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In Vitro Reconstituted Biotransformation of 4-Fluorothreonine from Fluoride Ion: Application of the Fluorinase

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SUMMARY

In this paper, we report that fluoride ion is converted to the amino acid/antibiotic 4-fluorothreonine 2 in a biotransformation involving five (steps a–e) overexpressed enzymes. The biotransformation validates the biosynthetic pathway to 4-fluorothreonine in the bacterium *Streptomyces cattleya* (Schaffrath et al., 2002). To achieve an in vitro biotransformation, the fluorinase and the purine nucleoside phosphorylase (PNP) enzymes (steps a and b), which are coded for by the *flA* and *flB* genes of the fluorometabolite gene cluster in *S. cattleya,* were overexpressed. Also, an isomerase gene product that can convert 5-FDRP 6 to 5-FDRibulP 7 (step c) was identified in *S. cattleya*, and the enzyme was overexpressed for the biotransformation. A fuculose aldolase gene from *S. coelicolor* was overexpressed in *E. coli* and was used as a surrogate aldolase (step d) in these experiments. To complete the complement of enzymes, an *ORF* coding the PLP-dependent transaldolase, the final enzyme of the fluorometabolite pathway, was identified in genomic DNA by a reverse genetics approach, and the *S. cattleya* gene/enzyme was then overexpressed in *S. lividans*. This latter enzyme is an unusual PLP-dependent catalyst with some homology to both bacterial serine hydroxymethyl transferases (SHMT) and C5 sugar isomerases/epimerases. The biotransformation demonstrates the power of the fluorinase to initiate C-F bond formation for organo-fluorine synthesis.

INTRODUCTION

Fluorinated natural products are extremely rare entities; only a handful of such compounds have been identified and characterized so far. In contrast, well over 4000 chlorinated, brominated, and iodinated natural products have been isolated (Neumann et al., 2008). *Streptomyces cattleya* is one of only two bacteria so far identified that elaborate fluorine-containing compounds (Deng et al., 2004). The bacterium possesses a biosynthetic pathway that generates the mammalian toxin fluoroacetate (FAc) **1** and the antibiotic 4-fluorothreonine (4-FT) **2** (Sanada et al., 1986). Because of the rare occurrence of fluorinated metabolites in nature and because selective fluorination is a significant activity in medicinal chemistry research, it is of interest to develop biotechnological methods for fluorochemical production. In this regard, we have been able to identify a number of enzymes on the fluorometabolite pathway to **1** and **2** in *S. cattleya*. The first enzyme is a 5′-fluoro-5′-deoxyadenosine synthase (the fluorinase), the C–F bond-forming enzyme (O'Hagan et al., 2002). This enzyme has been isolated and characterized and was shown to catalyze the reaction of S-adenosyl-L-methionine (SAM) **4** and fluoride ion to generate 5′-fluoro-5′-deoxyadenosine (5′-FDA) **5** (O'Hagan et al., 2002; Schaffrath et al., 2003; Dong et al., 2004). 5′-FDA **5** is then acted upon by a purine nucleoside phosphorylase (PNP), which mediates a phosphorolytic displacement of the adenine base to generate 5-fluoro-5-deoxy-D-ribose-1-phosphate (5-FDRP) **6** (Cobb et al., 2004). An isomerase then catalyzes the conversion of **6** to 5-fluoro-5-deoxy-ribulose-1-phosphate (5-FDRulP) **7** (Onega et al., 2007). Ribulose **6** is a substrate/product of an aldolase enzyme, and the next enzyme on the pathway cleaves 5-FDRulP **6**, in a retroaldol reaction to generate fluoroacetaldehyde (FAd) **3**. FAd **3** has been established as the last common intermediate feeding into each of the fluorometabolites **1** and **2** (Moss et al., 2000). An NAD^+-dependent fluoroacetaldehyde dehydrogenase has been purified (Murphy et al., 2001a) that oxidizes FAd **3** to fluoroacetate (FAc) **1**. Separately, a pyridoxal phosphate (PLP)-dependent enzyme activity has been identified that mediates an unusual transaldol reaction with the amino acid L-threonine and fluoroaceldehyde **3** to generate 4-FT **2** and acetaldehyde, in a unique PLP-dependent process (Murphy et al., 2001b). It was an objective of this research to overexpress each of the enzymes (enzyme steps a –e, Figure 1) involved in the pathway to 4-FT **2**, and then to recombine them in vitro to affect a synthesis of the amino acid 4-FT **2** from fluoride ion by biotransformation.

