SYNTHESIS OF L-[5-^{11}C]ORNITHINE

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SUMMARY

No-carrier-added L-[5-^{11}C]ornithine was synthesized in 25-40% radiochemical yield in a synthesis time of 50 min (EOB) and a specific activity >2.1 Ci/umol by the displacement reaction of potassium [^{11}C]cyanide with the functionally protected γ-bromohomoserine followed by selective reduction of the [^{11}C]nitrile with cobalt chloride-sodium borohydride complex, deprotection with 6M $HCl_{(aq)}$, and purification by HPLC. During the course of these studies it was found that cyanide ion is generated from acetonitrile in the presence of potassium hydroxide and kryptofix 222.

Key Words: carbon-11, L-[5-^{11}C]ornithine, ornithine decarboxylase.

INTRODUCTION

Ornithine decarboxylase (ODC) catalyzes the decarboxylation of L-ornithine to form the diamine, putrescine. This is the first and rate limiting step in polyamine biosynthesis. The activity of this enzyme, while normally low, is induced by a variety of growth stimuli.[1] For example, ODC has been found to be positively correlated with DNA synthesis[2] and grade of malignancy in brain tumors[3] and with the biochemical events accompanying ischemic injury and blood brain barrier breakdown.[4]

D,L-[1-^{11}C]Ornithine has been synthesized by [^{11}C]carboxylation of the corresponding α-lithioisocyanide and its accumulation into tumor tissue has been demonstrated.[5] In order to examine the potential of using positron emission tomography (PET) as a sensitive probe for tissue proliferation, ornithine labeled in the 5-position is required because the label would remain in putrescine, the product of ODC catalyzed decarboxylation, providing a

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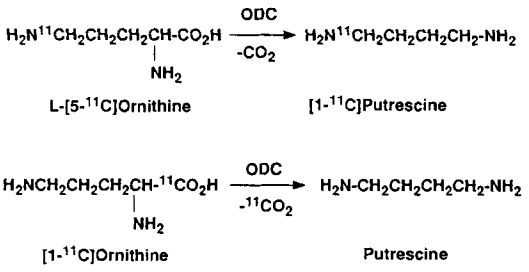
record of ODC activity. In contrast, the label from [1-^{11}C]ornithine would be lost as carbon-11 labeled carbon dioxide (Scheme I). Although the

SCHEME I

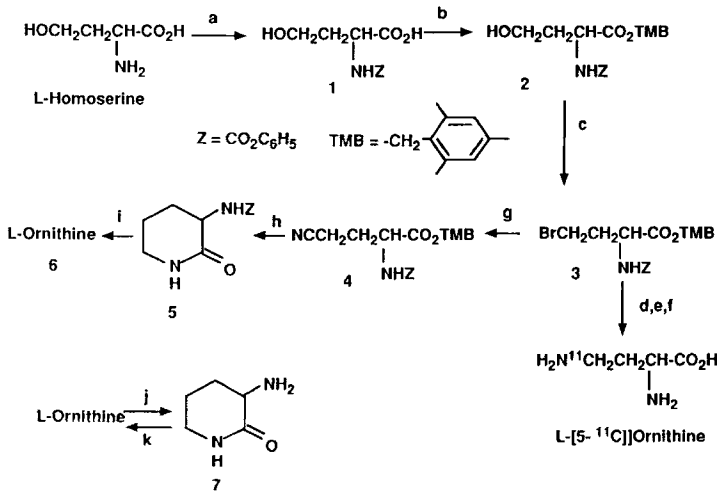


information which would be obtained with L-[5-^{11}C]ornithine and PET in the study of brain tumors may be similar to that obtained with [1-^{11}C]putrescine,[6] labeled ornithine may be a more sensitive indicator for tumors with minimal blood-brain barrier breakdown since it is known to cross the blood-brain barrier, transported by the basic amino acid carrier.[7] Furthermore, since L-ornithine is the substrate for the primary enzyme of interest, rather than a product, its uptake may correlate more directly with tumor grade. The tumor uptake of D,L-[5-^{14}C]ornithine and a comparative study of ODC activity in tumor with L-[1-^{14}C]- and L-[5-^{14}C]ornithine have recently been reported[8,9] thus supporting this approach.

