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Research Article

The first enzymatic method for C–^{18}F bond formation: the synthesis of 5′-[^{18}F]-fluoro-5′-deoxyadenosine for imaging with PET

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Summary

The use of the key enzyme involved in carbon–fluorine bond formation in *Streptomyces cattleya* catalysing the formation of 5′-fluoro-5′-deoxyadenosine (5′-FDA) from fluoride ion and *S*-adenosyl-L-methionine (SAM) was explored for its potential application in fluorine-18 labelling of the adenosine derivative. Enzymatic radiolabelling of [^{18}F]-5′-FDA was successfully carried out starting from SAM and [^{18}F]HF when the concentration of the enzyme preparation was increased from sub-mg/ml values to mg/ml values. The purity of the enzyme had no measurable effect on the radiochemical yield of the reaction and the radiochemical purity of [^{18}F]-5′-FDA. Copyright © 2003 John Wiley & Sons, Ltd.

Key Words: fluorinase; enzymatic radiolabelling; fluorine-18; [^{18}F]-5′-FDA

Introduction

Positron emission tomography (PET) is a non-invasive imaging technique that offers the highest spatial and temporal resolution of all

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nuclear medicine imaging modalities and can allow quantitation of tracer concentrations of labelled compounds in living tissues.[1] The technique involves the use of radiotracers labelled with positron emitting radionuclides that are designed to have *in vivo* properties which permit measurement of parameters regarding the physiology or biochemistry of a variety of living tissues.

The most commonly used positron emitting (PET) radionuclides are ^{11}C, ^{18}F, ^{15}O and ^{13}N, which are accelerator produced, and have half-lifes of 20, 110, 2 and 10 min, respectively. Due to their short half-lives ^{11}C, ^{15}O and ^{13}N labelled radiopharmaceuticals have to be synthesized in close proximity to a PET scanner. The use of ^{18}F offers a number of advantages over ^{11}C as a PET radionuclide, primarily because of its longer half-life. From a radiochemistry and radiopharmacy perspective, labelling with ^{18}F allows substantially more time for radiochemical synthesis, purification and quality control of radiopharmaceuticals for use in *in vivo* experiments. ^{18}F-labelled radiopharmaceuticals can be produced in quantities sufficient for the formulation of multiple doses from a single synthesis and for remote distribution to PET scanners at locations without on-site cyclotron facilities.

New methods for labelling compounds with fluorine-18 provide the possibility of developing new radiolabelled PET probes for *in vivo* imaging. The use of [^{18}F]fluoride ion as the synthetic precursor in the synthesis of fluorine-18 labelled radiopharmaceuticals has been extensively studied and successfully applied to the production of important PET *in vivo* probes.[2] It has been reported that adenosine derivatives labelled with fluorine-18 could have potential for the imaging of tumors. Indeed, 2′-fluoro-2′-deoxyadenosine labelled with fluorine-18 showed promising results in the evaluation of tumor cell proliferation.[3] Recent studies with another adenosine derivative, [^{18}F]-5′-fluoro-5′-deoxyadenosine have highlighted the difficulty of introducing [^{18}F]fluoride in the 5′-position when a series of 5′-halo and 5′-sulfonic acid alkyl and aryl esters failed to produce the expected radiolabelled adenosine derivatives in sufficient yield.[4] Indeed nucleophilic displacement of halogens in the 5′-position with [^{18}F]fluoride yielded the desired radiolabelled product, however radiochemical yields were found to be extremely low ($\leqslant 1\%$) suggesting that additional work should be done to find a more suitable production method. More recently further studies performed by the same group confirmed the difficulty of radiofluorination in the 5′-position when a series 5′-sulfonic acid alkyl and aryl esters failed to produce the expected radiolabelled adenosine derivatives in good yield

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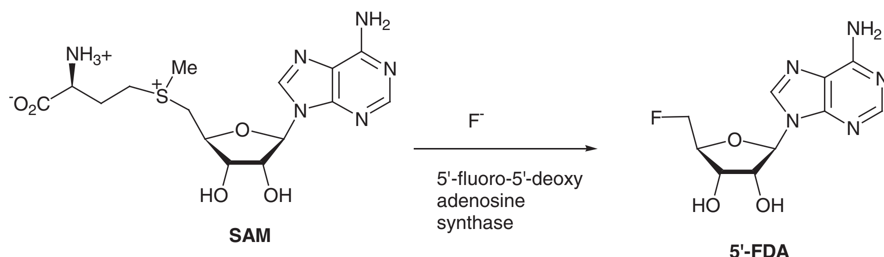
under commonly used nucleophilic substitution reaction conditions with [^{18}F]fluoride-ion.[5]