[^{18}F]-Fluoride ion is an excellent starting point for the incorporation of fluorine into organic compounds for positron emission tomography (PET) imaging studies (Tressaud and Haufe, 2008). [^{18}F]-Fluoride is prepared directly on the cyclotron without any "cold carrier" isotope and can be generated in very high specific radioactivity (GBq). Thus, it is attractive to develop a suitable synthesis that has the potential for optimization of a radiolabeled preparation of [^{18}F]-4-FT **2** from [^{18}F]-fluoride. In this study, we

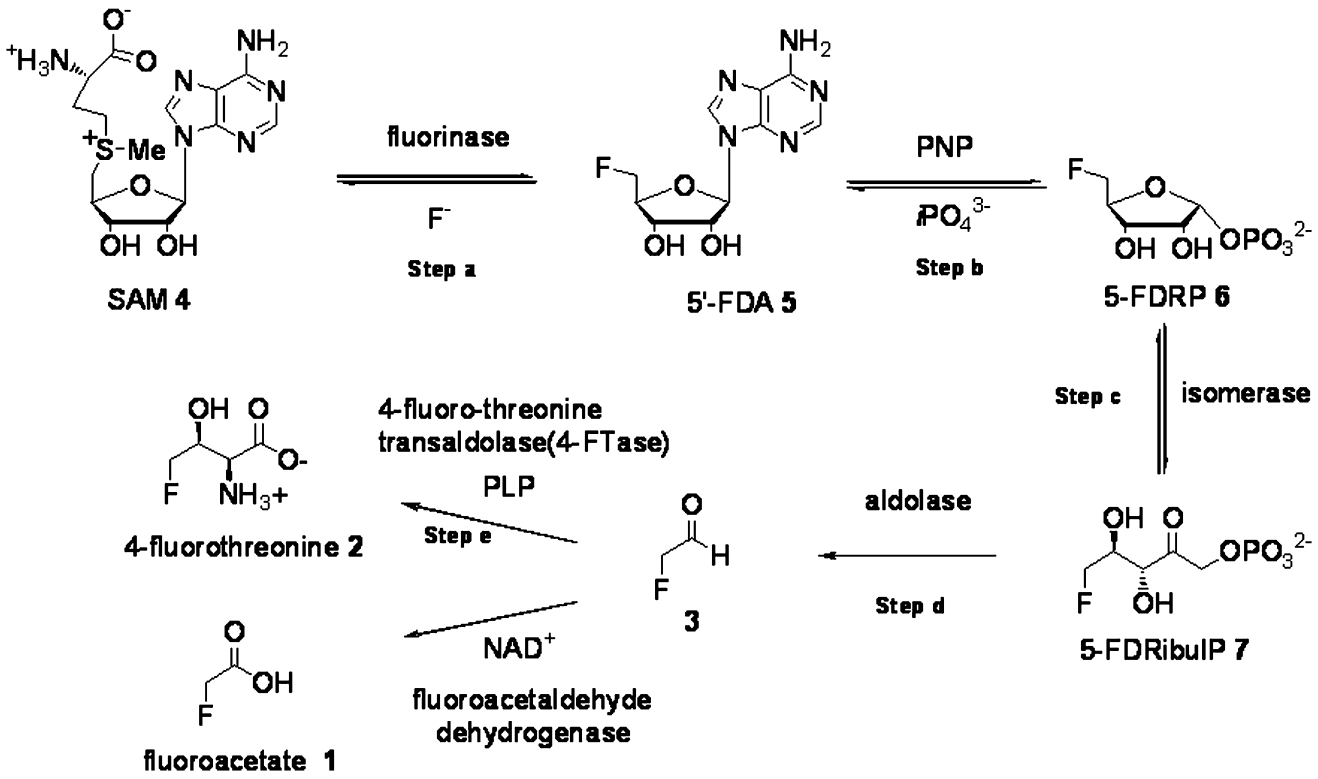
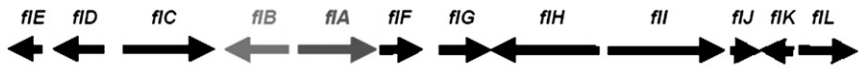


Figure 1. The Biosynthetic Pathway to Fluoroacetate and 4-Fluorothreonine
Current status of fluorometabolite biosynthesis in *S. cattleya* showing metabolic intermediates and the enzyme steps a–e and cofactors involved.

report an in vitro biotransformation combining four enzymes overexpressed from *S. cattleya* genes (fluorinase, PNP, isomerase, and PLP-transaldolase) with a surrogate aldolase from *S. coelicolor*.

In 2005, a 10 kb gene cluster was identified by targeting the fluorinase gene (*flA*) in the cosmid library from *S. cattleya* (Huang et al., 2006). Adjacent to *flA* were a number of genes that appear to be involved in the biosynthesis and regulation of the fluorometabolite pathway (Figure 2). The fluorinase *flA* gene is in the middle of the cluster, and the immediate upstream gene (*flB*) has been shown to express a purine nucleoside phosphorylase (PNP) enzyme that is selective for the conversion of 5′-FDA **5** to 5-FDRP **6** (Huang et al., 2006) (step b, Figure 1). Several regulatory and resistance genes were also identified, although their exact roles are unclear. However, the genes for the remaining three biosynthetic enzymes (enzyme steps **c–e**, isomerase, aldolase, and PLP transaldolase, Figure 1) do not appear in the gene cluster; thus, only the *flA* (fluorinase) and *flB* (PNP) gene products are available from this cluster. In this paper, we describe the cloning and sequence analysis of the PLP-transaldolase gene (step e, Figure 1) and a FDRP-isomerase gene (step c, Figure 1) from *S. cattleya*. These two gene