We report here a rapid synthesis of high specfic activity, L-[5-^{11}C]ornithine. The starting material 3 for radiosynthesis was prepared as shown in Scheme II. The amino group of L-homoserine was first protected as the benzyl carbamate,[10] followed by conversion of the carboxyl group to the trimethylbenzyl ester.[11] The resulting compound 2 was brominated[12] with carbon tetrabromide and triphenylphosphine. The overall yield was 60% from L-homoserine. In the cold synthesis, the bromide 3 was converted to the corresponding nitrile 4 by reacting with potassium cyanide in DMSO. Upon reduction with cobalt chloride-sodium borohydride complex,[13,14,15] the resulting amine was cyclized to give the lactam 5 which was then hydrolyzed to yield L-ornithine. L-[5-^{11}C]Ornithine was synthesized by a multistep synthesis: displacement reaction[12] of potassium [^{11}C]cyanide with the

functionally protected γ-bromohomoserine 3 followed by selective reduction of the [^{11}C]nitrile, deprotection[16] with 6M $HCl_{(aq)}$, and purification by HPLC.

SCHEME II



(a) NaOH, $ClCO_2C_6H_5$; (b) TMBCl, Et_3N; (c) CBr_4, $(C_6H_5)_3P$; (d) $K^{11}CN$, Kryptofix 222;

(e) $CoCl_2 \cdot 6H_2O$, $NaBH_4$; (f) HCl; (g) KCN, DMSO; (h) $CoCl_2 \cdot 6H_2O$, $NaBH_4$; (i) HCl

EXPERIMENTAL

Materials:

Homoserine, D,L-homoserine, benzyl chloroformate, 2,4,6-trimethylbenzyl chloride, carbon tetrabromide and triphenylphosphine were purchased from Aldrich Chemical Co. Potassium cyanide was purchased from Fisher Scientific Company. Kryptofix 222 was purchased from EM Industries, Inc. Cobalt (II) dichloride hexahydrate was purchased from Mallinckrodt, Inc. and sodium borohydride was purchased from Alfa Inorganics Division, Ventron, Inc. Marfey's reagent, 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA), was purchased from Pierce Chemical Company. N,N-Dimethylformamide was distilled from BaO; methylene chloride and acetonitrile were distilled from P_2O_5. Dimethyl sulfoxide (DMSO) was distilled from CaH_2 and stored over 4Å molecular sieves. The other substrates which are not commercially available were synthesized and characterized by standard methods.

Analyses:

Melting points were determined with a Fisher-Johns melting point

apparatus and are uncorrected. NMR spectra were recorded with a Brucker 300 MHz spectrometer in either chloroform-D, or D_2O. Gas-liquid chromatographic analyses (GLC) were carried out with a gas chromatograph equipped with a thermal conductivity detector. Mass spectra were recorded with a Finnegan-Mat GC-MS 5100 mass spectrometer using electron impact ionization at 70 eV. HPLC analyses were carried out with a Perkin-Elmer liquid chromatograph equipped with a radioactivity monitor and UV detector. Details about columns and solvent systems used in each case are given in the experimental section. Microanalyses were performed by Galbraith Laboratories, Inc.

Cyanide concentration was determined spectrophotometrically (Varian DMS 70 UV-visible spectrophotometer) with a pyridine-pyrazolone mixture[17] using a standard curve for cyanide solutions in the concentration range of 7.7 to 77 nmol.

