Preparation of [^{11}C]-Thymidine and [^{11}C]-2'-Arabino-2'-Fluoro-β-5-Methyl-Uridine (FMAU) Using a Hollow Fiber Membrane Bioreactor System

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Summary: A series of hollow fiber membranes containing immobilized enzymes were prepared and used in the synthesis of ^{11}C-labelled nucleosides. ^{11}C-Formaldehyde was produced in an alcohol oxidase/catalase bioreactor and circulated through a thymidylate synthase bioreactor with an appropriate substrate to produce the corresponding ^{11}C-nucleotide. These labelled nucleotides were subsequently dephosphorylated in an alkaline phosphatase bioreactor. The bioreactor approach was amenable to hot-cell conditions and yielded ^{11}C-products in higher yield and shorter synthesis times than conventional chemical approaches.

Key Words: bioreactor, nucleoside, thymidylate synthase, hollow fiber membranes

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INTRODUCTION

Unlike the diagnosis of bacterial infections, the specific diagnosis of viral diseases is less developed (1-3). The method by which a viral disease is diagnosed depends on the stage of the disease. Soon after viral infection, culture techniques can be used to demonstrate the presence of an antigen, although serological tests are often used during convalescence or recovery (4). The latter type of test depends on the presence of detectable amounts of proteins (viral antigen). When considering virologic specimens on a cellular level, the test needs to distinguish between transfected and normal cells. One of the ways to distinguish between these cells is through the differential identification of enzymes which are only expressed in the transfected cytoplasm. An example of an enzyme which demonstrates this selectivity is viral thymidine kinase (5). Several nucleoside thymidine analogs in which this enzyme is the target are pharmacological active following their metabolic entrapment into infected cells.

Our investigation into novel diagnostic approaches prompted us to design a rapid method by which to introduce the positron-emitting radionuclide carbon-11 into the chemical structure of nucleoside drug analogs. The nucleoside described in this study is 2'-arabino-2'-fluoro-β-5-methyl uridine (FMAU) which has been shown to bind to thymidine kinase (6). ^{14}C-labelled FMAU is known to accumulate within herpes simplex infected neuronal tissue (7). Thus, a positron-emitting analog of this compound is expected to be useful in the diagnosis of the herpes virus ex vivo.

The ideal synthetic method for ^{11}C-labelled compounds should be rapid (due to the short physical half-life (20.4 min) of ^{11}C), specific (to facilitate the isolation of the desired chemical species), safe (to reduce radiation exposure in the hot cell) and technically feasible. A system that lends itself to fulfill most of these criteria is enzyme-based bioreactors (8, 9). Most enzymes have a rapid turnover and are very specific, while their attachment to bioreactor membrane supports facilitate separation (similar to molecular sieving or filtration) and can even enhance enzyme activity (10). A detriment to the use of enzymes for the synthesis of complex

molecules is the lack of ^{11}C-labelled substrate sources for bioreactors. Several bioreactors in tandem might therefore be required to synthesize a single radiopharmaceutical.

Previously, we have shown that bioreactor methodology has the potential to increase the ease and convenience of radiopharmaceutical synthesis (8,9) and many others have demonstrated the used of enzymes in the synthesis of positron labeled compounds (11,12). In this paper, we describe the use of a multicomponent bioreactor to produce ^{11}C-labelled thymidine and FMAU.

EXPERIMENTAL

2-Deoxyuridine, 2-fluoro-2-deoxy-1,3,5-tri-O-benzoyl-α-D-arabinofuranose and uracil were obtained from Sigma Chemicals (St. Louis, MO) along with the enzymes alcohol oxidase, catalase and alkaline phosphatase. All other chemicals were obtained from Aldrich Chemicals (Milwaukee, WI). TLC analysis was conducted on silica plates (2.5 x 7.5 cm; Whatman, Hillsboro, OR). ^{1}H and ^{13}C-NMR spectra were obtained on a Varian VXR-300 MHz spectrometer; spectra were run at 21°C in DMSO-d_6 against a tetramethysilane internal standard. Elemental analyses (C,H and N) were performed by Atlantic Microlabs (Norcross, GA).

