Journal of Labelled Compounds and Radiopharmaceuticals
J. Labelled Cpd. Radiopharm. **44**, 287-294 (2001)
DOI: 10.1002/jlcr.454

ENZYME CATALYSED SYNTHESIS of L-[4-^{11}C]ASPARTATE and L-[5- ^{11}C]GLUTAMATE

Antoni G.[1,4], Omura H.[2] , Ikemoto M.[2], Moulder R.[4], Watanabe Y.[3,4]and Långström B.[1,4]
The Subfemtomole Biorecognition Project, Japan Science and Technology Corporation and Uppsala University PET-Centre,[1] Ikeda Food Research Co. Ltd. Hiroshima, Japan,[2] Osaka Bioscience Institute Osaka, Japan,[3] Uppsala University PET-Centre, Uppsala, Sweden.[4]

ABSTRACT

The nitriles β–[^{11}C]cyano-L-alanine **1** and γ-[^{11}C]cyano-α-amino-L-butyric acid **2** were obtained by enzymatic catalysis from *O*-acetyl-L-serine and *O*-acetyl-L-homoserine with carrier added hydrogen [^{11}C]cyanide (0.1 μmol), using *O*-acetyl-L-serine sulfhydrylase [EC.4.2.99.8] and *O*-acetyl-L-homoserine sulfhydrylase [EC.4.2.99.10], respectively. L-[4-^{11}C]Aspartate **3** and L-[5-^{11}C]glutamate **4** were then obtained by alkaline hydrolysis of **1** and **2**, respectively. Purification by semi-preparative HPLC yielded **3** and **4** with radiochemical purities higher than 95% and with decay corrected radiochemical yields of 60-70% and 50-60%, respectively based on the total amount of collected hydrogen [^{11}C]cyanide. The enantiomeric purities were determined, after derivatisation with *N*-α–(2,4-dinitrophenyl-5-fluoro-)-L-alaninamide, by HPLC to be higher than 97.5%. The total reaction times for **3** and **4**, counted from end of bombardment, were 50-55 min. The specific radioactivity of **3** and **4** were approximately 30 GBq/μmol at end of synthesis starting from 20 GBq hydrogen [^{11}C]cyanide.

Key words: ^{11}C-aspartic acid,^{11}C-glutamic acid, *O*-acetyl-L-serine sulfhydrylase [EC.4.2.99.8], *O*-acetyl-L-homoserine sulfhydrylase [EC.4.2.99.10].

Address correspondence and reprint requests to: Assoc. Prof. Gunnar Antoni, e-mail: Gunnar.antoni@pet.uu.se, FAX: +46 18 4715392

Received 22 September 2000
Revised 6 October 2000
Accepted 21 December 2000

INTRODUCTION

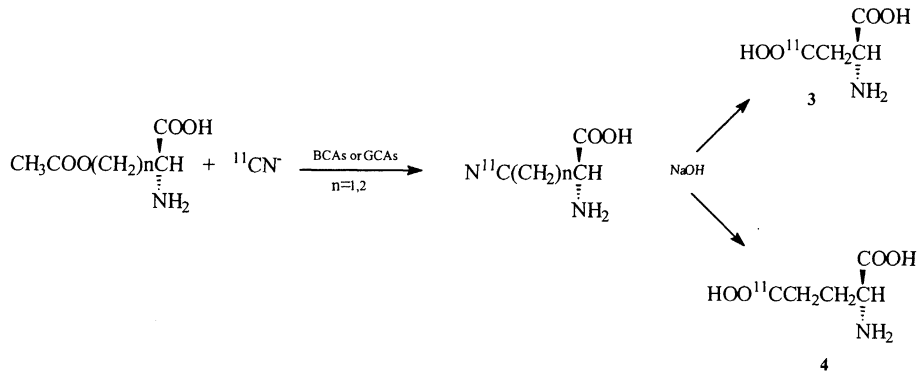
Amino acids labelled with the short-lived positron emitting radionuclides ^{11}C ($t_{1/2}$=20.4 min), ^{18}F ($t_{1/2}$=110 min) and ^{13}N ($t_{1/2}$ =10 min) have been applied as tracers in biomedical research and clinical applications utilising the Positron Emission Tomography technique (PET)(1). L-[Methyl-^{11}C]methionine (2) and 5-hydroxy[β-^{11}C]-L-tryptophan (HTP),(3) have been used for the detection, delineation and classification of tumours using the PET technology. Studies of *in vivo* neurotransmitter syntheses using the endogenous neurotransmitter precursors 3,4-dihydroxy-L-phenylalanine (DOPA) and HTP ^{11}C-labelled in the β-position have also been reported.(4)

