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The identification of 5′-fluoro-5-deoxyinosine as a shunt product in cell free extracts of *Streptomyces cattleya*

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Abstract

5′-Fluoro-5′-deoxyinosine (5′-FDI) is identified as an adventitious side product that accumulates in cell free incubations of SAM and fluoride ion in *Streptomyces cattleya*. 5′-FDI was identified by a combination of isotopic labelling studies and co-synthesis studies as well as enzymatic degradation. Although it is an efficiently generated end product of the cell free incubations, 5′-FDI is not a biosynthetic intermediate and it does not accumulate as a fluorometabolite with fluoroacetate and 4-fluorothreonine in whole cell incubations of *S. cattleya*. Clearly the purine deaminase which converts 5′-fluoro-5′-deoxyadenosine (5′-FDA) to 5′-FDI in the cell free extract does not come into contact with 5′-FDA in whole cells, suggesting some level of compartmentalisation in cells of *S. cattleya*. The biotransformation of 5′-FDI from fluoride ion extends the range of organofluorine products, beyond biosynthetic intermediates, that can be generated by this system, for applications such as enzymatic labelling with fluorine-18 for positron emission tomography applications.

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1. Introduction

Streptomyces cattleya has an unusual capacity in its ability to biosynthesis organofluorine compounds from inorganic fluoride. The bacterium forms the toxin fluoroacetate **1** and the antibiotic 4-fluorothreonine **2**, and these remain one of a very small group of naturally occurring fluorometabolites, particularly from bacteria [1]. The only other bacterial fluorometabolite identified so far is the antibiotic nucleocidin **3** which was isolated from *Streptomyces calvus* [2].

The biosynthetic pathway in *S. cattleya* to fluoroacetate **1** and 4-fluorothreonine **2** (Fig. 1) has been investigated in some detail and several of the enzymes including the fluorination enzyme have been purified and characterised [3]. The fluorination enzyme [4,5] mediates a reaction between *S*-adenosyl-L-methionine (SAM) **4** and fluoride to generate 5′-fluoro-5-deoxyadenosine (5′-FDA) **5**.

5′-FDA **5** is acted on by a purine nucleotide phosphorylase (PNP) to generate 5-fluoro-5-deoxyribose-1-phosphate **6** [6]. Fluoroacetaldehyde **8** has been shown to be a common precursor to both fluoroacetate **1** and 4-fluorothreonine **2** [7]. The enzymes and intermediates between 5-fluoro-5-deoxyribose-1-phosphate **6** and fluoroacetaldehyde **8** remain to be characterised however the evidence suggests an isomerisation of **6** to 5-fluoro-5-deoxyribulose-1-phosphate **7** followed by a retroaldol reaction mediated by a putative aldolase, to generate fluoroacetaldehyde **8**. Such a biochemical conversion has precedent in an analogous pathway in primary metabolism, which involves the conversion of 5-thiomethyl-5-deoxyribose phosphate to 5-thiomethyl-5-deoxyribulose phosphate [8]. A pyridoxal phosphate (PLP) dependent transaldolase has been purified from *S. cattleya* which mediates a reaction between L-threonine and fluoroacetaldehyde **8** to generate 4-fluorothreonine **2** and acetaldehyde [9]. Fluoroacetaldehyde **8** is also efficiently oxidised to fluoroacetate **1** by an NAD dependent alcohol dehydrogenase, which has been isolated and purified from *S. cattleya* [10]. In this paper, we describe the capacity of cell free extracts of *S. cattleya* to efficiently convert 5′-FDA **5** to 5′-fluoro-5′-deoxyinosine (5′-FDI) **9**, in a reaction which leads to the accumulation of this shunt product. It is not clear why this reaction is so prevalent in the cell free extract particularly as 5′-FDI **9** is never apparent in whole cell incubations of *S. cattleya*.