Preparation of L-2-[(Carbobenzyloxy)amino]-4-hydroxybutyric Acid (1). L-Homoserine (2.0 g, 16.8 mmol) was dissolved in 10 mL of 2N sodium hydroxide at 0° C. To the well-stirred solution was added benzyl chloroformate (3.2 g, 18.8 mmol) over 1 h together with 1 N sodium hydroxide to maintain the pH at 9.8-10.0. The suspension was then brought to room temperature and stirred for an additional 3 h and then extracted with ether (2 x 10 mL). The aqueous phase was cooled in an ice bath, carefully acidified to pH 3 with 3 N HCl, and extracted with ethyl acetate (2 x 20 mL). The extracts were dried over anhydrous $MgSO_4$ and evaporated. The resultant oil was crystallized from ethyl acetate/hexane to afford 3.57 g (80%) of 1. m.p. 100-101° C (Lit.[10], 99-100°). ^{1}H NMR ($CDCl_3$) δ 7.33 (s, 5H, C_6H_5), 5.91 (d, 1H, NH), 5.5 (br, 1H, OH), 5.11 (s, 2H, CH_2Ph), 4.51 (m, 1H, CH), 3.72 (m, 2H, CH_2O), 2.13, 1.85 (m, 2H, $CHCH_2$).

Preparation of Trimethylbenzyl L-2-[(Carbobenzyloxy)amino]-4-hydroxybutyrate (2). A solution of 1 (2.64 g, 10.4 mmol) in dry DMF (12 mL) was treated with Et_3N (1.6 mL) and 2,4,6-trimethylbenzyl chloride (1.9 g, 11 mmol). The mixture was kept overnight at room temperature and then diluted with 20 mL of 2.5% aqueous sodium bicarbonate. The precipitate was filtered off and washed with ethyl acetate. The combined filtrates were washed once with water, then dried over anhydrous $MgSO_4$ and concentrated *in vacuo* to

afford 3.84 g (96%) of crude 2. An analytical sample was obtained after flash chromatography. m.p. 115-116.5° C. ^{1}H NMR ($CDCl_3$) δ 7.35 (s, 5H, C_6H_5), 6.88 (s, 2H, C_6H_2), 5.65 (d, 1H, NH), 5.24 (d, 2H, $COOCH_2$), 5.11 (s, 2H, CH_2Ph), 4.55 (m, 1H, CH), 3.67 (m, 2H, CH_2O), 2.75 (br, 1H, OH), 2.33, 2.28 (2s, 9H, $3CH_3$), 2.2-2.5 (m, 2H, $CHCH_2$). MS, m/e (rel. intensity): 385 (M^+, 1), 294 (1), 235 (4), 208 (1), 164 (6), 132 (38), 108 (28), 91 (100), 86 (4), 79 (7), 77 (7), 65 (9). Anal. calcd. for $C_{22}H_{27}NO_5$: C, 68.55; H, 7.06; N, 3.63. Found: C, 68.27; H, 7.06; N, 3.59.

Preparation of Trimethylbenzyl L-2-[Carbobenzyloxy)amino]-4-bromobutyrate (3). To a magnetically stirred solution of crude 2 (466 mg, 1.21 mmol) and carbon tetrabromide (802 mmg, 2.42 mmol) in 20 mL of methylene chloride was slowly added a solution of triphenylphosphine (665 mg, 2.54 mmol) in 15 mL of methylene chloride over 20 min. After the addition was complete, the reaction mixture was stirred for an additional 0.5 h and the solvent was removed *in vacuo*. The residue was purified by flash chromatography to afford 380 mg (>70%) of 3. m.p. 86-88° C. ^{1}H NMR ($CDCl_3$) δ 7.35 (s, 5H, C_6H_5), 6.88 (s, 2H, C_6H_2), 5.45 (d, 1H, NH), 5.24 (d, 2H, $COOCH_2$), 5.10 (s, 2H, CH_2Ph), 4.50 (m, 1H, CH), 3.37 (m, 2H, CH_2Br), 2.33, 2.28 (2s, 9H, $3CH_3$), 2.2-2.5 (m, 2H, $CHCH_2$). MS, m/e (rel. intensity): 449 (0.1), 447 (0.1), 272 (0.2), 270 (0.2), 228 (1.3), 226 (1.4), 222 (1.4), 132 (100), 107 (6), 105 (6), 91 (99). HPLC (Phenomenex Spherisorb ODS 5 column, 4.6 x 250 mm, MeOH : H_2O = 75 :25, flow 1.5 mL/min, 254 nm) showed compound 3 had a retention time of 8.11 min. Anal. calcd. for $C_{22}H_{26}NO_4Br$: C, 58.93; H, 5.84; N, 3.12. Found: C, 59.54; H, 5.90; N, 3.13.