Organo-fluorine compounds are not abundant in nature and as a consequence there are very few naturally occurring enzymes involved in carbon–fluorine bond formation. *Streptomyces cattleya* is unusual in its capacity to generate both fluoroacetate and 4-fluorothreonine as secondary metabolites. Recently O'Hagan *et al.* have reported that the key enzyme involved in C–F bond formation in *Streptomyces cattleya* catalyses the formation of 5′-fluoro-5′-deoxyadenosine from fluoride ion and *S*-adenosyl-L-methionine (SAM) (Scheme 1).[6] This enzyme has recently been purified to homogeneity and the conversion of fluoride ion and SAM to 5′-FDA can be monitored conveniently by either HPLC or by ^{19}F-NMR.[9]



Scheme 1. The fluorination enzyme from *Streptomyces cattleya* mediates the conversion of *S*-adenosyl-L-methionine (SAM) to 5′-fluoro-5′-deoxyadenosine

With this novel biocatalyst available it became very attractive to explore its potential application for fluorine-18 labelling of the adenosine derivative. Successful examples of synthetic routes for PET radiopharmaceuticals combining conventional organic reactions with enzyme catalyzed reactions can be found in the literature. However the small number of biocatalysts available almost exclusively limits their use for the production of carbon-11 labelled amino acids.[7]

In this present communication we report, to our knowledge the first enzymatic formation of a C–^{18}F bond as exemplified by the radiolabelling of 5′-[^{18}F]fluoro-5′-deoxyadenosine ([^{18}F]-5′-FDA) with fluorine-18 by incubating a protein extract from *S. cattleya* with [^{18}F]HF and SAM.

Results and discussion

The synthetic route for the preparation of [^{18}F]-5′-FDA is shown in Scheme 2.

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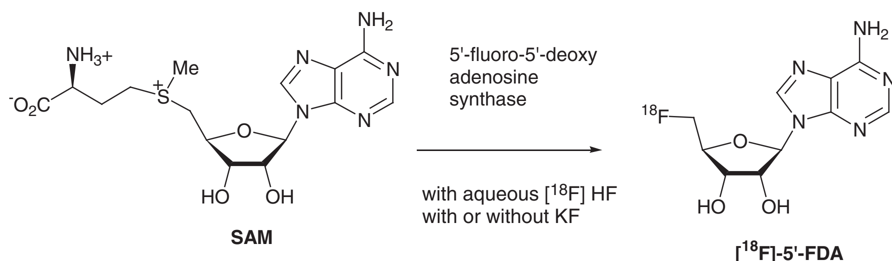

Scheme 2. Enzymatic radiolabelling of 5′-[^{18}F]fluoro-5′-deoxyadenosine

Table 1 shows reaction conditions used for the enzymatic radiolabelling assays. Several enzymatic preparations obtained by different methods of final purification (ion exchange fraction (F_B and F_D) vs gel filtration fraction (F_C)) and with different levels of purity: pure (F_D) vs partially purified (F_C) were tested. Both F_B and F_C were further concentrated to obtain two additional enzymatic fractions F'_B and F'_C containing 4 mg/ml of proteins. Each enzymatic radiolabelling assay was performed under no-carrier-added and carrier-added (KF, 0.5 M) conditions.

In a typical no-carrier-added experiment, the enzyme was incubated with 50 MBq of aqueous [^{18}F]HF (in a solution of [^{18}O]H_2O), and SAM at 40°C at pH 7.0. When carrier-added reaction conditions were investigated, KF was added to the reaction mixture as a 0.5 M solution in water immediately after the incubation with [^{18}F]-fluoride had started. Samples from the reaction mixture were collected, loaded onto the anion exchange column and eluted with water to separate unreacted fluoride from more lipophilic species. After the solid phase purification, aliquots not retained on the column were analysed using a high-

Table 1. A summary of the experiments and outcomes

Fraction	Protein concentration (in mg/ml)	KF (0.5 M)	[^{18}F]-5′-FDA	Run no.
F_C	0.4	+	–	1
		–	–	2
F'_C	4	+	+	3
		–	+	4
F_B	0.4	+	–	5
		–	–	6
F'_B	4	+	+	7
		–	+	8
F_D	0.2	+	–	9
		–	–	10

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performance liquid chromatography (HPLC) system coupled to a radioactivity detector. Radioactive products retained on the column were finally eluted with sodium carbonate and analysed by HPLC.