2. Materials and methods

2.1. General methods

NMR spectra were recorded in $CDCl_3$ or d_6-DMSO for preparative sample analyses and in D_2O for biological analyses on a Bruker AC 300 spectrometer. Observation of fluoride metabolism in cell free extracts of *S. cattleya* by ^{19}F NMR

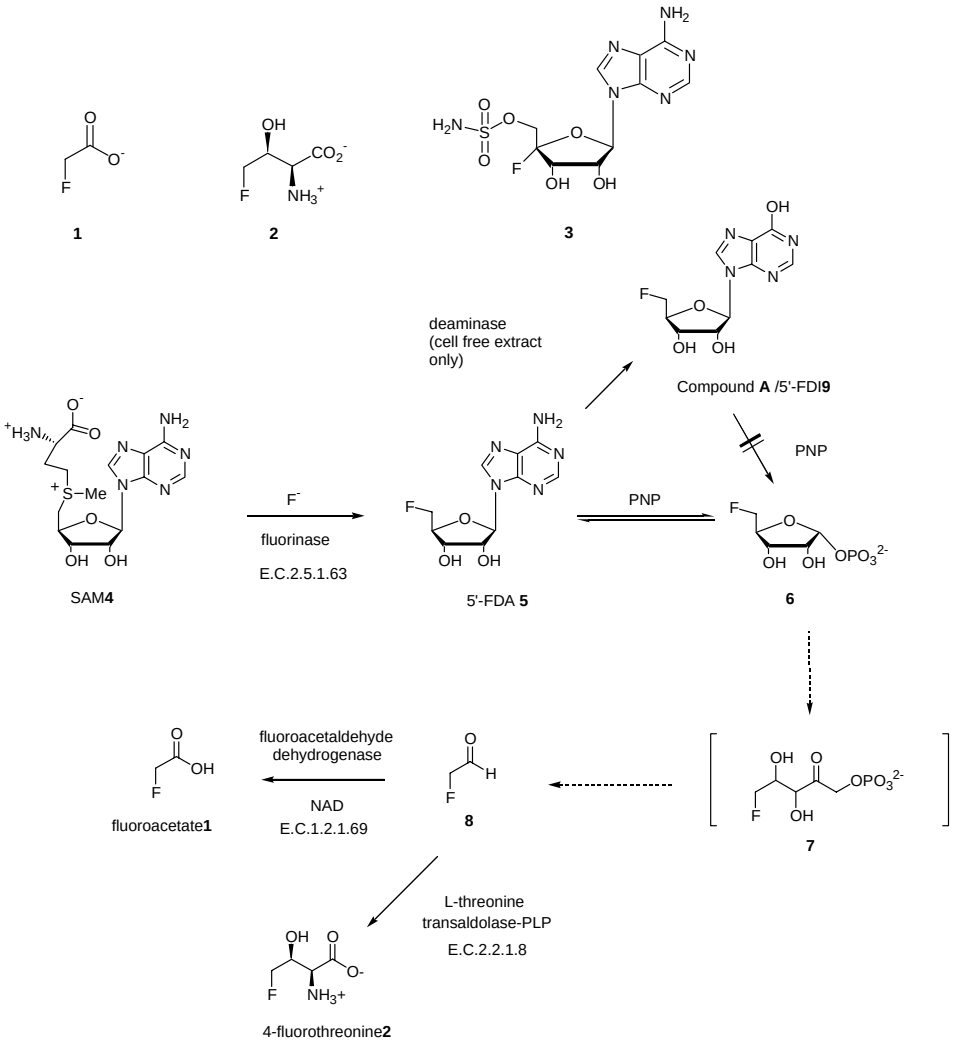

Fig. 1. Biosynthetic pathway to the fluorometabolites **1** and **2** in *S. cattleya* indicating intermediates, and the enzymes purified to date. In cell free extracts only, 5′-FDA **5** is converted to 5′-FDI **9** by the action of a deaminase. 5′-FDI **9** is not a substrate for the purine nucleotide phosphorylase (PNP).

was carried out using a Varian Inova 500 MHz spectrometer. Chemical shifts and coupling constants are reported in ppm and Hz, respectively. 5′-FDA was prepared as previously described [11]. GC–MS analyses were recorded on an Agilent 6890 gas chromatograph connected to an Agilent 5973 mass-selective detector using methods as previously described [12]. *N*-Methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) and [^{18}O]-water (97 atom %) were purchased from the Aldrich Chemical Company. Adenosine deaminase (EC 3.5.4.4) from calf intestinal mucosa and 5′-ade-

nylic acid deaminase (EC 3.5.4.6) from *Aspergillus* sp were purchased from the Sigma Chemical Company.

