REVIEW

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Fluorometabolite biosynthesis and the fluorinase from *Streptomyces cattleya*

Hai Deng,[a] David O'Hagan*[b] and Christoph Schaffrath[a]

[a] *School of Chemistry and Centre for Biomolecular Science, University of St Andrews, North Haugh, St Andrews, KY16 9ST, UK*

[b] *School of Chemistry and Centre for Biomolecular Science, University of St Andrews, North Haugh, St Andrews, KY16 9ST, UK. E-mail: do1@st-andrews.ac.uk*

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This review outlines the recent developments in uncovering the enzymes and intermediates involved in fluorometabolite biosynthesis in the bacterium *Streptomyces cattleya*. A particular emphasis is placed on the purification and characterisation of the fluorinase, the C–F bond forming enzyme which initiates the biosynthesis. Nature has hardly developed a biochemistry around fluorine, yet fluorinated organics are important commercial entities, therefore a biotransformation from inorganic to organic fluorine is novel and of contemporary interest.

Dr Hai Deng received his B.Eng. in polymer chemistry at South China University of Technology at Guangzhou City (1993). After a two-year research assistantship (1995) at the Wuhan Institute of Chemical Technology he studied for a Masters degree in Chemistry at Huazhong University of Science and Technology in Wuhan city (1998). He came to the UK in 1999 to carry out his Ph.D. at the University of Wales at Swansea with Dr Robert W. Lovitt in the area of biotransformations using membrane bioreactors. In 2002, he joined Professor O'Hagan's research group at the Centre for Biomolecular Sciences at St Andrews where he has been involved in the biochemical aspects of fluorometabolite biosynthesis in S. cattleya and ^{18}F-PET labelling studies with the fluorinase. His current research interests are in secondary metabolite biosynthesis and in new biotransformation technologies.

Professor David O'Hagan is a Glaswegian who studied for a degree in chemistry at the University of Glasgow (1982). He moved to the University of Southampton to carry out a Ph.D. (1985) with Professor John A. Robinson (now at Zurich) in the area of antibiotic biosynthesis in Streptomyces. After a postdoctoral year with Professor Heinz G. Floss at the Ohio State University he joined the University of Durham in 1986 as a Senior Demonstrator and progressed to Professor in 1999. He carried out research on the biosynthesis or secondary metabolites from bacteria, plants and fungi and he also developed a strong research interest in organo-fluorine chemistry. These interests are combined in the fluorometabolite project in S. cattleya. In 2000 he moved to the School of Chemistry at the University of St Andrews where he is Head of Organic Chemistry and has research labs in the Centre for Biomolecular Sciences. He is currently Chair of the Editorial Board of Natural Product Reports and is a founding member and Chairman of the Royal Society of Chemistry, Fluorine Subject Group.

Dr Christoph Schaffrath was born in 1971 in Germany and holds an Engineering degree in Biotechnology and Applied Chemistry (1998) from the University of Applied Sciences in Emden (Germany). After a short industrial position, Christoph started his Ph.D. in October 1999 under the supervision of Professor David O'Hagan at the University of Durham. In 2000, he moved together with Professor O'Hagan to the University of St Andrews, where he was involved in the isolation of the fluorinase enzyme. After completing his Ph.D. in 2002, he joined Stylacats Ltd. in Liverpool, conducting research into new biocatalytic methods. He is currently working as European Business Development Manager at Onyx Scientific Ltd. in Sunderland, UK. The work he carried out during his Ph.D. on the fluorinase enzyme led to a number of prizes and awards including the Judges Award from the IChemE (2002), the IUPAC Award (2003) for best international Ph.D. Thesis in Chemical Sciences and recently he has been named in the 2004 list of the world's 100 Top Young Innovators by the MIT Technology Review.

Hai Deng

David O'Hagan

Christoph Schaffrath

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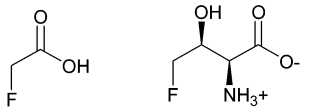
1 Introduction

In this review we summarise recent developments in our understanding of biological fluorination in *Streptomyces cattleya*. *S. cattleya* is a bacterium which has the unusual capacity to elaborate organo-fluorine metabolites in that it can biosynthesise fluoroacetate **1** and 4-fluorothreonine **2** from inorganic fluoride.[1] Over the last two years the enzyme involved in biological fluorination (C–F bond formation) from this organism has been identified and characterised.[2,3] The enzyme is a 5′-fluoro-5′-deoxyadenosine (5′-FDA) synthase (EC 2.5.1.63) which combines *S*-adenosyl-L-methionine **3** and fluoride to make 5′-FDA **4** (Scheme 1). The enzyme has been trivially termed the 'fluorinase'.[2]



1 **2**

The biosynthetic pathway from fluoride to fluoroacetate **1** and 4-fluorothreonine **2** has been substantially uncovered and this is reviewed in detail. In 1994, one of us (DO'H) was a co-author of a *Natural Product Reports* article which reviewed all of the fluorinated natural products and the limited information which was known about their biosynthesis.[4] Fluoroacetate dominated that article due to its high toxicity[5] and because it is the most ubiquitous fluorinated metabolite, found in many plants.[6,7] The biosynthetic hypotheses presented up to that date were speculative, without any supporting enzymology. Ten years ago the total number of fluorinated natural products reported in the literature was six and since that time there has only been one new fluorinated metabolite identified. This is 5-fluorouracil, and some derivatives which were isolated from a sponge from the South China Sea (see Section 2.1).[8] Clearly, a biological origin for 5-fluorouracil from the sponge must be questionable as the possibility of industrial contamination and accumulation by the sponge cannot be discounted. Fluorinated natural products remain extremely rare entities and there is no evidence that the frequency of their occurrence is increasing even as extraction, isolation and screening methods become more sophisticated.

The recent identification of several enzymes[2,9,10,11] on the biosynthetic pathway to fluoroacetate **1** and 4-fluorothreonine **2** from *S. cattleya* has revealed some novel metabolic intermediates carrying the C–F bond, and these are discussed below. These now contribute to the small list of fluorinated natural products, although they have only been detected *in vitro* as products of enzymology rather than metabolites which accumulate in the organism. Identification of the fluorinase, with its capacity to convert inorganic fluoride to organic fluorine, clearly offers new prospects in biocatalysis, particularly as organo-fluorine compounds are significant commercial entities in the agrochemicals, pharmaceuticals and fine chemicals industries.[12,13]

2 Fluorometabolites

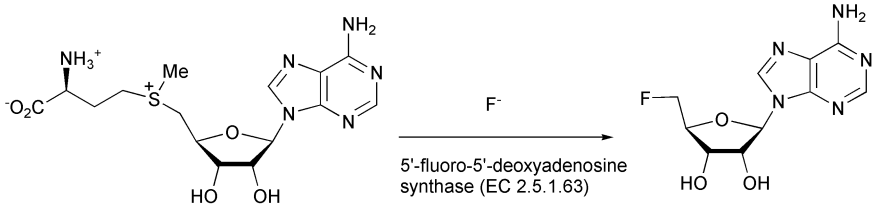
2.1 The known fluorinated metabolites

In 1943, fluoroacetate **1** was the first fluorinated metabolite reported, when it was identified by Marais from the South African shrub *Dichapetalum cymosum*.[6,14] The plant was known to be extremely toxic and its fresh leaves remain a hazard to grazing cattle and foraging animals around Pretoria in the early spring. Fluoroacetate **1** has subsequently been identified in many plant species, mostly in Africa but also in Australia and to a lesser extent South America. The identification of fluorometabolites from the bacterium *S. cattleya* is novel and its biosynthesis forms the basis of this review. It remains unclear if the biosynthetic pathway to fluoroacetate **1** in the bacterium is in any way related to its biosynthesis in plants.

Fluoroacetate's high toxicity lies in its *in vivo* conversion to (2*R*,3*R*)-fluorocitrate **5**. Sir Rudolph Peters was the first to draw attention to this 'lethal synthesis'[15] which involves the enzymatic conversion of fluoroacetyl-CoA and oxaloacetate to (2*R*,3*R*)-fluorocitrate **5**, catalysed by citrate synthase.[16] The resultant (2*R*,3*R*)-fluorocitrate **5** is a competitive inhibitor of aconitase, the subsequent enzyme on the citric acid cycle. Aconitase acts on (2*R*,3*R*)-fluorocitrate **5** by mediating a dehydration to reveal the allylic fluoride intermediate **6**. In a deviant reaction, aconitase then catalyses the S_N2' displacement of fluoride by hydroxide, resulting in 4-hydroxy-*trans*-aconitate **7**, a potent competitive inhibitor of aconitase (Scheme 2). The X-ray structure of a co-crystal of aconitase with the inhibitor **7** bound to the enzyme has been solved and provides experimental support for this mechanism.[7]

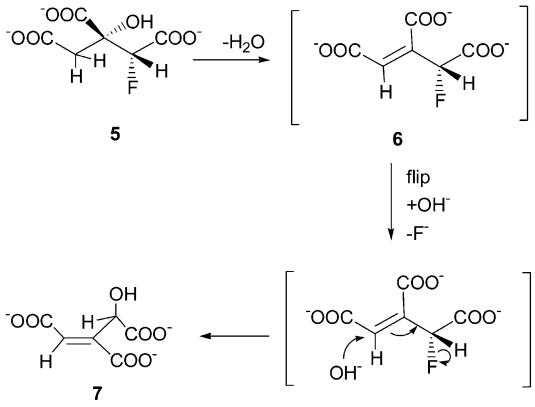
Sir Rudolph Peters (Fig. 1), working towards the end of his career as the first Director of the Babraham Institute in Cambridge,[17] made some other seminal contributions to the identification of fluorometabolites in plants.

