco, C-18 columns were pre-conditioned with 3 mL dichloromethane, 10 mL ethanol, and 20 mL water prior to use. Cation exchange resin, Bio-Rad laboratories, AG 50W-X4 200-400 mesh, was pre-conditioned with water.

The enzymes are commercially available and 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. 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. 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. L-Lactic dehydrogenase (L-LDH, EC 1.1.1.27) from porcine muscle, solution in 50% glycerol containing potassium phosphate buffer, pH 7.0. Catalase and GPT were frozen in small portions in order to preserve the catalytic activity of the enzymes.

The cofactors flavin adenine dinucleotide (FAD), pyridoxal 5-phosphate (PLP), and reduced β-nicotinamide adenine dinucleotide (NADH) were also purchased from Sigma and dissolved in 50 mM potassium phosphate buffer, pH 7.5, prior to use.

DL-[1-^{11}C]Alanine.- Hydrogen [^{11}C]cyanide was transferred in a stream of nitrogen gas to a septum-equipped glass vessel containing a solution of 20 mg (135 μmol) sodium 1-hydroxy-ethyl sulfite in 0.4 mL 5 M ammonia, cooled in an ice bath. The reaction mixture was heated at 70°C for 5 min after which the reaction vessel was opened and 1 mL concentrated hydro-chloric acid was added. The solution was heated and shaken at 175°C for 5 min. The DL-[1-^{11}C]alanine solution was diluted with 2 mL water before the radiochemical purity was analysed by HPLC using column A and the following conditions: solvents C/D, flow 2.0 mL/min, gradient 0-8 min 95-60% D, column temperature 40°C, wavelength 230 nm. The retention time was 6.1 min for [^{11}C]alanine.

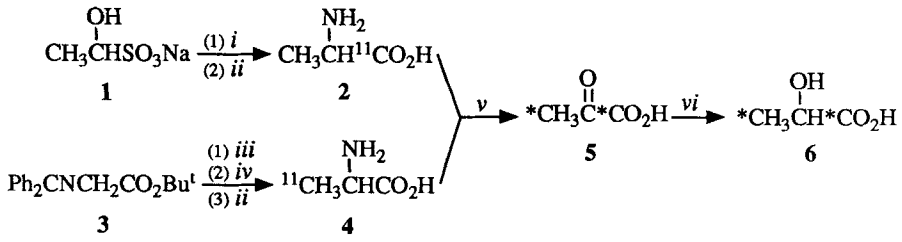
DL-[3-^{11}C]Alanine.- In a septum-equipped vial 3 mg (10 μmol) *N*-(diphenylmethylene)-glycine *tert*-butyl ester was dissolved in 0.5 mL dimethylformamide/dimethyl sulfoxide, 9:1 (v:v). After the addition of 2 μL 5.0 M potassium hydroxide, [^{11}C]methyl iodide was transferred to the reaction vessel in a stream of nitrogen gas. The reaction mixture was heated

at 80°C for 2 min and then transferred to a syringe, containing 20 mL water, connected to a SPE C-18 column. The mixture was passed through the column which was then washed with 3 mL water. The radioactivity trapped in the column was eluted with 2 mL dichloromethane into an open glass vessel containing 0.8 mL 6 M hydrochloric acid. The reaction mixture was heated and shaken at 130°C for 5 min during which the dichloromethane distilled off. After dilution with 2 mL water, the radiochemical purity of the racemic [3-^{11}C]alanine was analysed by HPLC using the same system as described above.

L-[1-^{11}C]Lactic acid and L-[3-^{11}C]lactic acid.- The racemic 1- or 3-^{11}C-labelled alanine solution was evaporated to dryness and redissolved in 0.8 mL 0.1 M tris(hydroxymethyl)-aminomethane/hydrochloric acid buffer, pH 8.5. The solution was transferred to a glass tube containing 50 µL 0.2 M α-ketoglutarate, 10 µL 1.7 mM FAD, and 10 µL 10 mM PLP after which the pH was adjusted to 8.5 with potassium hydroxide. To this solution was added 3.2 units D-AAO, 20 units GPT, 3600 units catalase, 500 units L-LDH, and 25 µL 40 mM NADH and the enzymatic reaction was left to proceed at 45°C for 5 min. The crude product was applied to a cation exchange column containing *ca.* 8 mL resin and the ^{11}C-labelled L-lactic acid was eluted with 4 mL water. After the addition of 1 mL 0.1 M phosphate buffer, pH 7.4, the pH was adjusted with sodium hydroxide to *ca.* 6 and the solution was filtered through a 0.22 µm pore filter into a sterile vial. The radiochemical purity was analysed by HPLC using column B and the following conditions: solvent E, flow 0.5 mL/min, column temperature 25°C, wavelength 230 nm. Retention times were 3.4, 4.6, and 5.6 min for ^{11}C-labelled alanine, pyruvic acid, and L-lactic acid, respectively.