Synthesis of 2-fluoro-2-deoxy-3,5-di-O-benzoyl-α-D-1-bromo-arabinofuranose (**2**)

Compounds (**2**) and (**4**) were prepared using a modification of the procedure described by Mansuri et al. (13). Two grams of 2-fluoro-2-deoxy-1,3,5-tri-O-benzoyl-α-D-arabinofuranose (**1**) were dissolved in dry dichloromethane (50 mL). Hydrogen bromide (80%) in acetic acid (1.25 mL) was slowly added to (**1**) under a nitrogen atmosphere and the mixture was stirred for 18 hours at room temperature. The solvent was removed under reduced pressure and the resulting solid was triturated in toluene and evaporated to a dry solid to yield (**2**).

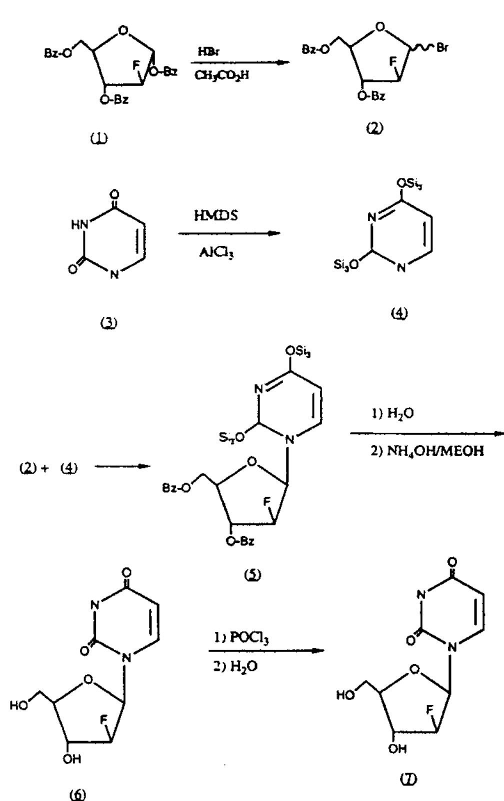

Figure 1. Synthesis of FAU-MP.

Synthesis of 2,4-bis-silylated uracil (4) Two hundred and fifty mg of uracil (3) were dispersed in a mixture of hexamethyl-disilazane (HMDS) and ammonium chloride (2 mg). This mixture was refluxed for 4 hours producing a clear solution which was reduced to a thick oil under vacuum by rotary evaporation. The product (4) was used without any further purification.

Synthesis of 1-β 2-4-bis silyl-uracil-2-arabino-fluoro-3-benzoyl-5-benzoyl-ribose (5) Compound (5) was prepared, using the procedure of Schinazi et al. (14) by dissolving 1.7 g of (2) with the product of the above reaction (4) in CH_2Cl_2 (30 mL) and refluxing for 24 hours. A solid (1.5 g) was obtained by solvent removal under reduced pressure and the product (5) was used without further purification.

Synthesis of 2'-arabino-2'-fluoro-β-uridine; FAU (6)

Compound (5) (1 g) was suspended in ammonium hydroxide/methanol 80:20 (70 mL) and stirred overnight. The solvent was removed under reduced pressure and the resulting oil added to water (25 mL). The aqueous phase extract was further diluted with water (100 mL) and extracted with n-BuOH/CH_2Cl_2 (1:9) (15 mL) three times. The solvent was removed under reduced pressure and 740 mg of a white powder was crystallized from ethanol. TLC analysis (mobile phase chloroform:methanol (9:1)) indicated complete absence of starting materials in the

reaction product. The ^{1}H-NMR spectrum of the product showed resonances of d 8.51 (s,1H, H-6), 6.16(dd 1H, H-1'), 5.16 (ddd 1H, H-2'), 4.26 (ddd 1H, H-3'), 3.85 (m 1H,H-4'), 3.71 (dd 1H-H-5), 3.59 (dd 1H, H-5) which are consistent with the proposed structure. A ^{13}C-NMR spectrum was also obtained to confirm stereochemistry. The resonances were (Hz) 12100.7 (C-4), 11323 (C-2), 10643 (C-6) 7200 (C-5), 6934 (C-2') 6150 and 6100 (C-1' and 3'), 5034 (C-4'), 4145 (C-5'). The purity of the product was established by elemental combustion analysis, and showed C, 43.9%, H, 4.9%, and N, 11.5% (theoretical values: C, 43.7 %; H, 4.8 % , N, 11.3 %).