2.2. Microbial growth and cell free extract preparation

All microbial work was carried out under sterile conditions in a Gallenkamp laminar flowhood and glassware was autoclaved in the routine manner. *S. cattleya* NRRL 8057 was obtained from the United States Department of Agriculture, Agricultural Research Service, Mid West National Regional Research Laboratories, Peroria, Illinois, USA. Cultures were maintained on agar slants containing soyabean flour (2% w/v), mannitol (2% w/v), agar (1.5% w/v) and tap water, and grown at 28 °C until sporulation was detected. The resultant static cultures were stored at 4 °C in an incubator. *S. cattleya* cells, competent in fluorometabolite biosynthesis, were grown from these slants and harvested after 6–8 days as previously described [13]. Cell free extracts were prepared after centrifugation (25 min, 14,000 rpm, 25 °C) of the harvested cells. Typically, the cell pellet was washed three times with KH_2PO_4 buffer (50 mM, pH 7.0). The final washed cell pellet was stored at −80 °C until required. Then frozen cells (0.2 g) were resuspended in Tris buffer (1 ml, 50 mM, pH 7.8) and the cells disrupted by ultrasonication (60% duty cycle for 30–60 s). Cell debris was removed by centrifugation (20,000 rpm, 25 min) and the resultant clear supernatant was used as the cell free extract for incubation experiments.

2.3. Cell free preparation of 5′-FDI from SAM and fluoride

The cell free extract (20 ml) prepared in Section 2.2 was supplemented with SAM (0.4 mM), KF (10 mM), and either with our without iodoacetamide (10 mM). The cell free extract was then divided into aliquots (20 × 1 ml) and the aliquots were incubated at 37 °C for 24 h. At the end of the incubation period, protein was precipitated by heating the vial to 90 °C for 3 min and the protein was then removed by centrifugation. The combined supernatants were lyophilised and the resultant powder was dissolved in D_2O (3 ml) for ^{19}F NMR analysis, or in water (3 ml) for HPLC purification of 5′-FDI. For HPLC, an aliquot of the solution was injected (10 × 300 μl) onto a reverse phase C-18 column (250 × 10 mm, Phenomenex) equilibrated with solvent (50 mM KH_2PO_4/acetonitrile, 95:5). The column was then eluted isocratically (20 min at 5 ml/min) with the same solvent monitoring at 260 nm (UV detection). 5′-FDI eluted from the column after 7.5 min. Repeated runs and collection resulted in a sample of 5′-FDI which had identical NMR and MS data to the reference compound prepared by biotransformation (see Section 2.3).

2.4. Oxygen-18 labelling of 5′-FDI by cell free biotransformation

Isotopically labelled (97 atom %)-[^{18}O]-water (0.25 ml) was added to an aliquot (1 ml) of the cell free extract prepared in Section 2.2. The extract was then supplemented with SAM (0.4 mM), KF (10 mM), and iodoacetamide (10 mM) and was incubated for 18 h at 37 °C. The reaction was monitored by HPLC and protein was

then precipitated by heating (90 °C/3 min). The supernatant was lyophilised and the sample treated with a large excess of MSTFA heating at 100 °C for 60 min. The sample was then analysed by GC–MS with the mass spectrometer operating in the chemical ionization mode with methane as the reagent gas, under the following conditions. Column HP Ultra 1 12 m × 0.2 mm × 0.33 μm, with a 1 μl splitless injection. The GC program started at 100 °C and was held for 1 min then the temperature was ramped at 10 °C/min to 300 °C. The mass spectrometer was operated in the selected ion monitoring mode using ions 487 and 489 to monitor the MH^+ of 5′-FDI and [^{18}O]5′-FDI, respectively. Each sample was analysed 10 times. A mean value was obtained for the percentage of MH^++2 ion 489 produced by the resultant 5′-FDI sample. The label in each replicate of the ^{18}O sample was calculated after correcting for the percentage of 489 ion in the unlabelled sample. A mean and standard deviation were then calculated for the percentage incorporation of label into the labelled sample of **9**. The mean MH^++2 (489 amu), representing oxygen-18 incorporation was 18.98759 with a standard deviation (SD) of 0.12734.