Results and Discussion

DL-[1-^{11}C]Alanine (**2**) was synthesised by nucleophilic substitution of sodium 1-hydroxy-ethyl sulfite (**1**) with [^{11}C]cyanide and ammonia followed by hydrolysis of the resulting aminonitrile with hydrochloric acid according to the method of Iwata *et al.*,[10] see Scheme 1. The method was modified and optimised to get a fast reaction for producing carboxy-^{11}C-labelled alanine, suitable for the subsequent enzymatic reactions.[15] The precursor solution was not preheated and a lower substrate concentration was used than described previously. The hydrolysis was carried out directly in the same reaction vessel without isolation of the aminonitrile. In this way high salt concentrations were obtained, but this did not influence the



Scheme 1. *Reagents: i*, $H^{11}CN/NH_3$; *ii*, HCl; *iii*, KOH; *iv*, $^{11}CH_3I$; *v*, D-AAO/Catalase and GPT; *vi*, L-LDH.

subsequent enzymatic reactions. DL-[1-^{11}C]Alanine was produced in a *one-pot* system from [^{11}C]cyanide in a 95% radiochemical yield, decay corrected, within *ca.* 15 minutes and with a radiochemical purity higher than 97%.

The synthesis of DL-[3-^{11}C]alanine (**4**) was performed by methylation of a glycine derivative, *N*-(diphenylmethylene)glycine *tert*-butyl ester (**3**), with [^{11}C]methyl iodide followed by hydrolysis using hydrochloric acid as described previously,[13] see Scheme 1. The method was modified by performing the alkylation in dimethylformamide/dimethyl sulfoxide instead of a phase transfer reaction. In this way the reproducibility of the subsequent enzymatic reactions was increased.[1] DL-[3-^{11}C]Alanine was produced in a 70% radiochemical yield, decay corrected, within 18 minutes with a radiochemical purity of over 99%.

The utilisation of enzymes as catalysts in organic synthesis is of increasing interest.[16] Due to the high stereospecificity and relatively fast reaction rates of many enzymes, they are well suited for labelling purposes. For the conversion of alanine to pyruvic acid (**5**) two enzymes, D-AAO and GPT, were used, see Scheme 1. In this way both of the enantiomers of alanine were utilized enabling multi-millicurie amounts of tracer to be produced, suitable for PET-investigations in man. The catalytic ability of these enzymes were then combined with L-LDH for the production of L-lactic acid (**6**), Scheme 1. In order to minimize synthesis time and technical handling, a *one-pot* synthesis for the enzymatic reactions was developed. Hence, optimisation of the reaction conditions were carried out with respect to all the enzymes involved. The synthesis was performed using D-AAO/catalase, GPT, and L-LDH simultaneously at 45°C with a pH of 8.5 for 5 minutes. It was found to be essential that the cofactor NADH was added after the pH-adjustment since NADH decomposes in acidic media. The total synthesis time including purification (and synthesis of hydrogen [^{11}C]-cyanide, in the case of L-[1-^{11}C]lactic acid) was 40 minutes, counted from release of [^{11}C]-

carbon dioxide. The radiochemical purities were analysed by HPLC and were found to be higher than 99%. The decay corrected radiochemical yields were *ca.* 80% for L-[1-^{11}C]lactic acid, based on hydrogen [^{11}C]cyanide, and *ca.* 60% for L-[3-^{11}C]lactic acid, based on [^{11}C]-carbon dioxide. In typical runs, 320 MBq (8.6 mCi) L-[1-^{11}C]lactic acid was obtained from 1.3 GBq (35 mCi) hydrogen [^{11}C]cyanide within 35 min and 400 MBq (10.8 mCi) L-[3-^{11}C]-lactic acid was obtained starting from 2.6 GBq (70 mCi) [^{11}C]carbon dioxide within 40 minutes.

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