Synthesis of FAU-MP (7)

FAU (5 mg) was dissolved in 3 mL of acetonitrile. Pyridine (0.1 mL) was added followed by the addition of water (50 mg). The reaction was cooled on ice followed by the addition of phosphoryl chloride (20 mg) with rapid stirring. The mixture was allowed to stir for one hour on ice followed by stirring for an additional 12 hours at 5°C. The reaction product was added to ethyl ether (20 mL) producing a white precipitate. The precipitate was collected by filtration and dried. Water (5 mL) was added to the precipitate and stirred for 30 minutes with mild heating (50°C) to facilitate the cleavage of the phosphorus-chlorine bond. At this point, the pH of the solution was adjusted to 8.0 with concentrated ammonium hydroxide. The freshly hydrolyzed nucleotide solution was loaded onto a 15 cm x 1 cm column packed with Biorad AG 1-X4 formate anion exchange resin, 200-400 mesh. The nucleotide (7) was eluted with a formic acid gradient (0-1M).

Preparation of Bioreactors

The scheme for attachment of enzymes to hollow fiber membrane solid supports is depicted in figure 2. Thymidylate synthase (TS), specific activity of 920 nmol/ min/ mg protein, was obtained from Biopure Company (Boston, MA). An aldehyde-containing hollow fiber membrane bioreactor obtained from Sepracor, (Marlborough, MA) had a residual volume of 0.4 mL and a membrane surface capable of binding up to 580 μg of protein / mL of residual volume. The cartridge was washed by circulating 100 mL of 0.03 M $NaHCO_3$, pH 7.4, at 2 mL / min. TS (890 mg) was added to 9 mL of a buffer consisting of 0.03 M $NaHCO_3$, pH 7.4 with 0.05 $MgCl_2$,

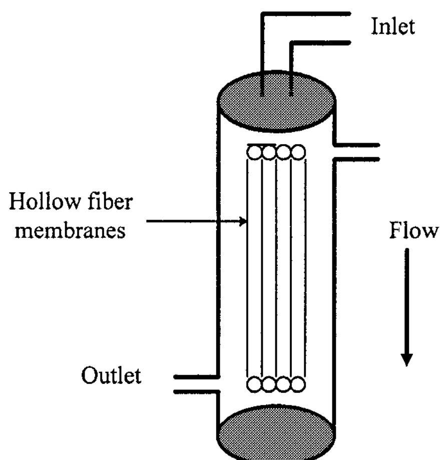

Figure 2. Schematic representation of the hollow fiber membrane cartridge used in the bioreactor synthesis of ^{11}C-nucleosides. The dimensions of the actual membrane cartridge are 10 x 2 x 2 cm.

and 3.5 mg dUMP (deoxyuridine monophosphate was added to the enzyme solution to protect key amino acids from becoming immobilized). The enzyme solution was recirculated (Rainin Rabbit Model pump) through the aldehyde module lumen to shell (1 mL/min) for one hour at room temperature. A sodium cyanoborohydride solution (0.05 M) was then circulated through the bioreactor (lumen to shell) at 1 mL/min for 1 hour to immobilize the enzyme. Ethanolamine (1.5 mL of a 1M solution) was subsequently circulated from lumen to shell at 1 mL/min for 30 min to mask unreacted aldehyde groups. The bioreactor column was finally washed with 100 mL of each of the following wash buffers at 2 mL/min lumen to shell: 0.03 M $NaHCO_3$ pH 7.4, Tris pH 8.0, and PBS with 0.1% azide pH 7.4 at 4°C.

A similar procedure was used for the alcohol oxidase/catalase (AO/CA) and alkaline phosphatase (AP) bioreactors. The AO/CA reactor was loaded with 100 units (30 U/mg) of AO and 50,000 units (40,000 U/mg) of CA. The AP bioreactor was loaded with 3000 U of enzyme (1500 U/mg).

Multistep Bioreactor Production of ^{11}C-Thymidine (**10**) *and ^{11}C-FMAU* (**11**)

The scheme for using three bioreactors for producing ^{11}C-labelled nucleosides is depicted in figure 3. ^{11}C-formaldehyde was generated from ^{11}C-methanol with the alcohol

X= H for dUMP (9) and thymidine (10)
X= F for FAU-MP (7) and FMAU (11)