*2.5. Biotransformation of 5′-FDA **5**-5′-FDI **9***

5′-Adenylic acid deaminase (50 mg, *Aspergillus* sp. Sigma Chemical, A-907) was added to a suspension of 5′-FDA (500 mg) in water (10 ml) and the mixture was stirred at 20 °C for 12 h. The reaction solution was then heated (90 °C/3 min) and centrifuged (14,000/15 min) to remove the precipitated protein. Lyophilisation of the supernatant afforded 5′-FDI (497 mg, 99%) as an off white solid. Mp softens at 156–159 °C and decomposes at 190 °C.

^{1}H NMR δ (d_6-DMSO) 4.13 (1H, dm, $^3J_{HF}$ 24.3, 4′-H), 4.20 (1H, m, 3′-H), 4.51 (1H, t, J 4.8, 2′-H), 4.64 (2H, dm, $^2J_{HF}$ 47.5, 5′-H), 5.92 (1H, m, 3′-H), 4.51 (1H, s, 2-H/8-H), and 8.23 (1H, s, 2-H/8-H);

^{19}F NMR δ (282 MHz; d_6-DMSO) −227.71 (dt, $^2J_{FH}$ 47.5 and $^3J_{FH}$ 24.2); ^{13}C NMR δ (75 MHz; d_6-DMSO) 69.3 (d, $^3J_{CF}$, 6.1, C-3′), 73.5 (d, $^4J_{CF}$ 1.6, C-2′), 82.5 (d, $^2J_{CF}$ 18.2, C-4′), 84.3 (d, $^1J_{CF}$ 168.6, C-5′), 87.6 (C-1′), 124.4 (C), 138.5 (C-2/C-8), 146.0 (C-2/C-8), 148.2 (C), 156.6 (C); *m/z* (ES) 269 (M^+−H, 100%); (ES-Tof) 293.0668 (M^++ Na. $C_{10}H_{11}N_4O_4FNa$ requires 293.0662).

3. Results and discussion

When cell free extracts of *S. cattleya* are incubated with SAM and fluoride they have the capacity to generate several organofluorine species as evinced by the ^{19}F NMR analysis over time as shown in Fig. 2A [11]. Fluoroacetate **1** accumulates (^{19}F NMR at −215 ppm), indicating that the cell free extract has all of the enzymes and co-factors necessary for complete fluorometabolite production from fluoride ion. Two other metabolites are apparent. The fluorinase product 5′-FDA (^{19}F NMR −229.4 ppm) is transient in the ^{19}F NMR spectra in Fig. 2A and it is overlayed by another metabolite, Compound A, with an identical coupling pattern (double of quartets $^1J_{HF}$ = 47 Hz and $^2J_{HF}$ = 29 Hz). When the cell free incubation with SAM