Assays using sub mg/ml concentrations of the enzyme preparation failed to give measurable amounts of radioactivity following elution of the ion exchange column with water. The radioactivity was entirely retained on the column and recovered after elution with aqueous sodium carbonate. The eluted material had a retention time (R_t) of 3.6 min on the reverse phase HPLC column which corresponds to the dead volume of the column and is consistent with either unreacted [^{18}F]fluoride or polar radiolabelled by-products. Similar results were observed when potassium fluoride (0.5 M, 10 μl) was added to the reaction mixture (runs 1, 5 and 9). Increasing the purity of the enzyme from partially purified (runs 1 and 2) to homogeneous (runs 9 and 10) had no measurable effect on the rate of conversion of SAM to [^{18}F]-5′-FDA.

Further concentration of the enzymatic preparations F_C and F_B using centrifugal concentrators gave two new fractions F'_C and F'_B with protein concentrations of 4 mg/ml. Experiments were carried out with the two new fractions using the same protocol as for runs 3, 4, 7 and 8. After a reaction time of 5 h, aliquots were collected and treated as described above. Elution of the ion exchange column with water gave fractions containing measurable amount of radioactivity. Analysis of the collected fractions by radio-HPLC showed two radioactive peaks. The retention time of the first radioactive signal eluting at 3.6 min on the reverse-phase column coincided with the dead volume of the column. The second radioactive product detected was the only radioactive non-polar material to elute off the reverse phase HPLC column with a retention time of 11.2 min (Figure 1). When aliquots were co-injected with an authentic sample of non-radiolabelled 5′-FDA, the UV signal of the reference compounds co-eluted with the radioactive peak.

Experiments have given [^{18}F]-5′-FDA in ca. 1% radiochemical yield in ca. 5 h from EOB decay corrected. The results suggest that although the enzymatic radiolabelling of [^{18}F]-5′-FDA cannot be yet regarded as a preferred option for its production and further studies to optimize the yield and the reaction time are needed we have established the feasibility of enzymatic radiofluorination with ^{18}F for the first time. In an effort to improve the yield and reaction time we have performed additional assays at higher temperature using fractions F_C and F_B. An increase of temperature from 40 to 80°C has failed to demonstrate conversion of

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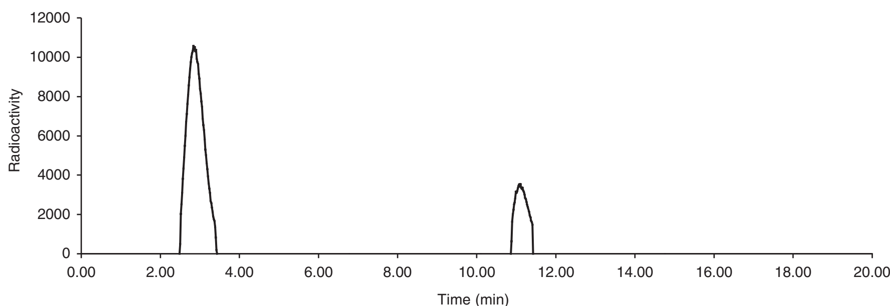

Figure 1. HPLC chromatogram of reaction mixture aliquots: 5–20% MeCN in 50 mM KH_2PO_4 and flow 1 ml/min

SAM to [^{18}F]-5′-FDA. Although the enzyme is very stable at its optimum temperature (ca. 40°C), some degradation is expected at 80°C. As demonstrated by the results generated using fractions F'_C and F'_B, enzyme concentration is a key factor influencing the radiochemical yield of the reaction. Therefore any further increase of the protein concentration could lead to better yields. The amount of native fluorination enzyme available from Streptomyces cattleya is limited; however the fact that the enzyme is from a class of bacterium in which the molecular biology is well developed offers biotechnological prospects for improving the utility of this approach. Indeed work has started to clone and over-express the fluorinase which will be used in future radiolabelling experiments to optimize radiochemical yields.