Figure 3. Synthesis of ^{11}C-Thymidine and ^{11}C-FMAU

oxidase/catalase bioreactor column beginning with ^{11}C-methanol produced at TRIUMF in British Columbia as previously described (8). Briefly, ^{11}C-carbon dioxide was produced by bombardment of a nitrogen target with 15 MeV protons at 10 μA for 10 minutes. $^{11}CO_2$ was collected in a liquid nitrogen trap and subsequently bubbled through a solution of 0.1M lithium aluminum hydride in tetrahydrofuran (3 mL). After removal of the solvent, the remaining methanolic-LAH complex was broken with the addition of 300 μL of 0.1M HCl. The reaction vessel was heated and the methanol bubbled into 3 mL of a 0.1 M TRIS buffer (pH 7.8). This solution was circulated through the AO/CAT bioreactor for 5 min. The contents of the bioreactor (3 mL) were pumped into a holding chamber (using a peristaltic pump), and 1 mg of tetrahydrofolic acid was added. After one minute of stirring, a solution (0.5 mL) containing magnesium chloride (10 mM), 2-mercaptoethanol (8 mM) and 1 mM of either deoxyuridine monophosphate (dUMP, **9**) or FAU-MP (**7**) was added to the product (^{11}C-methylene tetrahydrofolic acid; **8**). The resulting solution was circulated (with a peristaltic pump) through the TS bioreactor for 10 minutes at a flow rate of 1 mL/min. Dephosphorylation of the resulting nucleotides to produce the nucleosides ^{11}C-thymidine (**10**) and ^{11}C-FMAU (**11**) was

accomplished by circulating the products of the TS bioreactor through the AP bioreactor for an additional 5 minutes (1 mL/min).

The radiochemical purity of the products was determined using a silica thin layer chromatography plate and a chloroform:methanol (9:1) mobile phase. The product of the bioreactor synthesis was co-chromatographed with the unlabelled thymidine and FMAU, as well as the original solution added to the TS bioreactor. After development, the plate was cut into sections and each section was assayed in a gamma scintillation counter. The overall reaction efficiency was calculated by comparing activity in the sections corresponding to the R_f value of the nucleoside and dividing by the total activity spotted on the TLC plate.

RESULTS AND DISCUSSION

Various established nucleoside chemistry procedures (15) were employed to design the synthetic pathway of FAU (6). The multistepped chemical synthesis (figure 1) of this nucleoside resulted in a product yield of 64% based on compound (1). ^{13}C-NMR spectral analysis was used to confirm the anomeric position of the sugar relative to the nucleoside base. Whereas the α-anomer of nucleoside sugar normally have C-1'-F coupling constants ranging between 36.2 Hz and 35.5 Hz, the corresponding C-1'-F coupling constants for the β-anomer are in the 17.2 Hz to 16.3 Hz range (16). The C-1'-F coupling constant of FAU was found to be 16.6 Hz, which is consistent with the proposed structure of the β -anomer.

The conversion of the nucleoside FAU (6) to the nucleotide FAU-MP (7) is also illustrated in figure 1. Phosphoryl chloride has been extensively used to phosphorylate both protected and unprotected nucleosides. Selective phosphorylation at the 5'-position of unprotected nucleosides can be achieved when phosphoryl chloride in a trialkyl phosphate (e.g. triethylphosphate is used (17). We decided to use a modified agent for in situ phosphorylation, since it has been reported to be superior in phosphorylating non-traditional nucleosides (18). A 60% yield for the conversion of FAU to FAU-MP was achieved when following this procedure.

The immobilization of enzymes is an important part of extending their applications while conserving of enzymatic integrity. Upon immobilization, enzymes can catalyze continuous flow reactions. These immobilized enzyme modalities have several advantages such as being able to be used repetitively and rendering enzyme-free products. Since immobilization often stabilizes enzymes, they retain their biological activity longer (10). Immobilization may often result in steric changes to the enzyme, which may alter the chemical and physical properties of the system, thus enhancing its reaction rate, or allowing the enzyme to bind foreign substrates. Membrane supports are by definition more versatile than conventional particulate enzyme support media. Microporous membranes are of particular interest since they can be transformed into catalytically active systems (bioreactors) through the attachment of enzymes to the pore surfaces. The internal structures of these membranes are accessible to fluid via the convective passage of liquid through the membranes, and by molecular diffusion. As a result, surface processes that are normally restricted to diffusion are no longer subject to this energy-independent mode of transport when fluid is forced through the membrane by virtue of transmembrane pressure differences (10).

An aldehyde membrane support was used to immobilize the specific enzymes onto membranes through formation of Schiff bases (presumably with terminal amino groups of lysine moieties) followed by reduction. Unreacted aldehydes were blocked by treatment with excess ethanolamine. Each bioreactor (bearing a different enzyme) acted as a separate functional unit. The biosynthetic process of each bioreactor, alcohol oxidase/catalase [AO/CA], thymidylate synthase [TS] or alkaline phosphatase [AP] is outlined in Figure 3. The AO/CA bioreactor utilized ^{11}C-methanol as its substrate yielding ^{11}C-formaldehyde. The circulation time through this bioreactor was 5 minutes, which was previously shown to be adequate to convert up to 95% of ^{11}C-methanol to ^{11}C-formaldehyde (8). ^{11}C-formaldehyde was separately reacted with tetrahydrofolic acid, to reduce the process of hemithioacetal formation between thiols and formaldehyde (19).