Preparation of Trimethylbenzyl L-2-[Carbobenzyloxy)amino]-4-cyanobutyrate (4). Dry potassium cyanide (17.5 mg, 0.268 mmol) and 8 mL of DMSO were placed in a 50 mL flask. A condenser was fitted and the suspension was heated to 90° C under N_2 with stirring for 1 h. The apparatus was cooled to room temperature and a 5 mL DMSO solution of 3 (60 mg, 0.134 mmol) was slowly added over a period of 0.5 h. After an additional 8 h at room temperature, the mixture was diluted with water (50 mL) and extracted with ether (3 x 15 mL). The ether extracts were washed once with water and dried over anhydrous $MgSO_4$ and the ether removed at reduced pressure to yield 48 mg

of 4 (90%) as a white solid. m.p. 98-99.5° C. ^{1}H NMR ($CDCl_3$) δ 7.35 (s, 5H, C_6H_5), 6.88 (s, 2H, C_6H_2), 5.45 (d, 2H, $COOCH_2$), 5.10 (s, 2H, CH_2Ph), 4.45 (m, 1H, CH), 2.33, 2.28 (2s, 9H, $3CH_3$), 2.2-2.5 (m, 2H, CH_2CN), 2.0-2.3 (m, 2H, $CHCH_2$). MS, m/e (rel. intensity): 394 (M^+, 1), 222 (1), 173 (2), 134 (11), 133 (81), 132 (100), 107 (6), 105 (6), 91 (74). HPLC (Phenomenex Spherisorb ODS 5 column, 4.6 x 250 mm, MeOH : H_2O = 75 : 25, flow 1.5 mL/min, 254 nm) showed compound 4 had a retention time of 4.75 min.

Preparation of L-3-[(Carbobenzyloxy)amino]-2-piperidinone (5). Nitrile 4 (39 mg, 0.1 mmol) was dissolved in 10 mL of methanol. Cobalt chloride hexahydrate (48 mg, 0.2 mmol) and sodium borohydride (37.8 mg, 1 mmol) were added. The reaction was followed by means of TLC (50% ethyl acetate/ hexane); within 5 min the nitrile was consumed. The reaction mixture was filtered and evaporated to dryness. The residue was taken up in ethyl acetate and purified by column chromatography (kieselgel 60,0.04-0.063 mm, eluted with ethyl acetate) to afford 15 mg of 5 (60%). ^{1}H NMR ($CDCl_3$) δ 7.35 (s, 5H, C_6H_5), 6.88 (s, 2H, C_6H_2), 5.45 (d, 1H, NH), 5.24 (d, 2H, $COOCH_2$), 5.10 (s, 2H, $CH_2C_6H_5$), 4.45 (m, 1H, CH), 2.33, 2.28 (2s, 9H, $3CH_3$), 2.2-2.5 (m, 2H, CH_2CN), 2.0-2.3 (m, 2H, $CHCH_2$). MS, m/e (rel. intensity): 248 (M^+, 16), 141 (60), 113 (16), 108 (20), 99 (23), 91 (100), 79 (16), 65 (20), 43 (15). HPLC: Compound 5 had a retention time of 3.20 min on a Phenomenex C-18 column (4.6 x 250 mm, MeOH : H_2O = 75 : 25, flow 1.5 mL/min, 254 nm), and a retention time of 1.47 min on a Phenomenex NH_2 column (4.6 x 100 mm, CH_3CN : 0.01 M KH_2PO_4 = 60 :40, pH 2.90, flow 1.5 mL/min, 230 nm).