The excitatory neurotransmitters aspartate (ASP) and glutamate (GLU) have previously been labelled with ^{11}C by enzymatic reactions.(5,6) The potential use of L-[^{11}C]aspartate and L-[^{11}C]glutamate for the assessment of regional myocardial metabolism and as tracers for endocrine tumours has encouraged the development of reliable semi-automated chemo-enzymatic methods for the production of these amino acids.

Enzymatically catalyzed reactions usually exhibit high stereoselectivity, substrate specificity and high reaction rates and are thus of interest in labelling synthesis with short-lived radionuclides such as ^{11}C and ^{13}N. Due to the restricted access to labelled precursors and the short time-frame set by the half-lives of these radionuclides, enzyme catalysis may sometimes be the only feasible method to synthesise the desired compound labelled in a specific position, with sufficient radioactivity to be useful as a tracer in biomedical research utilizing the PET technology.

We have earlier presented a chemo-enzymatic method for the synthesis of 2,4-diamino-L-[4-^{11}C]butyric acid using *O*-acetyl-L-serine sulfhydrylase [EC.4.2.99.8] (BCAs) as catalyst in the key-reaction step producing the intermediate β-[^{11}C-CN]cyano-L-alanine (BCA).(7) This intermediate can also be converted to aspartic acid by alkaline hydrolysis. An analogous enzyme (*O*-acetyl-L-homoserine sulfhydrylase [EC.4.2.99.10], GCAs) catalysed reaction producing γ-[^{11}C-CN]cyano-α-amino-L-butyric acid (GCA) has been developed and from that glutamic acid is prepared by alkaline hydrolysis. The methods described here suggest that other amino acids can be synthesised from the common intermediates (labelled nitriles) obtained in the enzymatic reactions (Scheme 1).

In this paper the enzyme catalysed syntheses of L-[4-^{11}C]aspartic acid **3** and L-[5-^{11}C]glutamic acid, are presented.



Scheme 1

EXPERIMENTAL

General

[^{11}C]Carbon dioxide was prepared by the ^{14}N(p,α)^{11}C nuclear reaction using a nitrogen gas target and 17 MeV protons produced by the Scanditronix MC-17 cyclotron at the Uppsala University PET Centre. The [^{11}C]carbon dioxide was converted to hydrogen [^{11}C]cyanide using the Scanditronix RNP-17 radionuclide production system.(8) HPLC separations were performed by the use of a Beckman system consisting of a 126 pump and a Beckman 166 variable wavelengths UV detector in series with a β^+-flow detector (Bioscan Inc). Semi-preparative HPLC was performed using a Waters LC-NH_2 column (250x10 mm, 5 μm). Analytical HPLC was performed on the following columns: Beckman ultrasphere ODS (250x4.6 mm, 5 μm), Beckman LC-NH_2 (250x4.6 mm, 5 μm), Phenomenex Ultracarb (100x4.6 mm, 3 μm) C18 , Nucleosil LC-NH_2 (l00x4.6 mm, 5 μm) column or a Zorbax SAX (250x4.6 mm, 5 μm). A Gilson Aspec XL was used as an autoinjector and data collection was achieved on a personal computer using the Beckman System Gold software package. Mass spectrometry (LC-MS) was performed on a Fisons Instrument (Cheshire UK) VG Quattro triple quadrapole mass spectrometer with a pneumatically assisted electrospray probe. The system was interfaced with a Beckman HPLC system consisting of 126 and a 116 pump, a 166 variable wavelength UV detector in series with a β^+-detector.

The syntheses were carried out using the Synthia semi-automated synthesis system, consisting of a Gilson Aspec XL robot and a GE Fanuc PLC.

Chemicals

O-Acetyl-L-serine hydrochloride (OAS) was obtained from Sigma-Aldrich. O-Acetyl-L-homoserine hydrochloride (OAHS) was synthesized according to the literature.(9)

Potassium [^{13}C]cyanide (99%, ^{13}C) and pyridoxal-5-phosphate (PLP) were purchased from Sigma-Aldrich. Potassium cyanide (*p.a.*) and potassium hydroxide (*purissimum*) and sodium hydroxide (*purissimum*) were obtained from BDH Ltd. and Eka Nobel respectively. Sodium and potassium phosphate were obtained from Merck and sterile water from Kabi-Pharmacia.