ORF	Start/Stop (bp)	Length (aa)	Function/Homology
E	130-795c	222	DNA binding regulatory protein
D	857-1504c	216	Dehalogenase/Phosphatase
C	1845-3036	397	MFS permease
B	3057-3953c	299	5′-FDA phosphorylase
A	4173-5069	299	5′-FDA syntase
F	5197-5751	185	DNA binding regulatory protein
G	5951-6652	234	DNA binding regulatory protein
H	6652-8052c	467	Na^+/H^+ antiporter
I	8314-9780	489	Homocysteine lyase
J	9803-10195	131	DNA binding protein
K	10592-10176c	139	Thioesterase/acyltransferase
L	10700-11374	225	DNA binding regulatory protein

Figure 2. The genes Clustered Around the Fluorinase Gene in *S. cattleya*
Organization of the 10 kb gene cluster from *S. cattleya*, highlighting the fluorinase (*flA*) and the PNP (*flB*) genes, which mediate the first two enzymes (steps **a** and **b**) of fluorometabolite biosynthesis. The annotations for the remaining genes are deduced from sequence homologies. Gene *flK* is a fluoroacetyl-CoA thioesterase/acyltransferase that has been overexpressed and assayed, and may play a role in the resistance to fluoroacetate toxicity (Huang et al., 2006).

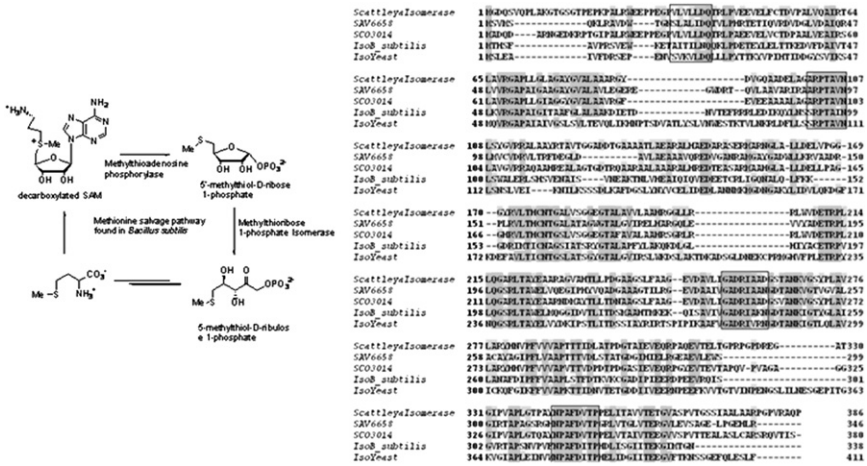


Figure 3. The L-Methionine Salvage Pathway and a Comparison of Some Isomerase Gene Sequences

Illustration of the L-methionine salvage pathway highlighting the methylthioribose-1-phosphate isomerase, which has analogy with the isomerase (**enzyme c**) on the fluorometabolite pathway. Comparison of the deduced amino acid sequences from isomerase genes identified in *S. cattleya, S. avermitilis, S. coelicolor, B. substilis*, and yeast. The peptide sequences in the black boxes were used to design degenerate primers for identification of the isomerase from *S cattleya*.

products in combination with the fluorinase, the PNP from the biosynthetic gene cluster, and an aldolase from *S. coelicolor* have been used to effect an in vitro biotransformation of 4-fluorothreonine **2** from inorganic fluoride.

RESULTS AND DISCUSSION

Overexpression of the Fluorinase and PNP Enzymes (Steps a and b)

The *flA* and *flB* genes from the 10 kb gene cluster of *S. cattleya* code for the fluorinase and the PNP enzymes, which catalyze the first two steps in fluorometabolite biosynthesis (Huang et al., 2006). The efficient overexpression of the fluorinase enzyme has been described elsewhere (Dong et al., 2004), and the enzyme is readily available and stable. Attempts at overexpression of the PNP enzyme in *E. coli* by PCR amplification of the *flB* gene from genomic DNA (*S. cattleya*) were only partially successful. Although the protein could be expressed successfully, it was largely insoluble (Huang et al., 2006), and it was difficult to obtain sufficient soluble protein for biotransformation assays. To circumvent the solubility problem on expression, the *flB* gene was fused to a modified pMAL vector coding for a maltose-binding protein (Guan et al., 1988). The vector was modified to include a His_6 tag terminus to aid purification. This fusion strategy has been used successfully to induce proper folding and generate soluble protein in cases where precipitation has been difficult. In the event that overexpression of this fused PNP-maltose-binding protein in *E. coli* gave rise to sufficiently active protein, it could be purified by Ni column elution. This PNP was used in subsequent biotransformations.