Synthesis of L-ornithine from 5. Compound 5 (8.5 mg, 0.03 mmol) was refluxed with 8 M $HCl_{(aq)}$ for 5-6 h at 70° C. The aqueous phase was washed with ether and evaporated to dryness. ^{1}H NMR analysis of the residue in D_2O was identical with that of an authentic sample of L-ornithine. The parent lactam (3-amino-2-piperidinone) (7) of L-ornithine was also prepared[18,19] for comparison with the α-carbobenzyloxyamino lactam (5). The experimental details for the preparation of (7) and hydrolysis to ornithine are given below.

Preparation of 3-Amino-2-Piperidinone (7). A three-necked reaction flask, equipped with a magnetic stirrer and a high efficiency reflux

condenser, was charged with L-ornithine hydrochloride (1.0 g, 5.9 mmol), acetonitrile (25 mL), and hexamethyldisilazane (12.5 mL). The reaction mixture was heated to reflux under a gentle stream of dry nitrogen for 48 h (the mixture became homogeneous after 3-5 h), then cooled, poured into cold methanol (50 mL), and evaporated to dryness under vacuum at room temperature. The residue was taken up with chloroform, the mixture was filtered through a Celite pad on a sintered glass funnel, and the filtrate was evaporated to dryness under vacuum at room temperature. The residue was then purified by crystallization from isopropyl ether or by column chromatography over silica gel (eluted with ethyl acetate). Yield: 605 mg (90%). m.p. 38-42° C (Lit.[19], 38-41° C). ^{1}H NMR ($CDCl_3$) δ 6.58 (br, 1H, NH), 3.28 (m, 3H, CH_2NH & $CHNH_2$), 1.5-2.2 (m, 6H, $2CH_2$ & NH_2).

Hydrolysis of (7) to L-ornithine. To crude parent lactam (7) (14 mg) was added 0.5 mL of methanol and 1 mL of concentrated hydrochloric acid at room temperature. The mixture was heated at 125-130° C for 30 min, cooled and then partitioned between ether and water. The aqueous phase was washed three times with ether and evaporated to dryness. ^{1}H NMR of the residue in D_2O was identical with that of an authentic sample of L-ornithine.

Synthesis of L-[5-^{11}C]Ornithine. Carbon-11-labeled hydrogen cyanide was produced according to a previously published method[20] and trapped in 0.2 mL of a 0.05 M potassium hydroxide solution. The solution was evaporated to dryness under vacuum. To the residue was added Kryptofix 222 (3mg) in acetonitrile (150 uL) and the reaction vessel was heated at 60° C for 1 min prior to addition of the protected γ-bromohomoserine (3) (5 mg) in acetonitrile (150 uL). The resulting solution was stirred for another 5 min at 60° C. After removal of the solvent *in vacuo*, the residue was taken up in 0.5 mL of methanol and transferred to a reaction vial. Cobalt (II) chloride hexahydrate (7 mg) and sodium borohydride (10 mg) were added sequentially and the mixture was stirred for 5 min at room temperature. After addition of 0.5 mL of concentrated HCl, the vial was closed (vial cap fitted with a 0.2 mm tube in order to release pressure) and heated for 10 min (125° C). The mixture was evaporated to dryness under a stream of nitrogen leaving a greenish residue which was taken up in 0.75 ml of water and injected onto a Phenomenex