In 1959, he and his collaborator Mary Shorthouse identified[18] ω-fluorooleic acid **8** as a constituent (3%) of the seed lipids of *Dichapetalum toxicarium*, a West African plant found mainly in Sierra Leone. Subsequent and detailed GC-MS analysis of these



3 **4**

Scheme 1 The fluorinase (5′-fluoro-5′-deoxyadenosine synthase) from *S. cattleya* mediates the conversion of SAM **3** to 5′-FDA **4**.[2,3]



5 6 7

Scheme 2 Proposed steps in the aconitase-mediated reaction leading to the synthesis of 4-hydroxy-*trans*-aconitate **7**, a competitive inhibitor of aconitase.[16]

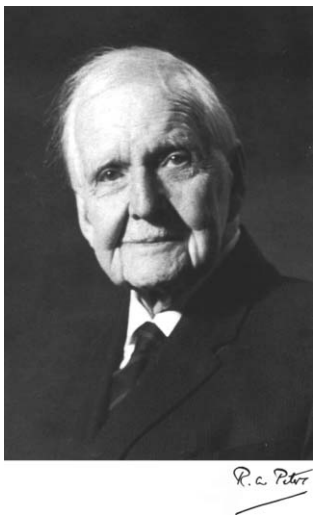
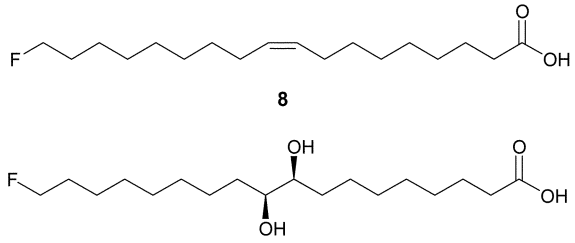


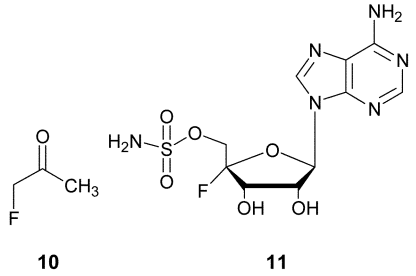
Fig. 1 Sir Rudolph Peters FRS (1889–1982), the pioneer of organo-fluorine natural products. Photograph reproduced by permission of Cambridge Central Library (Cambridgeshire Collection).

seeds,[19,20] mainly by David Harper and Jack Hamilton at Queen's University in Belfast, has revealed a range of ω-fluorofatty acids at much lower concentrations, but essentially mapping the metabolism profile of oleic acid in the seeds. Thus these seeds have the greatest number of fluorometabolites including ω-fluoro 16:1(Δ7), 16:1(Δ9), 18:0, 18:1(Δ9), 18:2(Δ9, Δ12), 20:0 and 20:1(Δ11), saturated and unsaturated lipids as well as ω-fluoro-9,10 dihydroxystearic acid **9**.[21] All of these metabolites appear to derive from metabolites of ω-fluorooleic acid **8**. The fatty acid most probably originates from the replacement of acetyl-CoA by fluoroacetyl-CoA as the starter unit in lipid biosynthesis, consistent with fluorine only ever being located at the terminal (ω) position of the lipids.



8 9

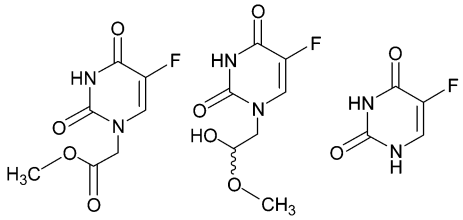
Rudolph Peters also reported some evidence for the presence of fluoroacetone **10**[22,23] as a metabolite of the Australian plant *Acacia georginae*, when the volatiles of the plant were passed through a dinitrophenylhydrazine (DNP) derivatising solution, although the authors admit some uncertainty on the molecular identity of the 'volatile' organo-fluorine metabolite. This analysis has never been revisited and therefore the reported identification of fluoroacetone should remain somewhat speculative.



10 11

Two bacteria have been reported to elaborate organofluorine metabolites. In 1969, the structure of the antibiotic nucleocidin **11** from the bacterium *Streptomyces calvus* was shown to contain a fluorine atom. This antibiotic had been isolated a number of years earlier but the first of two total syntheses[24,25] confirmed the presence of a fluorine atom at the 4′-position of the ribosyl ring system. The bacterial origin of this fluorometabolite makes it attractive for biosynthesis studies and a potential source of a novel C–F bond forming enzyme, however, several labs have tried and failed to re-establish the microbial production of this metabolite,[24,26] and to date more recent microbial production of nucleocidin **11** has remained elusive.

Most recently, in 2003, a series of 5-fluorouracil derivatives[8] **12**–**16** were purified from extracts of the marine sponge *Phakellia fusca* Schmidt, collected from the South China Seas. The structure of compound **16** was confirmed by X-ray diffraction analysis. It is not clear if and how these compounds have accumulated in the sponge, but they clearly have a structural relationship to the chemotherapeutic drug 5′-fluorouracil **14**, which raises the possibility that they are products of industrial contamination and metabolic derivatisation rather than *de novo* biosynthesis. These sponge isolates are the first organo-fluorine novelties to have been identified from a new source in 17 years, since the identification of **1** and **2** from *S. cattleya*,[1] and even then there is some uncertainty over their non-anthropogenic origin. There are no examples of organo-fluorine metabolites from animal or insect sources.



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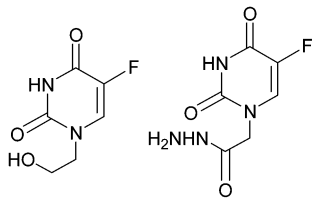


15 16

This low frequency of organofluorine compounds lies to a great extent in the chemical properties of fluoride. Although fluoride is abundant on the earth in mineral form (fluorspar), fluoride ion is a poor nucleophile in water due to its ease of hydration, and it is not oxidised like the other halogens to an X^+ species by haloperoxidases. These properties mitigate against its reactivity and they are coupled with an extremely low bioavailability for fluoride ion (1.3 ppm in surface water against

e.g. 3000 ppm for chloride ion) and accordingly Nature has had some difficulty evolving a biochemistry around fluoride.[4] Thus the identification of the fluorination enzyme[2,3] from *S. cattleya* offers the opportunity to study how this organism secures and activates fluoride ion and how it mediates a reaction to generate the C–F bond.

2.2 The fluorometabolites of *S. cattleya* and whole cell labelling studies

In 1986, a research group from Merck reported[1] the isolation of fluoroacetate **1** and 4-fluorothreonine **2** from extracts of *S. cattleya*. *S. cattleya* is the same organism that produces the β-lactam antibiotic thienamycin,[27] and during optimisation of the thienamycin fermentation they discovered a capacity of the organism to produce fluorometabolites, when a particular soya bean casein was used as a feed stock. This soya bean casein possessed high levels of fluoride, which triggered a latent capacity of the bacterium to elaborate these two metabolites. 4-Fluorothreonine **2** was identified during bioassays, as it displays mild and broad spectrum antibiotic activity against a variety of bacteria. The absolute stereochemistry of this metabolite was subsequently shown to be identical to L-threonine after a total synthesis.[28] This metabolite did not attract further attention as an anti-microbial, however during the early 1990's David Harper at Queen's University in Belfast began to evaluate more closely the capacity of the organism to secrete **1** and **2** under controlled laboratory conditions.[29] They established, using resting cell suspensions in Erlenmeyer flasks, that there was a lag phase of up to 5 days prior to fluorometabolite production (trophophase), and that during the production phase (idiophase) these metabolites could accumulated at mM concentrations. At up to 2 mM all of the fluoride could be converted to organic fluorine in these flasks (Fig. 2).