The Isomerase (Step c)

5-Methylthio-ribose-1-phosphate isomerase has been reported as a key enzyme involved in L-methionine salvage pathway (Ashida et al., 2003; Bumann et al., 2004; Tamura et al., 2008). In that pathway, shown in Figure 3, SAM **4** is converted to methylthioadenosine, and then a phosphorylase removes the adenine base in a similar manner to a PNP (step b). The resultant methylthioribose phosphate is isomerised to a ribulose. The isomerisation has clear similarities to that of the isomerase in fluorometabolite biosynthesis (step c), and, accordingly, the bacterial gene sequence to this methionine salvage enzyme acted as a start point for the identification of a similar gene/enzyme in *S. cattleya*. Homology of the *B. subtilis* isomerase gene with that of the *S. coelicolor* and *S. avermitilis* genomes highlighted two genes, SCO3014 (*S. coelicolor*) and SAV6658 (*S. avermitilis*), with ~35% amino acid identity, which until now have been annotated as "eukaryotic translation initiation factors 2B" (eIF2B). These are clearly putative *Streptomyces* methylthioribose-1-phosphate isomerases (Bentley et al., 2002). The SCO3014 gene was amplified by PCR from *S. coelicolor* genomic DNA, and the protein was overexpressed in *E. coli* for evaluation. The yield of recombinant protein was about 5 mg/L. Native gel filtration indicated that the protein is a dimer, and further purification gave a SDS monomer band of about 40 kDa. Upon incubation of the recombinant protein with 5-FDRP **6** (~1 mM, 5h) (Onega et al., 2007), 5-FDRP **6** was clearly converted to 5-FDRibulP **7,** as monitored by ^{19}F NMR.

A goal now was to identify an analogous gene in *S. cattleya*. Examination of the conserved domains of SCO3014 and SAV6658 allowed degenerate DNA primers to be designed for attempted PCR amplification using *S. cattleya* genomic DNA as the template. In the event, a 288 bp PCR product was amplified, and sequence comparison indicated a deduced amino acid sequence with 90% homology to SCO3014 and SAV6658. Chromosomal walking from this DNA fragment resulted in the sequencing of a 1161 bp ORF with a deduced amino acid sequence that had ~75% identity to the putative isomerase from *S. coelicolor*. The 1161 bp ORF was then amplified in full from *S. cattleya* genomic DNA and was overexpressed in *E. coli* in good yield (20 mg/L). The purified protein gave a SDS-page band of about 40 kDa, and the sequence was confirmed by MS-MS analysis. Biotransformation assays of in vitro prepared 5-FDRP **6** (by treatment of FDI with a commercial PNP [Deng et al., 2006]) revealed that this *S. cattleya* enzyme also catalyzes the conversion of 5-FDRP **6** into 5-FDRibulP **7**. It is most likely that this isomerase from *S. cattleya* serves the same function as that of SCO3014 and SAV6658, although its ability to catalyze the conversion of **6** to **7** may indicate that it has a dual function, particularly as there is no obvious isomerase closely associated with the *flA* and *flB* genes (Figure 2). This *S. cattleya* isomerase was used in the subsequent in vitro biotransformations (*vide infra*).

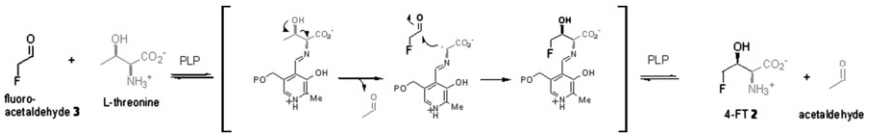


Figure 4. PLP-Dependent 4-Fluorothreonine Transaldolase
Minimal mechanism of the PLP-transaldolase involved in the final step of 4-FT **2** biosynthesis in *S. cattleya*. The enzyme has an obligate requirement for L-threonine and mediates a L-threonine/fluoroaceldehyde **3** to 4-FT/acetaldehyde cross-over reaction.

The Aldolase (Enzyme d)

The aldolase used in these experiments was a putative fuculose aldolase (SCO1844) from *S. coelicolor* (Bentley et al. 2002). Attempts to purifying the relevant aldolase from *S. cattleya* have failed so far. A partially purified cell free extract of *S. cattleya* clearly contains two aldolase activities, when assays are conducted by incubation of fluoroacetaldehyde and dihydroxyacetone phosphate (DHAP). Further fractionation resulted in the purification of a Type II Zn^{2+}-dependent fructose aldolase; however, this aldolase catalyzes the conversion of DHAP and fluoroacetaldehyde **3** to an alternative diastereoisomer, 5-fluoro-deoxyxylulose phosphate (5-FDXulP) (McGlinchey, 2006). Attempts to partition the more relevant aldolase activity have so far been frustrated by loss of protein activity on further fractionation. In order to achieve in vitro reconstruction of 4-FT biosynthesis, we have used a putative fuculose aldolase from *S. coelicolor* (SCO1844), which was amplified from genomic DNA and overexpressed from *E. coli*. In the event, a PCR product of ~700 bp was generated, which was ligated into pHISTev *E. coli* expression vector, and this led to good overexpression of a protein that demonstrated Zn^{2+}-dependent aldolase activity. Although the natural substrate for this enzyme is not 5-FDRulP **7**, it was nonetheless able to catalyze the efficient cleavage of **7** to fluoroacetaldehyde **3**. In the reverse direction, incubations of fluoroacetaldehyde (Fad) and dihydroxyacetone phosphate (DHAP) generated 5-fluororibulose phosphate, but as the minor diastereoisomer (2:1), with most probably 5-fluororhamnulose phosphate as the major product, illustrating further the lack of stereointegrity of this aldolase (Espelt et al., 2005). The overexpressed aldolase was, however, used for the conversion of 5-fluororibulose to fluoroacetaldehyde in the subsequent biotransformations acting only to generate FAd.