Enzyme purification

O-acetyl-L-serine sulfhydrylase [EC.4.2.99.8] and *O*-acetyl-L-homoserine sulfhydrylase [EC.4.2.99.10] were purified from *Bacillus stearothermophilus* from a cell-free extract by ammonium sulphate fractionation, ion exchange column chromatography, preparative polyacrylamide gel electrophoresis and hydrophobic column chromatography. The purified enzymes were confirmed to be homogeneous by SDS-polyacrylamide gel electrophoresis.(10)

Synthesis of L-[4-^{11}C]aspartate (Scheme l)

Hydrogen [^{11}C]cyanide was passed through 1 ml of 50% sulphuric acid heated to 50°C, was dried by passage through phosphorous pentoxide and trapped in potassium hydroxide (260 µl 50 mM) and potassium cyanide (10 µl, 10 mM, 0.1 µmol KCN). To the trapped hydrogen [^{11}C]cyanide was added, 360 µl 0.2 M dipotassium hydrogen phosphate, 20 µl of 10 mM PLP in 0.1 M potassium dihydrogen phosphate, 140 µl of a 0.1 M solution of *O*-acetyl-L-serine hydrochloride (OAS) in 0.1 M dipotassium hydrogen phosphate and 50 µl of a solution of BCAs (approximately 100 units/ml). The reaction mixture was heated in a sealed vessel at 65°C for 5 min. Following addition of 1.5 ml of 2.5 M NaOH, the reaction mixture was heated at 130°C for 12 min.

Synthesis of L-[5-^{11}C]glutamate (Scheme 1)

Hydrogen [^{11}C]cyanide was trapped as described above. To this was added, 325 µl 0.2 M dipotassium hydrogen phosphate, 20 µl of 10 mM PLP in 0.1 M potassium dihydrogen phosphate, 170 µl of a 25 mM solution of *O*-acetyl-L-homoserine hydrochloride (OAHS) in 0.1 M potassium dihydrogen phosphate and 50 µl of the solution of GCAs (approximately 100 units/ml). The reaction mixture was heated in a sealed vessel at 75°C for 10 min. Following addition of 1.5 ml of 2.5 M NaOH, the reaction mixture was heated at 130°C for 12 min.

Purification of L-[4-^{11}C]aspartate and L-[5-^{11}C]glutamate

The reaction mixture was mixed with 700 µl of 6M HCl and 1500 µl of acetonitrile/water (5/1, v/v) and was injected onto the semi-preparative HPLC column. Mobile phase composition: solvent A=75 mM NaH_2PO_4 (adjusted to pH 6.6 with phosphorous acid) and solvent B=acetonitrile/water 50/7 (V/V). A/B 35/70 isocratic for 2 min then a linear gradient 2-9 min to A/B 80/20 at a flow rate of 5 ml/min. The collected fractions were evaporated at 110°C to remove the acetonitrile. The residue was dissolved in sterile sodium phosphate buffer (0.1 M pH 7.4) and the solution was filtered through a sterilized 0.2 µm filter into a sterilized injection vial.

Synthesis of ^{11}C/^{13}C-labelled aspartate and glutamate

Combined ^{11}C/^{13}C syntheses were performed using the same procedures as already described for **3** and **4** apart from that 0.4 mg of K(^{13}C)CN was added instead of 10 µl of a 10 mM solution of KCN to the trapping solution. A solid phase extraction method was used to separate **3** and **4.** The residue from the alkaline hydrolysis was mixed with 7.6 ml of 50 mM sodium hydrogen phosphate and passed through a AG1-X8 column (780-825 mg). The anion-exchange column was washed with water and the product eluted with 0.5 M hydrochloric. This procedure was used to obtain **3** and **4** free from phosphate to avoid LC-MS contamination.

Analysis and identification

Chromatographic analysis

The outcome of the enzyme reactions were analysed using a Beckman ultrasphere ODS column, eluted with 10 mM KH_2PO_4 at a flow rate of 0.75 ml/min, (UV detector at 210 nm). The retention times both for **1** and **2** were 3.6 min with hydrogen [^{11}C]cyanide eluting at 4.5 min.