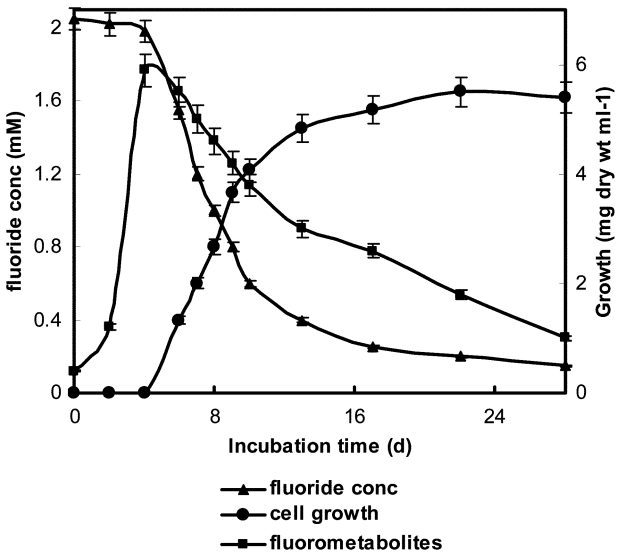
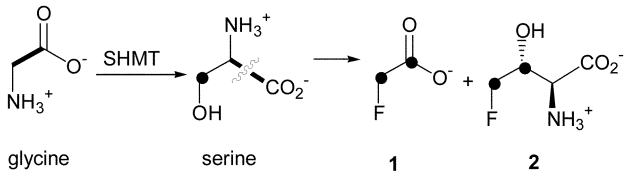


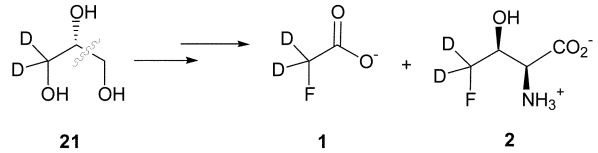
Fig. 2 Profile of fluoride uptake and fluorometabolite production in resting cells of *S. cattleya* grown on a defined medium in the presence of 2 mM fluoride ion.[29]

In the beginning of a collaboration which extended from the mid to the late 1990's our own laboratory (at that time at the University of Durham) and the Belfast group used isotopic labelling studies to explore the important metabolic pathways involved in fluorometabolite biosynthesis in *S. cattleya*. Appropriate labelling studies established that fluoroacetate **1** was not a precursor of 4-fluorothreonine **2** and *vice versa*.[29] For example, incubation of [2-^{2}H]-fluoroacetate did not label the fluoromethyl group of 4-fluorothreonine.[30] Isotopic labelling studies exploring the incorporation of ^{13}C-labelled glycine and serine showed that glycine labelled serine, and that serine always labelled fluoroacetate and 4-fluorothreonine in an identical manner (Scheme 3). Such incorporations are readily detected by ^{13}C–^{19}F coupling in the ^{19}F-NMR spectrum of the produced metabolites. Any ^{13}C-labelling pattern in C-1 and C-2 of fluoroacetate is always mirrored[31] by a similar pattern in C-3 and C-4 of 4-fluorothreonine. This also extended to studies involving deuterium incorporation into each of the fluoromethyl groups of **1** and **2**. Such incorporations are also readily detectable by heavy atom induced chemical shifts in the ^{19}F-NMR spectra of labelled products. Single or double incorporation of deuterium into the fluoromethyl group of fluoroacetate **1** from a given precursor is always mirrored[29] by the same pattern and level of incorporation of deuterium into the fluoromethyl group of 4-fluorothreonine **2**. It was clear that these two C_2 units have a common biosynthetic origin.



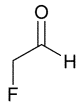
Scheme 3 Labelling patterns revealed the conversion of glycine to L-serine by the action of serine hydroxymethyl transferase (SHMT) prior to the incorporation of serine into fluoroacetate and C-3 and C-4 of 4-fluorothreonine, in *S. cattleya*.[31]

Soda's group in Japan demonstrated[32] in 1995 that [2-^{13}C]-glycerol efficiently labelled C-1 of fluoroacetate **1** in *S. cattleya*. This placed a focus on the involvement of the glycolytic pathway in fluorometabolite biosynthesis. In order to explore this further, a stereochemical study involving (2*R*)-[1-$^2H_2$]- and (2*S*)-[1-$^2H_2$]-glycerols was carried out.[33] In the event, only the (2*R*)-[1-$^2H_2$]-glycerol resulted in isotope incorporation into the fluoromethyl groups of **1** and **2**, indicating that it is the pro-*R* hydroxymethyl group of glycerol that becomes incorporated into the fluorometabolites and that the pro-*S* methyl group is metabolically cleaved during the biosynthesis (Scheme 4). A complete understanding of this glycerol labelling pattern emerged subsequently and will be discussed more fully in Section 4.1.



Scheme 4 Only the pro-*R* hydroxymethyl group of glycerol **21** is incorporated into the fluoromethyl groups of fluoroacetate **1** and 4-fluorothreonine **2** in *S. cattleya*.[33]

These isotope labelling experiments led to the firm conclusion that there must be a common, two-carbon fluorinated intermediate acting as a biosynthetic precursor to both of the fluorometabolites **1** and **2**. This intermediate was found to be fluoroacetaldehyde **17**.[34]

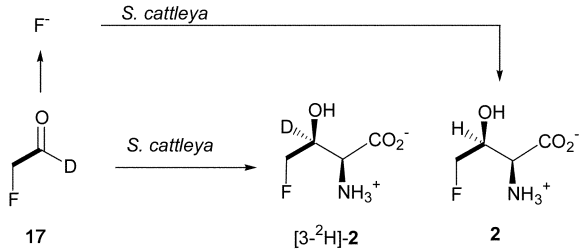


17

2.3 Fluoroacetaldehyde as a biosynthetic intermediate

Fluoroacetaldehyde **17** emerged as a clear candidate for the common C_2 intermediate to fluoroacetate **1** and 4-fluorothreonine **2**. Oxidation can deliver fluoroacetate **1**, and the condensation

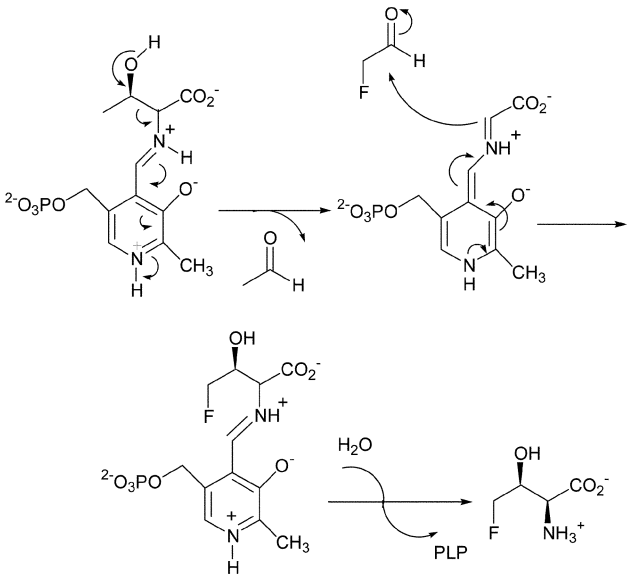
of fluoroacetaldehyde **17** with glycine, mediated by a threonine aldolase, offered a direct route to 4-fluorothreonine **2**. Support for this latter hypothesis was explored in the first case, in whole cell experiments,[34] after incubation of [1-^{2}H]-fluoroacetaldehyde with washed cell suspensions of *S. cattleya*. GC-MS analysis of the resultant 4-fluorothreonine **2** indicated a high level (up to 60%) of incorporation of the isotope into C-3 of 4-fluorothreonine **2**, consistent with its direct incorporation into 4-fluorothreonine **2** without prior oxidation of fluoroacetate **1**. The significant levels (up to 40%) of unlabelled 4-fluorothreonine **2** were also apparent in this experiment, and the unlabelled material was derived from *in vivo* defluorination and *de novo* biosynthesis of 4-fluorothreonine **2** from fluoride (Scheme 5).



Scheme 5 [1-^{2}H]-Fluoroacetaldehyde **17** is efficiently incorporated intact into 4-fluorothreonine **2**, although defluorination and *de novo* biosynthesis resulted in a dilution of the isotope incorporation with time.[34]

Cell-free protein extracts of *S. cattleya* were then analysed for their capacity to oxidise fluoroacetaldehyde **17** to fluoroacetate **1** in the presence of NADH. A highly active NADH-dependent aldehyde dehydrogenase was assayed and the enzyme purified to homogeneity.[9] *N*-Terminal amino acid sequence analysis indicated that the enzyme had a high level of homology to other bacterial acetaldehyde dehydrogenases, however, the kinetic parameters supported a particular selectivity for fluoroacetaldehyde **17** (FAc K_m = 0.08 mM), in that the aldehyde dehydrogenase could oxidise fluoroacetaldehyde much more efficiently than acetaldehyde V_{max} (Ac K_m = 0.81 mM). The enzyme was also able to oxidise glycoaldehyde and chloroacetaldehyde, and appears to prefer substrates with an electronegative substituent at the α-position to the aldehyde carbonyl. On balance this appears to be an enzyme on the fluoroacetate pathway, rather than an indiscriminate bacterial aldehyde dehydrogenase.

The cell-free extract was then explored for its ability to generate 4-fluorothreonine **2** by incubation of fluoroacetaldehyde with various amino acids and co-factors. Bacterial threonine aldolases utilise the co-factor pyridoxal phosphate (PLP) in condensations of acetaldehyde with glycine to generate L-threonine.[35] Accordingly, PLP was explored as a possible co-factor for the analogous transformation in *S. cattleya*. In the event, incubations of fluoroacetaldehyde **17** and PLP with the amino acid L-threonine, rather than glycine, resulted in the *in vitro* synthesis of 4-fluorothreonine **2**.[10] Glycine was not a substrate for this enzyme. The enzyme was purified and it was shown to mediate the interconversion of L-threonine and fluoroacetaldehyde **17** for 4-fluorothreonine **2** and acetaldehyde, in a transaldolation reaction. A minimal mechanism for this enzyme is represented in Scheme 6. It is intriguing that for every molecule of 4-fluorothreonine **2** generated by the bacterium, a molecule of L-threonine is sacrificed. This suggests that there is some selection advantage to the organism in producing 4-fluorothreonine **2** at the expense of threonine. Perhaps the organism incorporates a 4-fluorothreonine **2** in place of a threonine residue into a specific protein as a defence mechanism when it is under metabolic stress?