An alternative strategy to the enzymatic pathway would be to reinvestigate the nucleophilic radiofluorination of [^{18}F]-5′-FDA using different fluorination agents such as tetrabutylammonium fluoride (nBu$_4$NF) which has been widely used in fluorine-18 labelling reactions. Indeed the synthetic preparation of our 5′-FDA standard was achieved by treatment of 2′-*O*,3′-*O*-isopropylidene-5′-*p*-tosyladenosine with nBu$_4$NF followed by hydrolysis with dilute sulphuric acid.[6] Clearly the same strategy could be used for the preparation of [^{18}F]-5′-FDA starting from the same precursor and using [^{18}F]nBu$_4$NF as the radiofluoride source commonly prepared by reacting nBu$_4$NOH with the cyclotron target content.[8]

In conclusion, an enzymatic radiolabelling method for the production of [^{18}F]-5′-FDA has been successfully developed starting from SAM and [^{18}F]HF. To our knowledge this work is the first to report the use of an enzyme to mediate the reaction between the substrate and a radionuclide and is clearly the first example of incorporating ^{18}F fluoride into

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an organic product using an enzymatic method. With the help of newly isolated biocatalysts mediating carbon–fluorine bond formation we are now extending the range and versatility of the chemistry using short-lived isotopes. Preliminary experiments indicate that the approach described here is suitable for use with fluorine-18 but will need further developmental work to optimize the radiochemical yield and the time aspect of the rapid-labelling synthesis. The ability to over-express this bacterial enzyme offers a real biotechnological prospect for developing the utility of this novel approach to ^{18}F labelling.

Experimental section

Purification of 5′-fluorodeoxyadenosine synthase[9]

The enzyme was partially purified by adding solid $(NH_4)_2SO_4$ to cell free extracts until a 45% saturation and removing the precipitate by centrifugation. The supernatant was adjusted to 60% saturation with solid $(NH_4)_2SO_4$ and the precipitate was collected after centrifugation. For the partially purified fraction F_B (Figure 2), the pellet was then resuspended in Tris–HCl buffer (50 mM, pH = 7.8) and passed through a desalting column (HiTrap™ desalting, 5 ml, Amersham Pharmacia), which was equilibrated and eluted with the same buffer. The active fractions were pooled and subjected to a strong anion exchange column (10 ml Source 15 Q, Amersham Pharmacia) at 2 ml/min, with a linear gradient of 0–0.3 M KCl in Tris–HCl over 80 ml. The active fractions were termed as F_B (0.4 mg/ml). For an alternative preparation route to generate the fractions F_C and F_D (Figure 2), the $(NH_4)_2SO_4$ fraction (F_A) was then resuspended in Tris–HCl buffer (50 mM, pH = 7.8) with 1 M $(NH_4)_2SO_4$, and the protein was eluted from a phenyl sepharose HP (Amersham Pharmacia Biotech) column at 2 ml/min using an AKTA Prime FPLC system (Amersham Pharmacia Biotech) (First 40 ml Tris–HCl buffer with 1 M $(NH_4)_2SO_4$ and then a linear gradient from 1 to 0 M $(NH_4)_2SO_4$ in Tris–HCl buffer (50 mM, pH = 7.8) over the final volume of 80 ml). The active fractions were concentrated (1.5 ml) using a 10 kDa MACROSEP (Pall Folton) Centrifugal concentrator and the solution was applied to a HiLoad 16/60 Superdex 200 gel filtration column (Amersham Pharmacia biotech) with Tris–HCl buffer (50 mM, pH = 7.8). The fractions containing fluorination activity (F_C, 0.4 mg/ml) were pooled and subjected to a 10 ml Source 15Q strong anion exchange