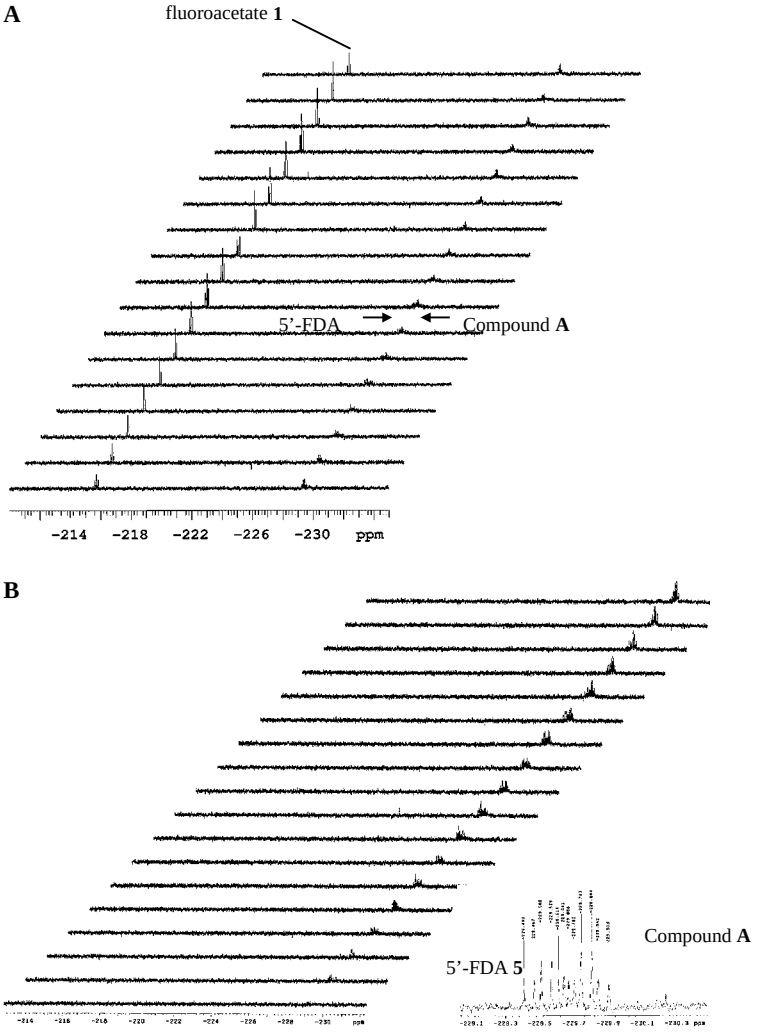

Fig. 2. ^{19}F NMR analysis (recorded every hour for 17 h progressing from the bottom to the top) of a *S. cattleya* cell free extract (A) after incubation with SAM (0.4 mM) and fluoride ion (10 mM) and (B) with iodoacetamide (10 mM) added. Fluoroacetate, 5′-FDA **5**, and Compound A (5′-FDI) are observed. [11] Compound A predominates over 5′-FDA **5** with time, and this is most clearly observed in the iodoacetamide incubation, when fluoroacetate production is arrested. The blown up insert (B) of the ^{19}F NMR signal shows the two signals (each are doublets of triplets $^{1}J_{HF} = 47$ Hz, $^{2}J_{HF} = 29$ Hz) after 10 h.

and fluoride ion is carried out with iodoacetamide added at 10 mM, then fluoroacetate production is arrested as shown in Fig. 2B.

Iodoacetamide appears to inhibit the conversion of 5′-FDA **5** to 5-fluoro-5-deoxyribose-1-phosphate **6**, presumably by covalent modification of the protein in a manner which disrupts is catalytic functioning. In the iodoacetamide incubation, 5′-FDA

5 and compound A are more apparent (Fig. 2B), and through time there is a steady and complete conversion to compound A. Partial purification of the cell free extract after an ammonium sulphate (60%) cut gave a protein fraction that no longer had the capacity to support fluoroacetate **1** biosynthesis after incubation with SAM and fluoride ion. However, this fraction retained the ability to convert 5′-FDA to compound A. This partially purified preparation was ideal for preparing a sample of compound A for analysis. Compound A was purified by HPLC after incubation of the protein preparation with 5′-FDA **5**. ^{1}H NMR and ^{19}F NMR analyses indicated that compound A was structurally very similar to 5′-FDA, and that it was clearly a 5′-fluoro-5′-deoxyribose nucleoside. However, electrospray mass spectrometry (ES-MS) analysis gave a mass ion for compound A which was *one atomic mass unit higher* than 5′-FDA **5** ($MH^+ = 269$ amu versus **5** $MH^+ = 268$ amu). The conversion of the NH_2 of adenine to an OH of inosine could account for this mass difference. To explore the possibility that compound A was 5′-fluoro-5′-deoxyinosine (5′-FDI) **9**, an experiment was carried out where the protein extract was incubated with 5′-FDA **5** in a buffer prepared using isotopically labelled (20 atom %)-[^{18}O]-water. The resultant product was then subjected to derivatisation by treatment with *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) followed by GC–MS analysis. The silyl derivatised 5′-FDA and 5′-FDI (Fig. 3) were analysed under chemical ionisation (CI) conditions, and gave mass ions of $MH^+ = 486$ and $MH^+ = 487$ amu's, respectively (Fig. 3). In the isotopically labelled experiment, there was a new mass ion of $MH^+ = 489$ which constituted 19% of the total mass ion and was two mass units higher than that observed in a control experiment with unlabelled buffer.