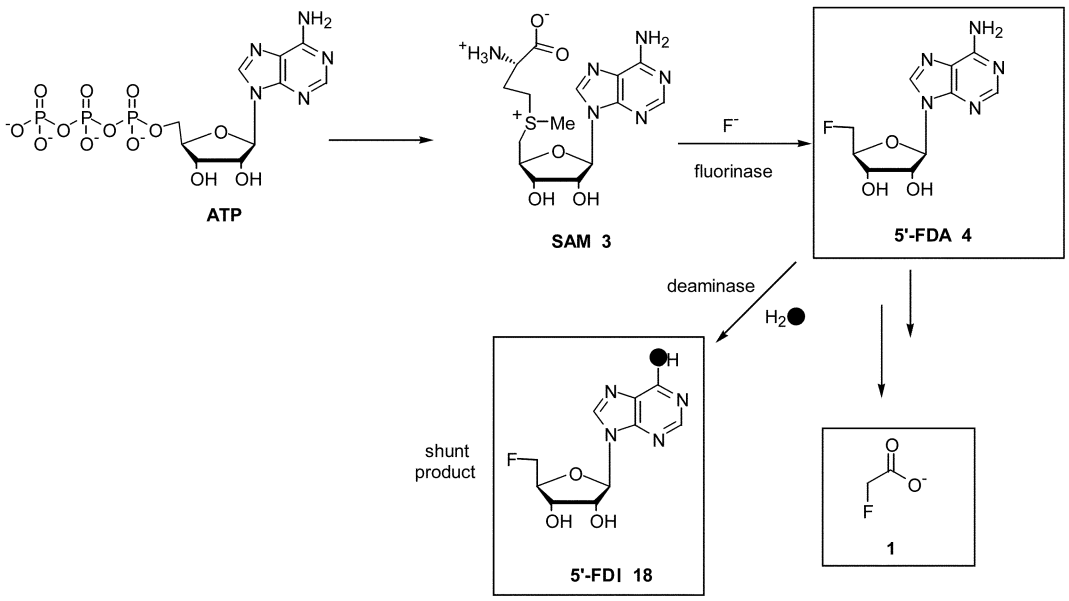
Scheme 6 A mechanistic proposal for the conversion of L-threonine and fluoroacetaldehyde **17** to 4-fluorothreonine **2** and acetaldehyde catalysed by the PLP enzyme threonine transaldolase (EC 2.2.1.8) from *S. cattleya*.[10]

3 Isolation of a fluorination enzyme

3.1 Cell-free fluorination and purification of the fluorinase

It was a clear objective to identify the C–F bond forming enzyme. Isotope labelling studies (Section 2.2) had revealed some general information indicating that glycine was being processed to serine *via* serine hydroxymethyl transferase, and that glycerol and serine were entering the glycolytic pathway.[31] However, the labelling studies did not reveal the immediate fluorination substrate. High field ^{19}F-NMR spectroscopy offers a very sensitive method for detecting new organo-fluorine compounds, and the prospects of identifying cell-free fluorination improved by application of this method. In 2002 we carried out a series of experiments in St Andrews incubating cell-free extracts, prepared under a variety of conditions with various co-factors, buffers and fluoride ion and the reactions were monitored by ^{19}F-NMR. It was extremely exciting to observe among many unsuccessful experiments the conversion of ATP and fluoride ion in the cell-free extract to three organic fluorine compounds. Clearly, the cell-free extract was converting inorganic to organic fluorine, in a novel biotransformation. Fluoroacetate **1** was the most prominent fluorometabolite of the three and clearly the experiment indicated that the cell-free extract had retained all of the activities required for the entire biosynthesis of fluoroacetate from inorganic fluoride and ATP. Further experiments revealed that *S*-adenosyl methionine (SAM **3**) was similarly competent to mediate cell-free biosynthesis of these organo-fluorine compounds in the cell-free extract under the same conditions. The ^{19}F-NMR spectra from a time course experiment with SAM **3** is shown in Fig. 3 and represents biological fluorination catalysed by a protein solution in an NMR tube and not within a cell. The relationship between ATP and SAM **3** is of course very close where the ubiquitious enzyme SAM synthetase mediates a reaction between L-methionine and ATP to generate SAM[37] (Scheme 7).

At this stage it appeared most reasonable that SAM **3** was converted to 5′-fluoro-5′-deoxyadenosine (5′-FDA) **4** by the fluorination enzyme. A synthetic sample of 5′-FDA **4** was prepared for analytical comparison with the product of the enzyme reaction. Initially the product from the crude cell-free extract appeared to have all of the NMR characteristics of 5′-FDA **4**, however ES-MS analysis indicated a variance of 1 amu.



Scheme 7 *In vitro* cell-free reactions of *S. cattelya* from ATP/SAM and fluoride ion. The three 'boxed' organo-fluorine products, 5′-FDA **4**, 5′-FDI **18** and fluoroacetate **1**, were observed during the incubation by ^{19}F-NMR spectroscopy (see Fig. 3).[48]

When the synthetic material was prepared it was clear that the HPLC retention times of synthetic 5′-FDA **4** and the material isolated from the cell-free extract were different. This raised the possibility that the cell-free extract had an unexpected adventitious deaminase activity which converted 5′-FDA **4** to 5′-fluoro-5′-deoxyinosine (5′-FDI) **18**. This was confirmed in an experiment using labelled water ($H_2{}^{18}O$) and it was found that the new product now had a mass ion which was two atomic mass units higher than that expected for 5′-FDA, indicating oxygen-18 incorporation to generate [^{18}O]-5′FDI **18** as illustrated in Scheme 7. The speedy resolution of this issue was important because purification of the fluorinase required a reliable HPLC indicator for the product peak, rather than a misleading by-product peak. Indeed, as the cell-free extract was partially purified then the deaminase activity could be removed and the fluorinase was shown to convert SAM **3** directly to 5′-FDA **4**. A revisitation of the ^{19}F-NMR spectra in Fig. 3 allows the two peaks at −229.4 ppm and −229.6 ppm to be assigned to 5′-FDA **4** and 5′-FDI **18** respectively, the latter accumulating due to the irreversible deaminase activity in the cell-free extract. Accordingly all three peaks in the original cell-free experiments with ATP and SAM **3** (Fig. 3) were now assigned.

With an HPLC-based assay in hand, production of 5′-FDA **4** could be monitored directly from SAM **3**. This allowed the enzyme to be purified by standard protocols. Kinetic analysis of the fluorinase revealed a high K_m (1.9 mM) for fluoride ion. Presumably this reflects the difficulty of securing fluoride ion from the bulk solution for catalysis on the surface of the enzyme due to its high heat of hydration. This high K_m also raises questions about the intracellular concentration of fluoride ion within *S. cattleya* cells for efficient turnover of the enzyme. There is some limited evidence based on fluoride ion uptake into the cells that *S. cattleya* has a process for actively uptaking fluoride, presumably to high intracellular concentrations.

Further investigations of the enzyme indicated that the fluorinase is inhibited by SAM analogues such as *S*-adenosylhomocysteine (SAH) **19**, the product of demethylation. SAH **19** is a potent inhibitor with a K_i of 29 μM, whereas sinefungin **20** showed weaker inhibition. Clearly these compounds are competitive inhibitors for the SAM binding sites.

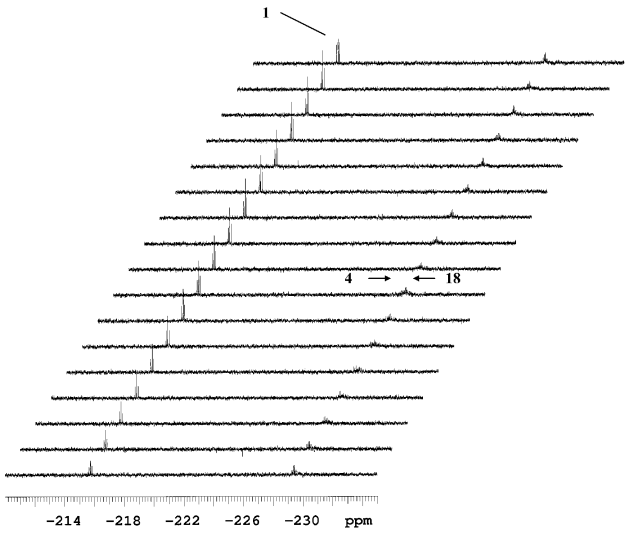
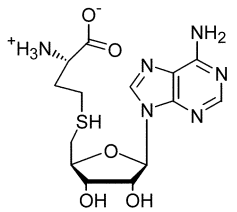
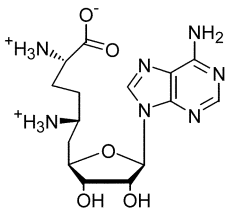