Identification of the 4-Fluorothreonine Transaldolase (4-FTase) Gene from *S. cattleya* (Enzyme e)

The final step in 4-FT biosynthesis in *S. cattleya* involves a pyridoxal phosphate-dependent transaldolase enzyme that mediates a cross-over reaction between L-threonine and fluoroacetaldehyde to give 4-fluorothreonine and acetaldehyde (Murphy et al., 2001b). The few bacterial PLP threonine aldolases that have been identified to date utilize acetaldehyde and glycine in a direct condensation reaction (Herbert et al., 1993). The *S. cattleya* enzyme does not utilize glycine but instead performs a mechanistically more elaborate reaction. A minimal mechanism is shown in Figure 4.

It follows that for every molecule of 4-fluorothreonine **2** generated by the bacterium, a molecule of L-threonine is sacrificed. This suggests that perhaps there is a selection advantage to the organism in producing 4-fluorothreonine **2** at the expense of L-threonine. The gene for this 4-FT transaldolase enzyme is not in the 10 kb *flA* gene cluster, and it became a research objective to identify and clone this gene. This was achieved by a reverse genetic approach after purification of the wild-type 4-FT transaldolase. The 4-FTase was purified to homogeneity using an HPLC assay, as previously reported (Scholze, 1985). It proved difficult to obtain a significant amount of protein; however, the accumulated protein after several purification protocols was pooled and was subjected to in-gel trypsin digestion followed by MS-MS amino acid sequencing. This generated 9 identifiable peptide fragments of 7–15 aa residues, and these peptide fragments were used to design degenerate DNA primers for trial and error amplification from genomic *S. cattleya* DNA. This trial and error process led to the amplification of a 240 bp piece of DNA. A BLAST (NCBI) database search of the deduced 80 aa sequence indicated ~40% homology to L-serine hydroxymethyl transferase (SHMT) proteins from other microorgansims. Although this finding initially suggested that the SHMT gene had been amplified, the homology to SHMT from *S. coelicolor* and *S. avermitilis* (Actinomycetes, which have been the subject of full genome sequencing) was ~30%, indicating a more tenuous relationship to that gene/enzyme. Chromosomal gene walking from this locus was then conducted until a 2.2 kbp sequence of DNA was established, which contained a complete open reading frame (*FTase*) of 1.905 kbp. The *FTase* coded for a 634 aa protein composed of two domains, as illustrated in Figure 5. The larger domain (440 aa) is homologous to the PLP-binding domain of SHMT enzymes in such microorganisms as archaea and thermophilic bacteria (~35% aa identity). The smaller domain (145 aa) has homology with the phosphate-binding domain of bacterial ribulose-1-phosphate-4 epimerases (*araD*) or L-fuculose aldolases (>28%). There are about 35 aa between the SHMT-like and araD-like domains that have no obvious functional correlation. Thus, this novel PLP transaldolase appears to have a hybrid construction with key binding motifs from these distinct enzymes. The *araD* enzymes as a superfamily catalyze reversible aldol/retro-adol carbon-carbon bond cleavage, often leading to epimerization (Luo et al., 2001; Lee et al., 2000). Such an activity has a connotation to the PLP-transaldolase reaction.

With the PLP transaldolase gene identified, overexpression of the protein was investigated. The *FTase* gene was introduced into the *Streptomyces lividans* TK24 strain via the *E. coli*/*Streptomyces* shuttle vector pXY200 (Cone et al., 2003). Apramycin-resistant clones were grown in the YEME medium, and the FTase was induced and expressed by the addition of thiostrepton (10 μg/mL) (Cone et al., 2003). Incubation of the cell-free extract with L-threonine (1 mM), PLP (10 μM), and fluoroacetaldehyde (~1 mM) generated 4-fluorothreonine as monitored by ^{19}F-NMR. Assays conducted in cell-free extracts of wild-type *S. lividans* TK24 did not give rise to any 4-fluorothreonine