spherisorb NH_2 HPLC column (250 x 10 mm, 5um) using a mixture of acetonitrile and 0.01 M KH_2PO_4 (55:45, pH 5.5) as the mobile phase. The elution process was monitored by a NaI-detector and a refractometer. The radioactive peak corresponding to the retention time of ornithine (ca. 10 min) was collected in a total volume of about 4 mL with a flow rate of 2.5 mL/min. The solvent was evaporated at reduced pressure and the residue was made isotonic by the addition of a sterile saline solution. The resulting solution was filtered (sterile 0.22 um millipore) into a sterile vial. The total reaction time was ca. 50 min and the radiochemical yield was 25-40% calculated for the end of bombardment (EOB) and based on starting activity of hydrogen [^{11}C]cyanide produced at the end of cyclotron bombardment. In a typical experiment using 80-90 mCi of hydrogen [^{11}C]cyanide, 3-4 mCi of [5-^{11}C]ornithine was obtained after a reaction time of 50 min.

Radiochemical and chemical purity were assayed by thin layer chromatography (TLC) using MN polygram G/UV 254 silica plastic TLC plates by spotting [5-^{11}C]ornithine with authentic carrier material and developing with ethanol:water:ammonia (15:5:2 v/v) followed by visualization with ninhydrin. The radiochemical purity was also assayed by HPLC as ornithine and also as the o-phthalaldehyde (OPA) derivative of ornithine.[21,22] For ornithine, a Phenomenex NH_2 column (100 x 4.6 mm, particle size 5um) was used with a 60:40 mixture of CH_3CN :0.01M KH_2PO_4 (pH 2.90, flow 1.5 mL/min) as the mobile phase. Ornithine was visualized at 230 nm and had a retention time of 3.6 min. For the OPA derivative of ornithine, a Hewlett packard Hypersil ODS column (100 x 4.6 mm, 5um) and a FD-100 Filter Fluorometer (excitation filter 330 nm, emission filter 418 nm) were used with a 40: 60 mixture of 50 mM sodium acetate: methanol as the mobile phase (flow 1.5 mL/min). The OPA derivative of ornithine had a retention time of 3.25 min. In both cases, authentic carrier was added and the elution profile of the radioactivity was congruent with the carrier.

Enantiomeric purity analysis. The enantiomeric purity of L-ornithine was assayed by examining the Marfey's derivative of ornithine prepared with optical active Marfey's reagent FDAA[23] as described below. The sample (5 μmol in a total volume of 100 μL) was placed in a 1 mL Reacti-Vial and 200 μL of a

1 % solution of FDAA (Marfey's reagent) in acetone was added. Then 40 µL of 1.0 M sodium bicarbonate solution was added and the reaction mixture was heated at 40° C for 1 h. The reaction mixture was cooled and 20 µL of 2 M HCl was added to degas the solution. An aliquot was analyzed using a reverse phase HPLC column (Spherisorb ODS, 250 x 4.6 mm, 5um) eluting with (A) 50 mM ammonium formate pH 3.5 (B) MeOH, gradient: 0-15 min 20-50% B at a flow 2.0 mL/min, 340 nm. The derivative of D-ornithine had a retention time of 16 min while that of L-ornithine had a retention time of 18 min and Marfey's reagent had a retention time of 12 min. A non-labeled experiment using protected bromide or nitrile as starting material and carried out under the same conditions described for the synthesis of [5-^{11}C]ornithine afforded L-ornithine of high enantiomeric purity (ca. 90% e.e.; determined by HPLC of the Marfey's derivative). The enantiomeric purity of [5-^{11}C]ornithine was also assayed by HPLC of the Marfey's derivative, prepared in the presence of authentic D,L-ornithine. Upon HPLC separation of the diastereomeric derivatives, only the peak corresponding to the derivative of L-ornithine was enriched with radioactivity.

Specific activity determination. Specific activity was determined by analytic HPLC analysis (Phenomenex NH_2 column, 100 x 4.6mm, 5um) using a calibration curve of peak area of the UV trace at 230 nm as compared to nmol concentration of a standard solution of L-ornithine. An aliquot (200 uL from a total of 4 mL) containing the L-[5-^{11}C]ornithine (2.54 mCi, corrected to EOB) after separation by HPLC was analyzed and there was no visible mass associated with the product. Since the minimum amount of L-ornithine that we could detect was 0.2 ug/injection, the specific activity would be greater than 2.1 Ci/umol.