Fig. 3 *In vitro* enzymatic fluorination. A time course showing ^{19}F-NMR spectra indicates the production of three fluorine-containing products fluoroacetate **1**, 5′-FDA **4** and 5′-FDI **18** during incubations of SAM **3** and fluoride ion in a *S. cattleya* cell-free extract.[48]





3.2 Cloning and over-expression of the fluorinase *flA* gene

The fluorinase *flA* gene was cloned[36] in collaboration with Joe Spencer and his co-worker Fanglu Huang at the University of Cambridge, using a reverse genetics strategy. *N*-Terminal amino acid analysis and trypsin digestion of the wild type enzyme allowed sufficient amino acid sequence to design PCR primers. The *S. cattleya* genomic DNA was then used as a template to amplify the fluorinase gene *flA*. Subsequent sequencing revealed that the *flA* gene is 897 base pairs in size and codes a protein of 299 amino acids corresponding to a protein monomer with

a molecular weight of 32 kDa. The DNA and amino acid sequences of the *flA* gene and enzyme are shown in Fig. 4.

```
  1 atggctgccaacagcacacgtcgccccatcatcgcgttcatgagc
    M  A  A  N  S  T  R  R  P  I  I  A  F  M  S
 46 gacctggggaccacggacgactccgtcgcccagtgcaaggggctc
    D  L  G  T  T  D  D  S  V  A  Q  C  K  G  L
 91 atgtacagcatctgcccggacgtcacggtggtggacgtctgccac
    M  Y  S  I  C  P  D  V  T  V  V  D  V  C  H
136 tcgatgacgccctgggacgtcgaggagggcgcccgctacatcgtg
    S  M  T  P  W  D  V  E  E  G  A  R  Y  I  V
181 gaccttccgcgcttcttccccgagggaacggtcttcgccaccacc
    D  L  P  R  F  F  P  E  G  T  V  F  A  T  T
226 acctatccggcgaccggcaccaccacccgctcggtggcggtgcgc
    T  Y  P  A  T  G  T  T  T  R  S  V  A  V  R
271 atcaagcaggccgccaagggcggtgcccgcggccagtgggcgggc
    I  K  Q  A  A  K  G  G  A  R  G  Q  W  A  G
316 tcgggggccggcttcgagcgcgccgagggctcgtacatctacatc
    S  G  A  G  F  E  R  A  E  G  S  Y  I  Y  I
361 gcgcccaacaacgggctgctgaccaccgtgctggaggagcacggc
    A  P  N  N  G  L  L  T  T  V  L  E  E  H  G
406 tacctggaggcgtacgaggtcacctcgccgaaggtcatccccgag
    Y  L  E  A  Y  E  V  T  S  P  K  V  I  P  E
451 cagcccgaaccgaccttctacagccgggagatggtggccatcccc
    Q  P  E  P  T  F  Y  S  R  E  M  V  A  I  P
496 tccgcgcacctggccgccggcttcccgctgtccgaggtcggccgt
    S  A  H  L  A  A  G  F  P  L  S  E  V  G  R
541 ccgctggaggaccacgagatcgtccgcttcaaccgcccggccgtc
    P  L  E  D  H  E  I  V  R  F  N  R  P  A  V
586 gagcaggacggggaggcgctggtgggcgtggtctccgccatcgac
    E  Q  D  G  E  A  L  V  G  V  V  S  A  I  D
631 cacccgttcggcaacgtgtggaccaacatccaccgcaccgacctg
    H  P  F  G  N  V  W  T  N  I  H  R  T  D  L
676 gagaaggcgggcatcggctacggcgcccggctgcggctgacgctg
    E  K  A  G  I  G  Y  G  A  R  L  R  L  T  L
721 gacggcgtgctgccgttcgaggcgccgctgaccccgacgttcgcc
    D  G  V  L  P  F  E  A  P  L  T  P  T  F  A
766 gacgccggtgagatcggcaacatcgccatctacctcaacagccgc
    D  A  G  E  I  G  N  I  A  I  Y  L  N  S  R
811 ggttacctgtccatcgcgcgcaacgcggccagcctcgcctacccg

    G  Y  L  S  I  A  R  N  A  A  S  L  A  Y  P
856 taccacctcaaggagggcatgtccgcccgggtcgaggcccgctga 900
    Y  H  L  K  E  G  M  S  A  R  V  E  A  R  *
```

Fig. 4 The *flA* gene and amino acid sequences of the fluorinase from *S. cattleya*.[36]

The *flA* gene was cloned into the Pet28a(+) plasmid and inserted into *E. coli* BL21(DE3) for over-expression. The resultant protein was catalytically competent and could mediate the efficient conversion of SAM to 5′-FDA. Access to the over-expressed enzyme clearly opens up many opportunities for exploring the mechanism of the enzyme, generating mutants and extending its prospects as a biocatalyst. It became immediately attractive to obtain an X-ray derived structure of the fluorinase as the first step in understanding its mode of action.

3.3 Crystallisation and X-ray structure of the fluorinase

Crystallisation was initiated with the wild type enzyme. Jim Naismith and his co-worker Changjiang Dong at St Andrews were able to obtain the first X-ray diffraction data on the wild type enzyme, which was solved to 1.8 Å resolution.[38] The successful solution of the structure was completed using protein from the *E. coli* over-expression system received from the Spencer lab in Cambridge. This allowed a seleno-methionine enriched sample of the fluorinase to be crystallised and the structure was then solved by the anomalous diffraction method. The structure revealed a hexamer, constructed as a dimer of trimers. The fold of the monomeric unit of the trimer is novel and has no obvious relationship to any other protein superfamily or previously characterised enzyme (Fig. 5).

3.4 The structure of the fluorinase with SAM and 5′-FDA

The X-ray structure revealed that the trimer had three SAM **3** molecules bound at the subunit interfaces, with the top side of SAM **3** contacted by one monomeric subunit and the bottom side by another. The monomer, trimer and hexamer structures are shown in Fig. 5. The ribose ring of SAM **3** is held in an unusually planar conformation by hydrogen bonding contacts between the 2′- and 3′-hydroxyl groups and the carboxylate side group of Asp-16 (Fig. 7). This results in an unusual eclipsing interaction between the 2′-C–O and 3′-C–O bonds. All of the heteroatoms of the adenine ring are involved in hydrogen bonding donor or acceptor contacts, and the amino acid residues of the methionyl moiety are compensated by interactions to the surface of the protein. It is perhaps noteworthy that the ribosyl ring ether oxygen is not involved as a hydrogen bond acceptor and it is the only heteroatom of SAM **3** not to make such a contact. The possible stereoelectronic consequences of this are discussed below.

When the fluorinase was co-crystallised with SAM **3** in the presence of fluoride ion, the enzyme catalysed the conversion of the substrate to the products, 5′-FDA **4** and L-methionine (Fig. 6). A comparison of this product structure with the substrate (SAM, **3**) structure was particularly informative in suggesting a mechanism for the reaction.[34] 5′-FDA **4** and L-methionine now occupied the SAM **3** binding sites. A comparison of the diffraction data, both before and after the fluorination reaction, revealed very little difference between the two structures except in the bond forming/breaking region. The sulfur atom is displaced 1.8 Å and the orientation of the new C–F bond is approximately antiperiplanar (~167°) to the old C–S bond, indicative of a substitution reaction (S_N2) occurring with an inversion of configuration. This conclusion is reinforced by the stereochemical study described in Section 4.1.

Other features of the product structure provide a clue as to how the enzyme may activate fluoride ion for substitution (Fig. 7). The fluorine atom of 5′-FDA **4** forms two short hydrogen bonding contacts to Ser-158. The contact between the fluorine and the backbone NH hydrogen of Ser-158 is particularly short (~1.9 Å). The other contact is between fluorine and the OH hydrogen of the Ser-158 side chain. The hydrogen of this OH is involved in a bifurcated hydrogen bond to the 3′-OH group of the ribosyl ring of the product 5′-FDA **4** (Fig. 8). This 3′-OH group of the substrate/product is clearly an important residue in catalysis. Perhaps these two hydrogen bonds are important contacts to fluoride ion just prior to the reaction with SAM **3**, and these interactions, which are residual in the product structure, represent the last contacts to fluoride ion as the enzyme turns over.