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1       MPSSVNRTSRTEPAGHHREFPLSLAAIDELVAEEEAEDARVLHLTANETVLSPRARAVLA
61      SPLTSRYLLEHLDMRGPSPARLGNLLLRGLDRIGTIEESATEVCRRLFGARYAEFRCLSG
121     LHAMQTTFAALSRPGDTVMRVATKDGGHFLTELICRSFGRRSCTYVFDDTMTIDLERTRE
181     VVEKERPSLLFVDAMNYLFPFPIAELKAIAGDVPLVFDASHTLGLIAGGRFQDPLREGAD
241     LLQANTHKTFFGPQKGIILGNDRSLMEELGYTLSTGMVSSQHTASTVALLIALHEMWYDG
301     REYAAQVIDNARRLAGALRDRGVPVVAEERGFTANHMFFVDTRPLGSGPAVIQRLVRAGV
361     SANRAVAFNHLDTIRFGVQEITRRGYDHDDLDEAADLVAAVLLERQEPERIRPRVAELVG
421     RRRTVRYTGDPASAAGPPARERYAPPTAPAGHPARPRWIGVRLTPLPEPVTEAECAGAQR
481     LGRLAGAFPHQIDSSGNVSFTSTDGRLFVTGSGTYIKDLAPGDFVELTGAEGWTLHCRGD
541     GPPSAEAYLHHLLRERVGARYVVHNHCIPGRALETSGALVIPPKEYGSVALAEAVADACQ
601     DSQVMYVRRHGLVFWAHSYDECLALIEDVRRITG
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Figure 5. The Amino Acid Sequence of the PLP-Dependent Transaldolase

Full amino acid sequence of the PLP-transaldolase (Figure 4) from *S. cattleya*. The color coding illustrates the three domains. Bold sequence: 1–438, putative SHMT-PLP binding domain; 439–480, putative linker domain and bold sequence; and 481–634, putative phosphate-binding domain of bacterial epimerase/aldolase. The short peptides identified by MS-MS from wild-type 4-FTase are underlined.

production, indicating clearly that the biotransformation is a result of the inserted gene. The enzyme was then partially purified by affinity column chromatography. Although enzyme expression in *S. lividans* was low, gene product expression was confirmed by MS-MS sequencing of the partially purified protein. The overexpression of *Streptomyces* genes (e.g., in *E. coli*) is often problematic and leads to insoluble protein (inclusion bodies). This then requires that such genes are expressed in *Streptomyces* hosts, and even then expression is often poor. This PLP-transaldolase is a case in point. However, we have demonstrated unambiguously that the cloned gene product was expressed in the *S. lividans* host and that it was catalytically active. This preparation was subsequently used in the recombination biotransformations described below.

In Vitro Recombination Biotransformations to 4-Fluorothreonine

The biosynthetic pathway from fluoride ion and SAM **4** to 4-FT **2** in *S. cattleya* requires five enzymes (steps a-e). Access to each of the individual enzymes in an overexpressed form, either from *S. cattleya* or from a surrogate source, has been described above. A series of experiments were now conducted where either all of the enzymes were combined (experiment F), or for control reactions, one enzyme was omitted (experiments A–E) from the in vitro recombination biotransformations. All of the biotransformations were assayed by $^{19}F\{^{1}H\}$-NMR, and the spectra in Figure 6 illustrate conversions after 16 hr. All of the intermediates on the pathway have previously been isolated and characterized, and their ^{19}F-NMR resonances are already established. Identities were further reconfirmed by add-mixing reference compounds of 4-FT **2**, fluoroacetaldehyde **3**, 5′-FDA **5**, 5-FDRP **6**, 5-FDRulP **7,** and 5′-FDI **10** into product solutions of the relevant experiments for further analysis by $^{19}F\{^{1}H\}$-NMR.

In experiment A, all of the enzymes and cofactors (SAM, F^-, PLP, and threonine) except the fluorinase were combined, and as expected there was no organo-fluorine production. In experiment B, only the PNP was omitted from the reaction. Consistent with this, 5′-FDA **5** accumulates along with 5′-fluoro-5′-deoxyinosine (5′-FDI) **10**, a product that arises from enzymatic deamination of 5′-FDA **5** (Cobb et al., 2005). This conversion has

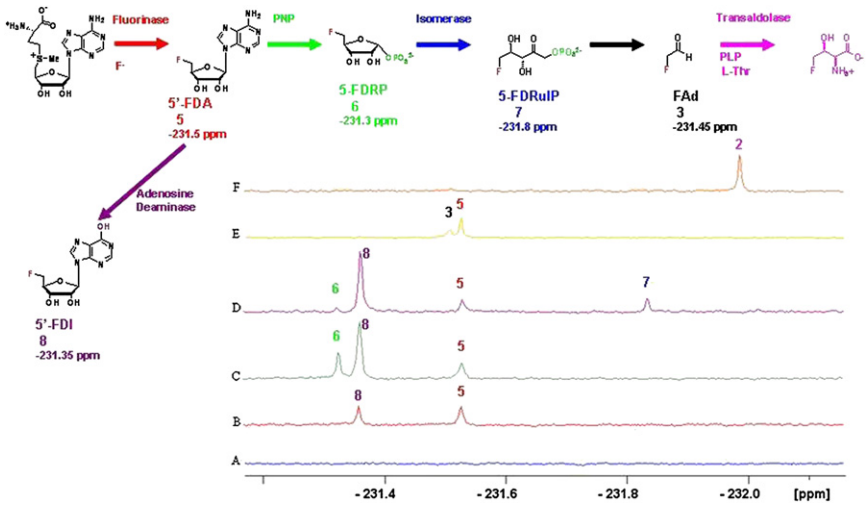
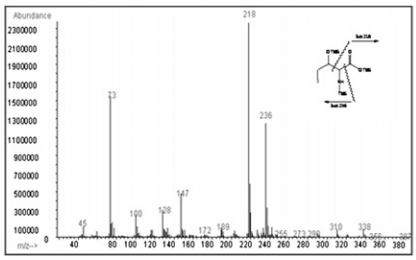