L-[^{11}C]Aspartate and L-[^{11}C]glutamate were analysed on both a LC-NH_2 column and a Zorbax SAX column. On the latter column were **3** and **4** eluted with 10 mM KH_2PO_4 a flow rate of 2 ml/min, (UV detector at 210 nm). The retention times for **3** and **4** were 9.3 min and 6.5 min, respectively. Using the LC-NH_2 column and solvent A=10 mM KH_2PO_4 and solvent B=acetonitrile/water 50/7 (V/V) as mobile phase, the retention times for **3** (linear gradient from A/B 50/50 to A/B 80/20, 0-5 min) and **4** (linear gradient from A/B 35/65 to A/B 80/20 0-5 min) were 6.5 min and 7.5 min, respectively.

Enantiomeric analysis

The enantiomeric purities of labelled aspartate and glutamate were determined by derivatisation with Marfey's reagent.(11) Two hundred microliters of a 1% solution of *N*-α-(2,4-dinitro-5-fluorophenyl)-L-alaninamide in acetone was mixed with 50 µl of product solution (adjusted to approximately pH 9) and 50 µl of a 50 mM solution of either aspartate or glutamate. The reaction mixture was heated at 50°C for 30 min, quenched with 50 µl of 2 M hydrochloric acid and 200 µl of ethanol, and then analysed by HPLC using a Beckman ultrasphere ODS column eluted with solvent A=50 mM ammonium formate pH 3.5 and solvent B=methanol, at a flow rate of 2 ml/min, linear gradient from A/B 75/25 to 40/60 during 1-10 min, (UV detector at 340 nm). The retention times for the diastereomeric Marfey derivatives of L- and D-aspartate were 2.2 min and 2.8 min, and for L- and D-glutamate 2.3 min and 3.3 min, respectively.

LC-MS analysis

The identities of ^{11}C-labelled aspartate and glutamate were confirmed by LC-MS analysis. Positive electrospray mass spectrometry was performed scanning in the range of 120-180 m/z. The LC eluent was split 1:70 between the mass spectrometer and a series coupled UV and radioactivity detector. The HPLC analysis was performed on two separate columns A: Phenomenex Ultracarb C-18 column and B: Nucleosil LC-NH_2 column. The following mobile phases were used: solvent A= nanopure water, B= acetonitrile, C= acetonitrile/aqueous solution of 5 mM ammonium formate and 5 mM acetic acid 1/1. Column A was eluted isocratic with a mobile phase consisting of 98% A, 1% B and 1% C using a flow rate of 1 ml/min monitored at 210 nm. Column B was eluted with a mobile phase consisting of 6.5% A, 92.5% B and 1% C linear gradient to 5% A, 94% B and 1% C during 3 min, at a flow rate of 1 ml/min, monitored at 210 nm.

RESULTS AND DISCUSSION

The radiochemical yields of the labelled nitriles BCA and GCA were 88-97%. The enzymatic reaction was optimised with respect to pH, temperature, buffer-, cyanide-substrate-, and enzyme concentration. The working pH range for the *O*-acetyl-L-serine sulfhydrylase [EC.4.2.99.8] and *O*-acetyl-L-homoserine sulfhydrylase [EC.4.2.99.10] was approximately 7.4-8.5 with a maximum enzymatic activity at pH 8 using potassium phosphate buffer. Other buffers (e.g. ammonium chloride/ammonium hydroxide or

tris(hydroxymethyl)aminomethane) gave lower radiochemical yields due to lower enzymatic activity.

As the buffer capacity of the phosphate is low (around pH 8) it was important to remove ammonia (used in the catalytic process for production of labelled hydrogen cyanide from methane) before trapping the cyanide in the reaction mixture to ensure a reproducible pH in the reaction mixture. This was done by passing the H[^{11}C]CN through 50% sulphuric acid followed by phosphorous pentoxide before trapping in potassium hydroxide. Using standardized amounts of trapping solution, substrate and buffer, a reproducible pH between 7.8-8.2 was obtained in the reaction mixture. This was important due to the technical problems encountered when trying to adjust the pH of a small volume of a highly radioactive solution.