The presence of this higher mass ion, two mass units greater than that of compound A prepared in unlabelled buffer, and three mass units higher than 5′-FDA, is consistent with the identity of compound A as 5′-FDI **9**. Oxygen had become incorporated into the molecule from the buffer during the hydrolytic biotransformation of 5′-FDA to 5′-FDI mediated by a deaminase. Further confirmation of the identity of 5′-FDI was secured by preparing a sample of 5′-FDI **9** by biotransformation using a commercially available deaminase from an *Aspergillus* sp. bacterium (Sigma Chemi-

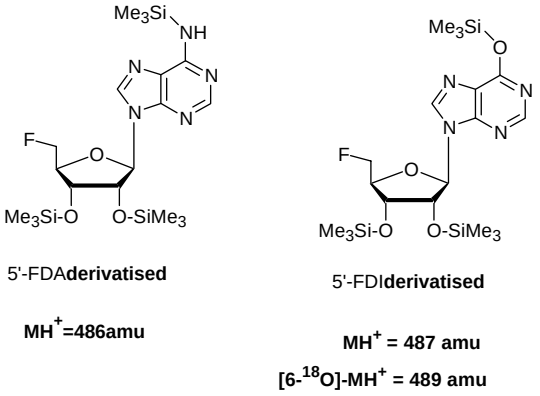

Fig. 3. Silyl derivatives analysed by GC–MS (CI) of 5′-FDA and 5′-FDI after treatment with MSTFA.

cal). Incubation of 5′-FDA **5** with the deaminase resulted in the rapid conversion of 5′-FDA to 5′-FDI. This product was isolated and characterised, and was identical in all respects to the product of the *S. cattleya* protein-mediated biotransformation. Thus by isotope labelling and comparative synthetic approaches, the identity of compound A was secured as 5′-FDI **9**.

When a sample of purified 5′-FDI **9** was incubated with a cell free extract competent in fluoroacetate synthesis from fluoride or 5′-FDA, it remained inert over the incubation period of 24 h. The conversion of 5′-FDA **5** to 5′-FDI **9** by the endogenous deaminase was irreversible and it was not converted to fluoroacetate or any other fluorometabolite. Also when a sample of 5′-FDI **9** was assayed with the purine nucleotide phosphorylase (PNP), the enzyme which converts 5′-FDA to 5-fluoro-5-deoxyribose-1-phosphate **6** (see Fig. 1), there was no activity. It was not a substrate for this enzyme, thus 5′-FDI **9** cannot re-enter the biosynthetic pathway via **6**. All the evidence suggests that 5′-FDI **9** is a stable shunt product of the cell free extract in the biotransformation of fluoride to fluoroacetate.

4. Concluding remarks

The accumulation of 5′-FDI **9** by cell free extracts of *S. cattleya* is perhaps surprising, particularly as it is demonstrated that **9** is not a biosynthetic intermediate on the fluorometabolite pathway. Also, 5′-FDI **9** does not accumulate in whole cells of *S. cattleya*. The efficient conversion of 5′-FDA **5** to 5′-FDI **9** in the cell free extract suggests that in whole cells there is a degree of compartmentalisation of enzymatic activity and that the endogenous deaminase does not come into contact with 5′-FDA **5**. The ability of the cell free extract to generate 5′-FDI **9** from fluoride ion extends the versatility of fluorination enzymology and a current focus involves using the extract to prepare a range of organofluorine compounds, including 5′-FDI in isotopically labelled [^{18}F]-form, from [^{18}F]fluoride, for positron emission tomography applications [14].

Acknowledgments

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