As mentioned above, it is noteworthy that the ribose ring ether oxygen is not involved in a hydrogen bond to the surface of the protein. It is well known that β-oxygens slow down the rate of S_N2 substitution reactions by up to an order of magnitude, largely because the electron withdrawing effect of the oxygen strengthens the bond to the leaving group, in this case the C–S^+R_2 bond, and increases the activation energy for substitution.[39] If the ether oxygen became involved in hydrogen bonding this would further increase the electron withdrawing power of the oxygen and further suppress the reaction rate. A similar effect would occur if the ether oxygen was involved in an anomeric interaction with the adenine base, but the conformation of the bound substrate on the enzyme mitigates

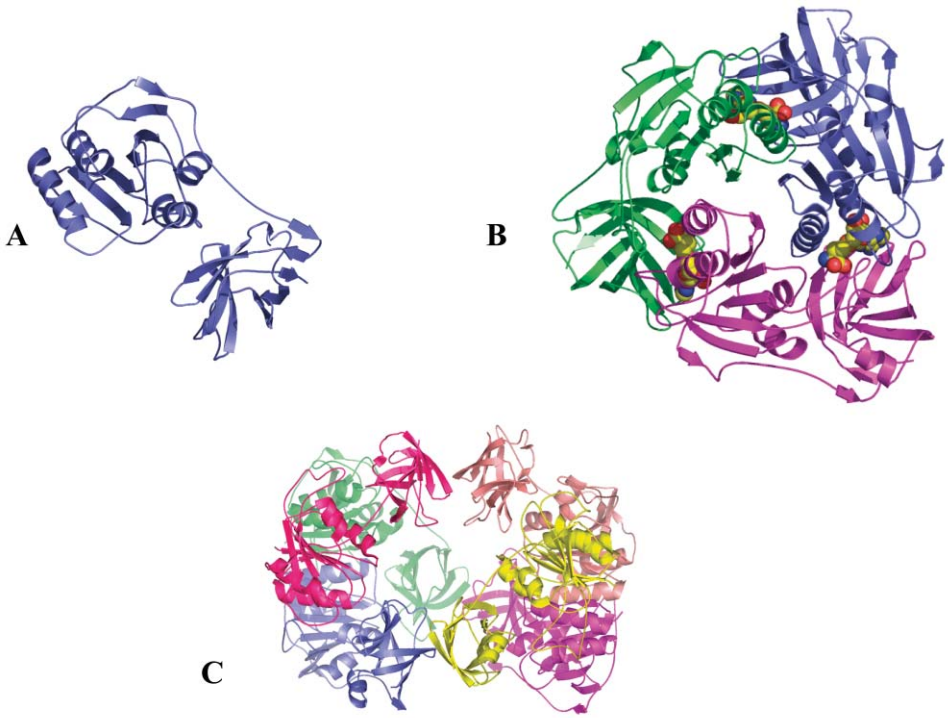


Fig. 5 The structure of the fluorinase. A. Monomer. B. A trimer with SAM **3** bound at the subunit interfaces. C. The overall hexamer structure which is a dimer of the trimer.[36]

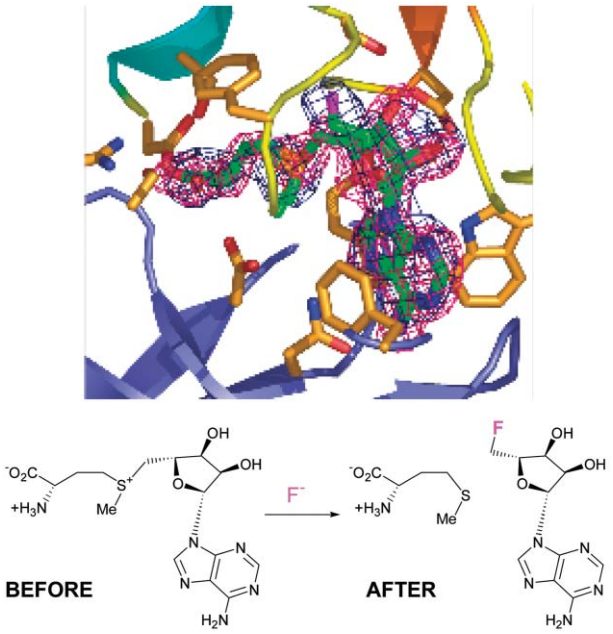


Fig. 6 The superimposition of the X-ray diffraction data of SAM **3** (BEFORE) and 5′-FDA **4** (AFTER) bound to the fluorinase enzyme, indicating an inversion of configuration (S_N2) as a consequence of the fluorination reaction.

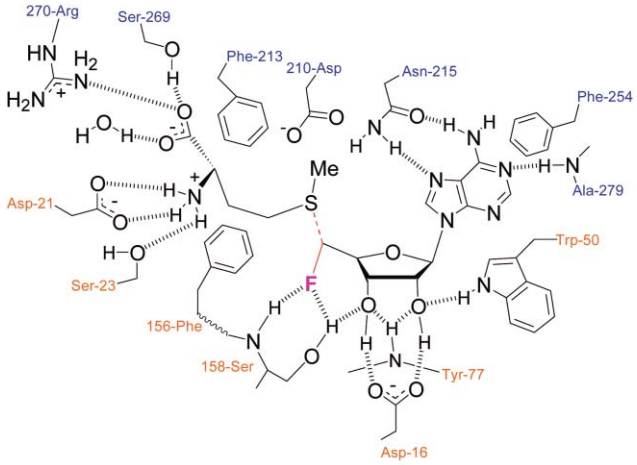


Fig. 7 Topographical image of the hydrogen bonding contacts of 5′-FDA on the surface of the fluorinase derived from the X-ray structure data. The two hydrogen bonds between Ser-158 and the organic bound fluorine at C-5′ form notable contacts.[36]

against this too. There is no doubt that this is a relatively difficult S_N2 process, but it would appear that the geometry and nature of SAM binding is optimised to accommodate this substitution reaction.

4 Stereochemical course and mechanism of enzymatic fluorination

An analysis of the stereochemical course of the fluorination enzyme was explored using deuterium isotope labelling, firstly in whole cell feeding experiments[40] and subsequently with the purified enzyme.[41] The approach envisaged the incubation of a

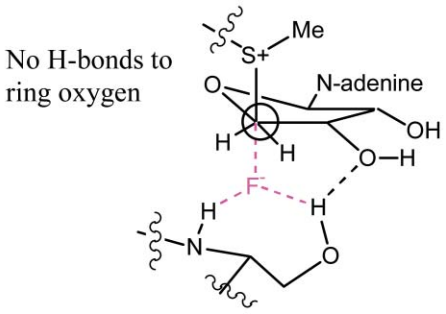
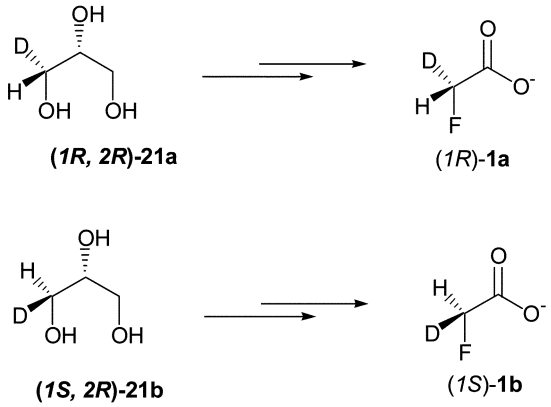


Fig. 8 The geometry and interaction of the substituents prior to fluoride substitution on the fluorinase. The absence of both a hydrogen bond to the ribose ring oxygen and an anomeric interaction between this oxygen and the adenine promote the substitution reaction.[36]

substrate carrying a single stereospecifically labelled deuterium atom attached to the carbon atom that eventually becomes fluorinated. This results in a 'chiral' fluoromethyl (F^2HHC-) group. Thus the first experiments explored the incorporation of the isotopically labelled glycerols **21a** and **21b** in resting cell incubations of *S. cattleya*. These glycerols were labelled with a single deuterium only on the pro-*R* hydroxymethyl arm of glycerol,[42] the pro-chiral arm that was already established to undergo fluorination (Section 2.2).[33] The OH group is formally replaced by F during the biosynthesis and therefore the resultant fluoroacetate should clearly posess a chiral fluoromethyl group. If the biosynthetic process is stereospecific, then the fluoromethyl groups in **1a** and **1b**, after feeding each of the glycerols **21a** and **21b**, should have opposite absolute configurations (Scheme 8).



Scheme 8 The incorporation of chiral deuterium labelled glycerols in whole cell incubations with *S. cattleya* results in the production of chiral fluoromethyl (F^2HHC-) groups on the resultant fluoroacetates **1a** and **1b**, which indicates an overall retention of configuration.[42]

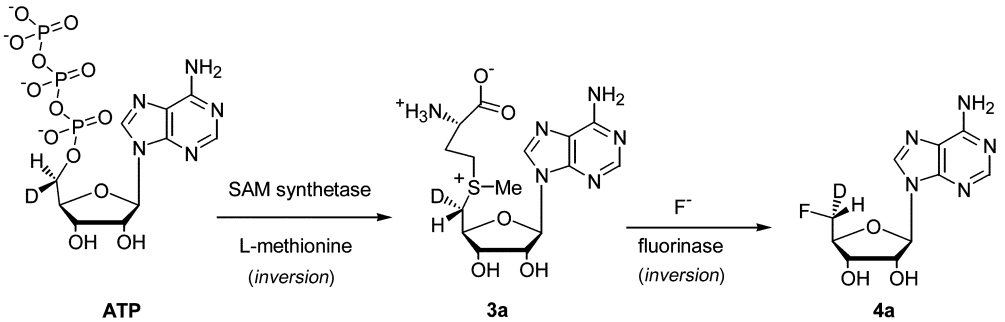
A key challenge was to find a reliable stereochemical assay to report the absolute configuration of the chiral fluoromethyl groups of fluoroacetate **1** after the feeding experiments with the labelled glycerols. This was particularly important in the whole cell studies as extremely small amounts (μg) of fluoroacetate are recovered from each experiment, and even then the material is only enriched to a level of about 10% with deuterium. This problem was solved in a collaboration with Jacques Courtieu and Abdelkrim Meddour at the Paris XI University in Orsay. The Orsay group have developed[43] a sensitive method of ^{2}H-NMR analysis in a lyotropic liquid crystalline phase generated from a homochiral amino acid derived polymer (*poly*-benzyl-L-glutamate, M.W. ~100 000, dissolved in chloroform or DMF). The quadropolar deuterium nucleus interacts strongly with the electric field gradient of the helical liquid crystal, orientating the nucleus either with or against the field and generating two resonances (a doublet) in the ^{2}H-NMR spectrum. If the deuterium sits at a stereogenic centre then the quadropolar deuterium of each enantiomer has a separate diastereomeric interaction with the electric field of the helix and generates two separate pairs of doublets, one for each enantiomer. Enantiomeric excesses can be calculated from the relative intensities of these doublets. In the event this method worked well for 'chiral' (F^2HHC-) fluoromethyl groups and formed the basis of the enantiomeric assay to explore the stereochemical course of the fluorinase.[40,41]