The enzyme reaction was also sensitive to the concentration of cyanide. Concentrations below 70 μM gave irreproducible radiochemical yields of **1** and **2** in the range of 30-80%. A standard amount of potassium cyanide (0.1 μmol) was added giving a total concentration of cyanide of approximately 180 μM. The optimum temperature for BCAs and GCAs was 45°C and 55°C, respectively, but for short reaction times (< 15 min) higher radiochemical yields was obtained by heating at 65°C and 75°C respectively. As the enzymatic reaction was relatively insensitive to the concentration of OAS and OAHS, a concentration range of 5-50 mM for the former and 0.5-5 mM for the latter could be used. Around 5 units (one unit is defined as the amount of enzyme that catalyse the formation of 1 μmol of BCA or GCA/min during standard assay conditions) of BCAs and GCAs were used. A reaction time of 5 min was thus sufficient to convert the cyanide in 90-97% radiochemical yield to BCA whereas 10 min was needed to obtain 88-95% radiochemical yield of GCA (not isolated yields and based on the trapped amount of [^{11}C]cyanide). Apart from unreacted cyanide, small amounts of unidentified impurities (3-5%) could be detected.

Hydrolysis of BCA with hydrochloric acid produced aspartate in quantitative yield whereas the acidic hydrolysis of GCA initially produced glutamate but during the course of the reaction this was converted into a new unknown by-product. However, alkaline hydrolysis gave labelled aspartate and glutamate in 90-95% radiochemical yields counted from the amount of BCA and GCA within 12 min reaction time. Shorter reaction time and lower temperature resulted in reduced yields of labelled aspartate and glutamate.

Purification by semi-preparative HPLC gave radiochemical purities ranging from 95% to 99%. The total syntheses times for L-[4-^{11}C]aspartate and L-[5-^{11}C]glutamate, from end of the radionuclide production were 45 and 55 min, respectively. The decay corrected radiochemical yields for L-[4-^{11}C]aspartate and L-[5-^{11}C]glutamate were 60-70% and 50-60%, respectively (based on trapped amount of [^{11}C]cyanide).

The enantiomeric purity was determined by derivatisation with Marfey's reagent and subsequent HPLC separation of the diastereomers to be 97.8% e.e. (L/D, 98.9/1.1) for **3** and **4**. The reason for the small amount of D-enantiomer present is not known but the rather drastic condition (2.5 M NaOH 135°C, 12 min) during the hydrolysis may have caused some racemisation.

Performing combined ^{11}C/^{13}C syntheses and analysing the products with LC-MS verified the identities of the obtained products. Mass spectrometric analysis of aspartate and glutamate standards (positive electrospray) showed major ions at 134 and 148 $(m+l)^+$, respectively. LC-MS analysis of the [^{11}C/^{13}C]aspartate and [^{11}C/^{13}C]glutamate showed major ions at 135 and at 149, respectively. Good correlation was obtained between the mass and radioactive peaks.

Labelled aspartate and glutamate were delivered in an isotonic phosphate buffer (pH 6-7.5) and the solutions were found to be sterile and free from pyrogens and were thus approved for intravenous administration in animals and humans.

ACKNOWLEDGMENTS

We are indebted to the Japan Science & Technology Corporation for financially supporting this project.

REFERENCES

1. Phelps M., Mazziotta J. and Schelbert. (eds), *Positron Emission Tomography and Autoradiography*: Principles and applications for the brain and heart. Raven Press, New York (1986).
2. Lilja A. *et. al., A.J.N.R.*, 6:505 (1985).
3. Eriksson B., *et. al., Q. J. Nucl. Med.* 44:68-76, (2000).
4. Hartvig P., *et. al., J. Neural. Transmiss. (Gen. Sect.)* 94:127-135 (1993).
5. Barrio J.R., *et. al., J.Med. Chem.* 25:93. (1982).
6. Cohen M.B., *et. al., Int. J. Appl. Radiat. Isot.* 33:613. (1982).
7. Antoni G., *et. al., Nuc. Med. Biol.* 24:595-601 (1997).
8. a). Christman D.R., *et. al., Int. J. Appl.Radiat. Isot.* 26, 435-442 (1975), b). Iwata R., *et. al., Appl.Radiat. Isot.* 38:97-102 (1987).
9. Nagai S., *et. al., J. Biol. Chem.* 242:3884-3895 (1967).
10. Watanabe *et. al.,* European Patent: EP 0 733 374 A2
11. Marfey P., *Carlsberg Res, Commun.* 49:591 (1984).