The substrates tested in the TS hollow fiber modules were 2'-deoxyuridine monophosphate (dUMP (9); the natural substrate), and 2'-deoxy-2'-fluoro-arabino-uridine monophosphate (FAU-MP (7)). The reaction was conducted in the presence of mercaptoethanol and magnesium both substrates were converted by the TS enzyme to their respective products. The TS bioreactor was shown to be the rate-limiting step in the biosynthetic route we investigated, although the time the substrate spent in this bioreactor was kept to a maximum of 10 minutes. This short time did not allow the complete conversion of either substrate to its corresponding product, but was necessary to reduce the amount of ^{11}C-activity loss due to physical decay.

The final component in the series of the three bioreactors contained AP which, when immobilized, has been demonstrated to rapidly dephosphorylate nucleotides (20). This enzyme is non-specific, and hydrolyzes native nucleotides and their analogs efficiently. When dUMP and FAU-MP were employed as substrates for the TS bioreactor, and the products (the monophosphates of ^{11}C-thymidine and ^{11}C-FMAU, respectively), were passed through the AP bioreactor, the desired products ^{11}C-thymidine (**10**) and ^{11}C-FMAU (**11**) were obtained. The native substrate for TS (dUMP) yielded ^{11}C-thymidine in a 13% radiochemical yield after a total synthesis time of 21 min from the end of bombardment (producing $^{11}CO_2$). FAU-MP has been postulated to be another substrate for TS, and this was corroborated by our results (21), although the overall radiochemical yield was only 4% (average of 3 experimental runs).

These experiments were designed to identify a practical application of bioreactor systems, and we consequently did not fully optimize the reaction conditions. In each bioreactor, an excess of protein (enzyme) was added to the hollow fiber matrix to ensure maximum attachment to the surface. The residence time of substrate through each reactor was somewhat arbitrarily set to comply with limitations due to the physical half-life of ^{11}C. The use of the enzyme TS for the introduction of ^{11}C is a rapid method to produce ^{11}C-thymidine and its analogs (22). This technique has several advantages over other methods reported in the literature to accomplish the same. Most of the previously reported methods are chemically based, usually involving a protected base which has a modified C-5 position with a good leaving group. These methods

introduce the ^{11}C-radionuclide by various means, and the resulting compound is then deprotected. Typically, yields obtained in the chemical synthesis of ^{11}C-thymidine are in the range of 2% to 5% within a period of 50 to 120 minutes (23, 24). Most of the methods for synthesizing ^{11}C-thymidine suffer from low yields and employ expensive precursor molecules.

The use of membrane bound enzymes eliminates the requirement for protected nucleoside bases. The monophosphate ester of uridine and its analogs can be easily prepared. The enzymatic method accurately introduces the label at the C-5 position of the pyrimidine base. Once functional, the bioreactor could be used to circulate many substrates through it in order to produce the corresponding ^{11}C-thymidine monophosphate analog. The first report for using a thymidylate synthase preparation was by Christman et al (22). In that investigation, ^{11}C-thymidine was produced with a yield of less than 2% and a reaction time exceeding 2 hours (starting with the production of 11C-formaldehyde). Nonetheless, this was a pioneering study since it demonstrated the potential of enzymes in the production of radiopharmaceuticals.

To prove the validity of our concept and the subsequent reactor design, we prepared a ^{11}C-labelled thymidine analog (2'-fluoro-2'-arabino-5-methyl-uridine; FMAU) as well as ^{11}C-thymidine itself. The latter has been used increasingly over the last few years in the detection of neoplastic disease, whereas ^{14}C-FMAU has been shown to have high focal uptake in herpes simplex encephalitis (7). This disease does not have a simple diagnostic test yet, and it was thought that ^{11}C-FMAU might be a good candidate for further clinical investigation in nuclear medicine.

In conclusion, the bioreactor approach using hollow fiber membranes proved to be a feasible approach for the synthesis of ^{11}C-nucleosides. This approach has the advantage in that it can be fully automated, and because it is based on convective flow, is not restricted by rate-limiting diffusion of the substrate to the enzyme.

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