(1*R*,2*R*)-Labelled glycerol gave rise to a sample of fluoroacetate with an (*R*) configuration and (1*S*,2*R*)-labelled glycerol gave the complementary (*S*) configuration in the resultant fluoroacetate, as shown in Scheme 8. Thus there was an overall retention of configuration in breaking the C–O bond of the pro-*R* arm of glycerol and forming the C–F bond of fluoroacetate. Such a retention of configuration is consistent either with a retaining enzyme or with two inversions of configuration. A full interpretation of these whole cell experiments had to await the stereochemical study with the purified fluorinase enzyme. With the fluorinase enzyme available, an experiment where (*R*)-[5′-^{2}H]-ATP was incubated in a coupled enzyme system with SAM synthetase and the fluorinase was conducted.

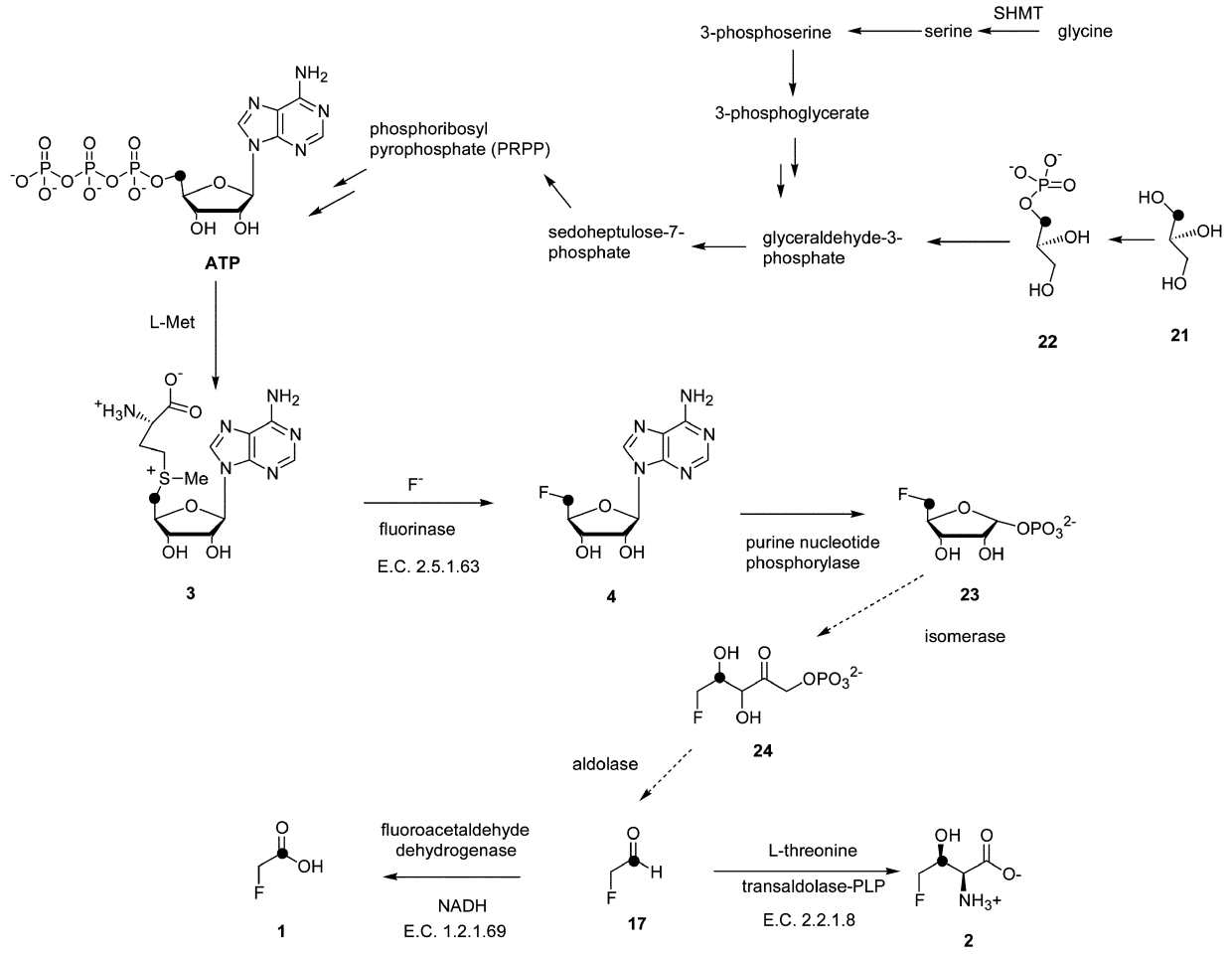
SAM synthetase is known to combine ATP and L-methionine to generate SAM, displacing triphosphate, and this reaction proceeds with an inversion of configuration.[44] In the event, ^{2}H-NMR spectroscopy using the Orsay method established that (*R*)-5′-FDA was generated in the coupled enzyme reaction from (*R*)-[5′-^{2}H]-ATP, again with an overall retention of configuration. However, the knowledge that SAM synthetase mediates a configurational inversion at C-5′ of ATP forced the conclusion that the fluorinase also proceeds with an inversion of configuration (Scheme 9).[41] This configurational inversion at the 5′-carbon implies that fluoride ion displaces L-methionine by an S_N2 reaction mechanism, a conclusion also supported by the X-ray structural study (Fig. 6).[36]

5 Overview of fluorometabolite biosynthesis in *S. cattleya*

The early isotope labelling experiments demonstrated the efficient incorporation of glycine, serine and glycerol into the fluorometabolites and these observations can now be rationalised with a knowledge that the biosynthesis proceeds *via* SAM **3** and 5′-FDA **4**. Glycine is related to serine *via* serine hydroxymethyl transferase (SHMT), and serine can undergo phosphorylation and can feed into the glycolytic pathway. Glycerol also feeds directly into the glycolytic pathway, and poignantly it is the pro-*R* hydroxymethyl arm of glycerol that becomes phosphorylated to generate *sn*-glycerol-3-phosphate **22**. Conversion of *sn*-glycerol-3-phosphate **22** to glyceraldehyde-3-phosphate and then, *via* sedoheptulose-7-phosphate, attachment to the ribose ring of ATP and SAM[45] accounts for the regiochemical incorporation of the pro-*R* hydroxymethyl carbon of glycerol into the C-5′ ribose carbon of ATP and SAM. The fluorinase then mediates the conversion of SAM to 5′-FDA. The next transformation involves



Scheme 9 Treatment of stereospecifically labelled (5′*R*)-[5′-^{2}H]-ATP with SAM synthetase and the fluorinase generates (5′*R*)-[5′-^{2}H]-5′-FDA as the product. This indicates that there are two sequential inversions of configuration resulting in an overall retention of configuration. Accordingly the fluorinase operates with an inversion of configuration.[41]



Scheme 10 An overview of the metabolite relationships and enzymes involved in the biosynthesis of fluoroacetate **1** and 4-fluorothreonine **2** in *S. cattleya* derived from isotope labelling experiments and biochemical studies. The dot traces the fate of the pro-*R* hydroxymethyl group of glycerol through to the fluorometabolites **1** and **2**.

the action of a purine nucleotide phosphorylase (PNP).[46] This enzyme converts 5′-FDA to 5-fluoro-5-deoxyribose-1-phosphate (FDRP) **23** (Scheme 10).

The product FDRP **23** has been isolated and characterised in partially purified extracts of *S. cattleya*.[11] The transformations of FDRP **23** to fluoroacetaldehyde remains to be characterised at the biochemical level. A clue to the nature of this biotransformation comes from the known metabolism of 5′-methylthioadenosine **25**, a metabolite of SAM in mammals. 5′-Methylthioadenosine is also degraded by the action of a PNP to generate 5-methylthio-5-deoxyribose-1-phosphate **26** in mammals. Interestingly, Abeles *et al.*[47] showed that this sugar phosphate is acted upon by an isomerase which converts it to the ring-opened 5-methylthio-5-ribulose-1-phosphate **27** (Scheme 11). An analogous reaction in *S. cattleya* would take FDRP **23** to the fluororibulose-1-phosphate **24**. Such sugars are well known products of aldolases and the reverse aldol reaction could clearly generate fluoroacetaldehyde **17**. Fluoroacetaldehyde **17** would then be converted as described above, to both fluoroacetate **1** and 4-fluorothreonine **2**.