RESULTS AND DISCUSSION

A potentially efficient approach to L-ornithine entails the nucleophilic displacement by cyanide of a leaving group (X^-) from a L-homoserine derivative[12] followed by catalytic reduction. To test this, trimethylbenzyl L-2-[(carbobenzyloxy)amino]-4-bromobutyrate 3 was prepared. Interestingly, we found that unlabeled cyanide ion was generated in the acetonitrile solvent

system in the presence of potassium hydroxide and kryptofix 222 (20 mg) (see Table 1). Moreover, each of these reagents was required for the generation of unlabeled cyanide. This reduced the specific activity of the final labeled L-[5-^{11}C]ornithine. Other solvent systems (DMSO, DMSO/DMF (50/50), DMF, MeOH) did not give satisfactory results, nor did the use of $Li^{11}CN$[24] (trapping $H^{11}CN$ in LiOH). When the displacement reaction was carried out in acetonitrile without kryptofix 222 the radiochemical yield was not reproducible, ranging between 20-73%. Fortunately, a satisfactory radiochemical yield (85-90%) and

Table 1. Formation of unlabeled cyanide from acetonitrile in the presence of potassium hydroxide and kryptofix 222.[a]

Run	KOH (mmol)	CH_3CN (mL)	Kryptofix (mg)	Irradiation[b] (^{11}CN)	CN^- produced[c] (ug)
1.	-	0.4	-	no	<0.05
2.	0.01	-	-	no	<0.05
3.	0.006	0.4	-	no	<0.05
4.	0.006	0.4	20	no	8.0
5.	0.01	-	-	yes	1.7
6.	0.006	0.4	20	yes	10
7.	0.01	0.4	20	no	41
8.	0.01	0.15	20	no	22
9.	0.05	0.2	2	no	2

a. All samples were finally dissolved in 0.5 mL of H_2O and the cyanide concentration was determined.[17]
b. Fifteen microamp beam for 15 min.
c. Follow the procedure for the synthesis of L-[5-^{11}C]-ornithine. See experimental section for details.

specific activity of the first step displacement reaction could be obtained when less than 2 mg of kryptofix and 5 mg of bromide 3 were used (see Table 2). This would generate less than 2.0 ug of unlabeled cyanide ion which would account for 13 ug of ornithine mass, if there were 100% conversion.

Selective reduction of nitrile 4 to lactam 5 was performed in methanol by addition of solid cobalt chloride and sodium borohydride. The reaction proceeded smoothly and afforded a quantitative yield of 5. Acid hydrolysis of 5 gave L-[5-^{11}C]ornithine in 35-50% overall radiochemical yield. The specific activity of the solution containing L-[5-^{11}C]ornithine after separation by HPLC was >2.1 Ci/umol. Derivatization of the final product using Marfey's reagent confirmed that little racemization (< 5%) occurred under the reaction conditions.

Table 2. Yield of [^{11}C]nitrile 4 from the reaction[a] of γ-bromohomoserine 3 and potassium [^{11}C]cyanide.

Run	KOH	Kryptofix(mg)	3(mg)	Radiochemical Yield[b] of 4
1.	c	20	7	73%
2.	c	20	6	65%
3.	c	10	7	80%
4.	c	5	3	42%
5.	c	7	3	31%
6.	c	5	5	53%
7.	c	3	5	85-90%
8.	c	2	4	80-84%
9.	c	1	5	83-89%
10.	c	0	7	20-73%

a. Total reaction time was 6 min at 60° C. See experimental section for details.
b. Percentage of activity for product [^{11}C]nitrile 4 from TLC assay, decay not corrected.
c. 0.05 M, 0.2 mL.

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