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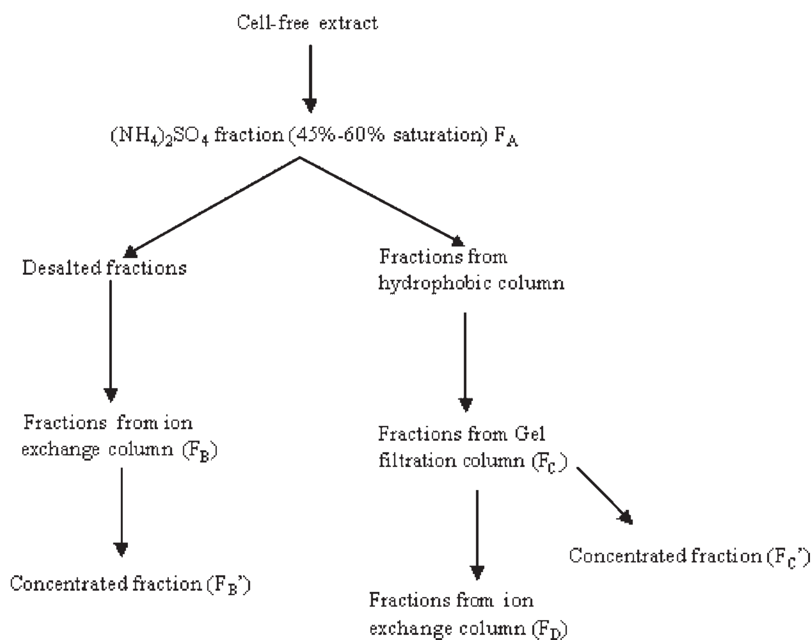

Figure 2. Overview of the purification steps for 5′-fluoro-5′-deoxyadenosine synthase from *S. cattleya*

column (Amersham Pharmacia biotech) at 2 ml/min, with a linear gradient of 0–0.3 M KCl in Tris–HCl over 80 ml. The active fractions were collected (8 ml, 0.2 mg/ml) to give fraction F_D. To obtain fractions F_B' and F_C', F_B and F_C (1 ml) were concentrated using 10 kDa MACROSEP (Pall Folton) Centrifugal concentrator (3000 g for 15 min). All purification steps were conducted at 4°C.

Enzymatic radiolabelling assays: A reaction mixture containing F_{IV} (100 μl), SAM 20 mM (20 μl), and $[^{18}F]HF$(50 MBq in ^{18}O-water, 75 μl) was incubated at 40°C. After 5 h, the reaction mixture was applied to the top of the packing bed of an anion exchange column (Alltech, SAX, 50 mg) conditioned with 10 ml of deionized water and 10 ml of air). $[^{18}F]$-5′-FDA was washed off the column with deionized water to produce an aqueous fraction containing $[^{18}F]$-5′-FDA. The radioactive fraction was loaded onto a reverse phase Hypersyl H5ODS 25 cm × 4.6 mm column (Hichrom) and chromatographed at 1 ml/min by linear gradient elution from a starting mobile phase of 50 mM KH_2PO_4 and acetonitrile (95:5 v/v) to a final mobile phase consisting of 50 mM KH_2PO_4 and acetonitrile (80:20 v/v) within 20 min. The UV

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trace was monitored at 260 nm (Beckman Coulter, System Gold 168 Detector) and radioactivity signal was recorded and analysed using a Packard Flow Scintillation Analyzer.

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References

1. Goodman MM. Automated Synthesis of Radiotracers for Positron Emission Tomography Applications. In *Clinical Positron Emission Tomography (PET)*, Hubner KF, Buonocore E, Collmann J, Kabalka GW (eds). Mosby-Year Book, Inc.: St. Louis, MO, 1991; 110–122.
2. Kilbourn MR. *Fluorine-18 labeling of Radiopharmaceuticals*, National Academy Press: Washington, DC, 1990.
3. Kim CG, Yang DJ, Kim EE, Chrif A, Kuang LR, Li C, Tansey W, Liu CW, Li SC, Wallace S, Podoloff DA. *J Pharmaceutical Sci* 1996; **85**: 339–344.
4. Lehel Sz, Horvath G, Boros I, Mikecz P, Marian T, Tron L. *J Radioanalyt Nucl Chem* 2000; **245**: 399–401.
5. Lehel Sz, Horvath G, Boros I, Marian T, Tron L. *J Radioanalyt Nucl Chem* 2002; **251**: 413–416.
6. O'Hagan D, Schaffrath C, Cobb SL, Hamilton JTG, Murphy CD. *Nature* 2002; **416**: 279.
7. Langstrom B, Antoni G, Bjurling P, Fasth KJ, Gee AD, Nagran K, Ulin J. *Acta Radiol* Suppl 1990; **374**: 147–151.
8. Martarello L, Greenamyre JT, Goodman MM. *J Label Compd Radiopharm* 1999; **42**: 1039–1051.
9. Schaffrath C, Deng H, O'Hagan D. FEBS Letters 2003; **547**: 111–114.

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