There is experimental evidence to support the conversion of 5-FDA **4** to fluoroacetaldehyde **17** and fluoroacetate **1** in cell-free extracts.[48] When 5-FDA was incubated with a cell-free extract from *S. cattleya* it was bioconverted to many fluorinated intermediates and end products. This is shown most clearly in the ^{19}F-NMR time course profile of such an experiment (Fig. 9). The ^{19}F-NMR spectrum in Fig. 9 shows that after addition of 5′-FDA, fluoroacetate appears. Fluoroacetaldehyde (as a complex with Tris buffer) is also obvious after three hours or so. In addition the cell-free extract had the capacity to reduce

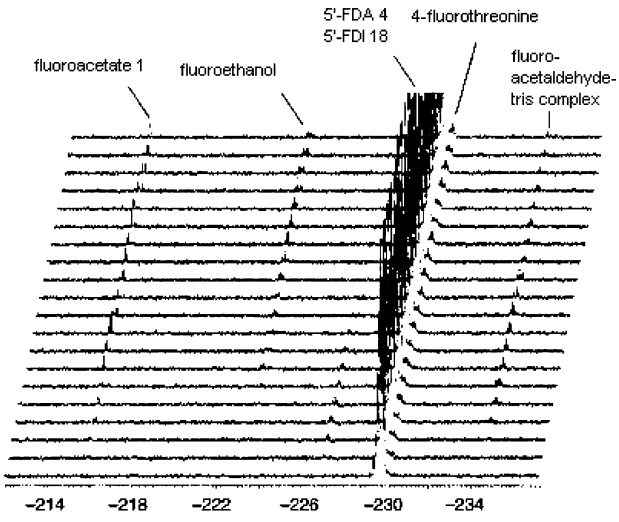
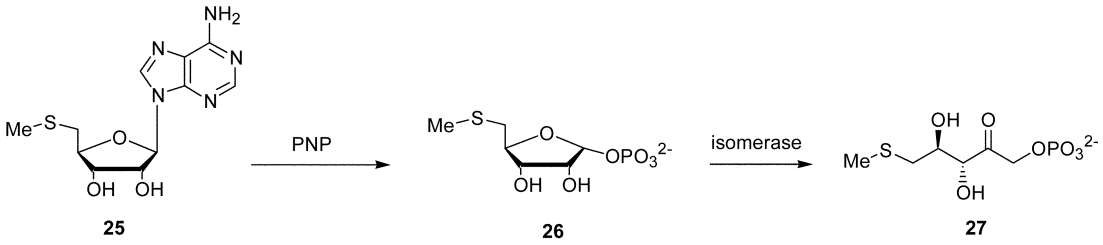


Fig. 9 ^{19}F NMR spectra of fluorine-containing products generated in the *S. cattleya* cell-free extract when incubated with 5′-FDA.[48]

fluoroacetaldehyde to fluoroethanol, and much of the 5′-FDA is converted to FDI by the action of the deaminase, as discussed above. Some transient fluorinated intermediates are visible in this experiment. For example, the peak at –227.2 ppm may be 5-fluororibulose-1-phosphate **24**, although this remains to be unambiguously determined.



Scheme 11 Summary of the pathway of the conversion of 5′-methylthioadenosine **25** to the ribose phosphate **26** and ribulose **27** in mammals as described by Abeles.[47]

6 The fluorinase as a catalyst for PET labelling with ^{18}F

Positron emission tomography (PET) continues to gain importance as a non-invasive diagnostic method in the clinic for imaging tumors, monitoring the distribution of drugs and identifying cell and receptor degeneration in the brain.[49] The two most prominent positron-emitting isotopes in this arena are ^{11}C ($t_{1/2}$ = 11 min) and ^{18}F ($t_{1/2}$ = 110 min). One of the current challenges in PET technology is the development of novel methods for the introduction of fluorine into organic molecules starting from either fluoride ion or hydrogen fluoride, the sources of the ^{18}F-isotope generated by the cyclotron. Ideal synthesis methods for PET are rapid, involve few steps and have straightforward purification protocols. Enzymatic methods offer an attractive prospect in PET synthesis because they are chemospecific and they generate few side products. However there are few examples in PET synthesis where enzymes have been used to introduce isotopically labelled atoms, largely because suitable enzymes are not available. In this regard, the fluorinase has immediate prospects for the incorporation of fluorine-18 into organic compounds, and the enzyme has been investigated as a catalyst for the synthesis of [^{18}F]-FDA (Scheme 12).[50]

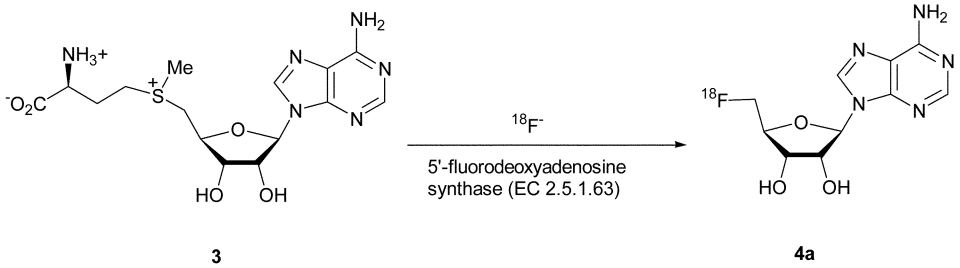
Incubation of wild type fluorinase from *S. cattleya* with [^{18}F]-fluoride resulted in the successful synthesis of radiolabelled [^{18}F]-FDA, with a radiochemical yield of ~1%. Although this is a modest conversion, the availability of the fluorinase, now in mg quantities, has significantly improved this synthesis, with radiochemical yields in excess of 50%. The utility of the fluorinase to form the C–^{18}F bond opens up future possibilities not only for the synthesis of [^{18}F]-FDA, but also for the synthesis of fluorine-18 labelled downstream metabolites such as 5-fluororibose-1-phosphate, 5′-FDI and fluoroacetate **1**.[51]

7 Final comments

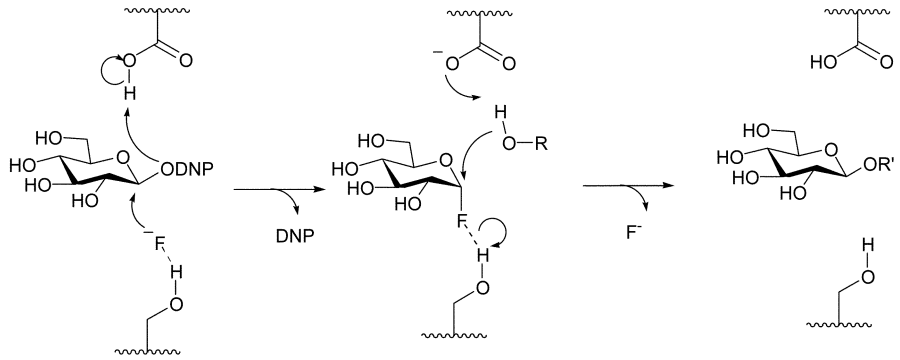
There are some other enzymatic systems which have been shown to utilise fluoride ion, most notably the observations by Withers, Zechel and co-workers,[52,53] who showed that mutant glycosyl transferase enzymes could generate α-fluoroglycosides as transient intermediates from DNP activated sugars. This was the first report of enzymatic C–F bond formation (Scheme 13).

These intermediates, although unstable, were then competent to act as activated substrates for glycosylation. Much earlier, Ochoa[54] had shown that fluorophosphate could be made from ATP when fluoride was incubated with pyruvate kinase. Again, this was an adventitious reaction when fluoride ion was added into the enzyme assay. Indeed, we have observed fluorophosphate and fluorophosphonucleosides in freeze-dried preparations of *S. cattleya* cell-free extracts after fluoride incubations with ATP,[55] however it is not clear if these metabolites are of physiological significance or if they are the consequence of indiscriminate reactivity of the pyrophosphates with relatively high concentrations of fluoride ion.

The fluorinase from *S. cattleya* is the only native fluorination enzyme to have been identified, and it has given the first insight into the biosynthesis of a fluorinated natural product. The identification of this enzyme has also opened up new prospects



Scheme 12 Enzymatic synthesis of [^{18}F]-5′-FDA from [^{18}F]-fluoride.[50]



Scheme 13 A mechanistic interpretation of enzymatic C–F bond formation in mutant glycosidase enzymes.[51,52] DNP = 2,4-dinitrophenyl.

for the preparation of organofluorine compounds by biotransformation. The possibilities have clearly been improved in this regard by the cloning and over-expression of the enzyme. It is intriguing that the structure and gene sequence of the fluorinase bear no obvious relationship to any known enzymatic activity, indicating a rather unique enzyme of limited distribution. It will be exciting now to explore biotransformations with this enzyme and also experiments where the gene is inserted into other *Streptomyces* organisms to try to induce the biosynthesis of novel organofluorine metabolites by combinatorial biosynthesis approaches.

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