Figure 6. In Vitro Biotransformations of Fluoride Ion Using Combinations of Overexpressed Enzymes

Resultant $^{19}F\{^{1}H\}$ NMR spectra of in vitro reconstituted biotransformations when fluoride ion was incubated at 37°C for 16 hr with cloned and overexpressed enzyme combinations. Control experiments were performed by removing one enzyme each in a stepwise manner. Experiment A, minus the fluorinase. Experiment B, minus the PNP. Experiment C, minus the isomerase. Experiment D, minus the fuculose aldolase (*S. coelicolor*). Experiment E, minus the PLP transaldolase (expressed in *S. lividans*). Experiment F, complete pathway.



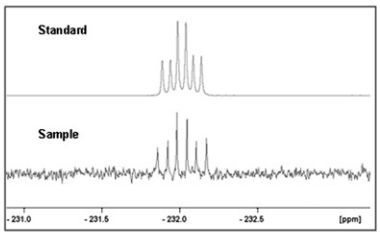


Figure 7. Analytical Data Supporting 4-FT Production

(A) GC-MS analysis of the persilylated 4-FT **2**. Two mass ions are prevalent, 218 amu and 236 amu [9]. (B) ^{19}F NMR of 4-FT **2** compared with a synthetic standard of 4-FT *2 (dt,* δ_F −232.0, $^2J_{HF}$ 46.9 Hz, $^3J_{HF}$ 25.0 Hz).

previously been identified in cell-free extracts of *S. cattleya*, where it is particularly active. In this case, it arises from a low activity in the *S. lividans* PLP transaldolase.

Experiment C has the PNP enzyme added but has no isomerase. In this case, the biotransformation moves one more step forward and now 5-FDRP **6** begins to accumulate. 5′-FDA **5** and 5′-FDI **10** are also present in the product mixture as a consequence of incomplete conversion. Experiment D is devoid of the aldolase and now 5-FDRulP **7** accumulates in the product mixture, along with all of the earlier metabolites. The isomerase is a reversible reaction (Saito et al., 2007); thus, all of the intermediates are apparent up until 5-FDRulP **7**. The shunt product 5′-FDI **10** remains a significant product because it is the result of an irreversible process and is acting as a product sink (Cobb et al., 2005). In Experiment E, the PLP-transaldolase (and adenine deaminase) activity is absent from the biotransformation, and, thus, all of the activities are in place to proceed as far as fluoroacetaldehyde **3**, which accumulates, along with residual 5′-FDA **5**. In this case, the aldolase is essentially irreversible under the conditions and pulls the equilibrium over to fluoroacetaldehyde **3** such that **6** and **7** do not appear to accumulate. Experiment F has all of the component enzymes and cofactors added, and, in the event, the biotransformation gives 4-FT **2** as the only organo-fluorine compound from fluoride ion. This complete reconstitution of all activities results in an efficient conversion with a single organo-fluorine product. The identity of 4-FT **2** was unambiguously confirmed by lyophylisation of an aliquot of the biotransformation solution and then GC-MS analysis after derivatization by persilylation. The inset in Figure 7A illustrates the fragmentation pattern in the resultant mass spectrum, which was identical to that from a synthetic reference sample of 4-FT **2** (Moss et al., 2000). Also ^{19}F-NMR (500 MHz) but without {^{1}H}-decoupling, revealed an identical ^{19}F-NMR signal at −232.0 ppm with a characteristic multiplicity (d.t $^2J_{HF}$ 46.9 Hz, $^3J_{HF}$ 25.0 Hz) to that of the synthetic standard (see Figure 7B).

SIGNIFICANCE

The paper describes the in vitro biotransformation of a fluorinated bioactive natural product from inorganic fluoride. 4-Fluorothreonine is an antibiotic secreted by the bacterium *Streptomyces cattleya*, and it is among the very rare fluorinated natural products. A combination of enzyme isolation studies, molecular biology, and metabolite identification have demonstrated that 4-fluorothreonine (4-FT) is biosynthesized along a pathway involving five enzymatic steps. Some of these enzymes are unique—for example, the fluorinase is an enzyme that unusually forms the C–F bond, and a novel pyridoxal-dependent (PLP) enzyme catalyzes a cross-over reaction between L-threonine and fluoroacetaldehyde to generate 4-FT and acetaldehyde. The remaining three enzymes are analogous to other bacterial enzymes, and thus it is not clear whether these enzymes are coded exclusively for secondary metabolite biosynthesis or whether they are recruited from primary metabolism and serve a dual function. Validation of the full biosynthetic pathway and the role of the individual enzymes is secured in an experiment involving a cocktail of the five overexpressed enzymes and appropriate cofactors. Fluoride ion is transformed to 4-FT in the in vitro recombination experiment. A series of experiments is also conducted where one enzyme is excluded from the cocktail and then the relevant fluorinated intermediate accumulate, further validating the role of the individual enzymes and the identity of the metabolic intermediates on the pathway.

The study extends the range of metabolites that have been assembled in vitro, by overexpression of all biosynthetic enzymes and in this particular case (Sattely et al., 2008), to a fluorinated antibiotic. Fluorinated compounds are becoming increasingly important as pharmaceuticals, with ~20% of licensed drugs containing a fluorine atom. The significance of this study, beyond validating the biosynthetic pathway, is that it demonstrates that the fluorinase enzyme can be used, in combination with other enzymes, to generate bioactive molecules by an in vitro biotransformation process, and thus there are prospects now for applying it more widely as a biotechnological strategy to organo-fluorine compounds—for example, as a novel approach to the radiolabeled [^{18}F]-synthesis of [^{18}F]-4-FT or of the intermediate fluorinated compounds, for application in positron emission tomography.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Culture Conditions

Streptomyces cattleya was grown in a defined medium (Reid et al., 1995). *Streptomyces lividans* TK24 and *Streptomyces coelicolor* were a gift from G. Challis (Warwick) and were grown in YEME medium. *E. coli* strains *DH5a* (Life Technologies Inc.) and JM109 (Stratagene) were routinely used as hosts for *E. coli* plasmids and the *E. coli*/*Streptomyces* shuttle vector pXY200. The pGEM-T easy cloning vector was obtained from Promega. The *E. coli* expression plasmid pHISTev (Liu et al., 2007) and *E. coli*/*Streptomyces* shuttle vector pXY200 (Cone et al., 2003) were obtained from H. T. Liu and J. H. Naismith (St. Andrews), and X. H. Yin and M. Zabriskie (Oregon State University), respectively. The pET28a(+) expression plasmid was purchased from Novagen. *E. coli* BL21 (DE3) Gold (Stratagene) and C43 (Novagen) were used as

expression hosts. Standards of 4-fluorothreonine and 5′-fluoro-5′-deoxyribose-1-phosphate were synthesized according to previous reports (Cobb et al., 2004; Moss et al., 2000).

DNA Isolation and Manipulations

Isolation of chromosomal DNA from *S. cattleya* and *S. coelicolor* was performed by standard procedures (Kieser et al., 2000), and QIAprep Spin Miniprep kits (QIAGEN) were used to prepare plasmids from *E. coli* strains. Restriction endonucleases, DNA ligase, DNA polymerase, and alkaline phosphatase were purchased from various sources and used according to the manufacturers' recommendations. DNA fragments were purified using Wizard PCR preps DNA purification system (Promega). The protoplast of *S. lividans* was generated according to standard procedures (Kieser et al., 2000). For degenerate primers, PCRs were performed with 6% DMSO and GoTaq DNA polymerase (1.5 unit, Promega) (20 μl). PCR conditions involved preheating at 98°C for 5 min, followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 58°C for 1 min, and extension at 72°C for 1–5 min depending on the size of DNA amplification, with 7 min infilling at 72°C. The PCR products were purified and ligated into the pGEM-T easy vector for the blue-white screening. The resultant plasmids were subjected to DNA sequencing. For ORF amplification, PCRs were conducted with 6% DMSO and pfu DNA polymerase (2.5 units, Fermentas) (50 μl). DNA sequencing was performed by The Sequencing Service (College of Life Sciences, University of Dundee, Scotland, www.dnaseq.co.uk) using Applied Biosystems Big-Dye Ver 3.1 chemistry on an Applied Biosystems model 3730 automated capillary DNA sequencer.

Degenerate PCR for Isomerase and 4-FTase from *S. cattleya*

Four degenerate primers (MWG-Biotech) were designed and used for the amplification of the corresponding gene fragment by PCR—IsoSca-dF1, IsoSca-dF2, IsoSca-dR1, and IsoSca-dR2. Only a pair of primers (IsoSca-dF2 and IsoSca-dR1) gave a desirable PCR product. Eight degenerate primers (MWG-Biotech) were designed on the basis of the MS-MS peptide sequences information (see Supplemental Data available online) and were used for the amplification of the corresponding gene fragment by PCR. Only one pair of primers, 625-dF and 528-dR, gave a desirable PCR product. PCRs were performed by using *S. cattleya* genomic DNA as the template.

Chromosomal Walking of FTase and Isomerase

The APAgene GOLD Genome Walking Kits (BIO S&T, Canada) were used for chromosomal walking of FTase and isomerase from *S cattleya*. For the FTase, two primers were used for forward and reverse direction gene walking experiments, FTase-F1 and FTase-R1, respectively. For the isomerase from *S cattleya*, four primers were used for gene walking—IsoSca-F1, IsoSca-F2, IsoSca-R1, and IsoSca-R2. PCRs were performed according to the manufacturer's instructions. ApE (v1.12) was used for sequence editing. Database searches used the BLAST algorithm. FramePlot (ver 2.3) was used for the sequence assembly (Ishikawa and Hotta, 1999).

Expression and Purification of the Isomerase from *S. cattleya*

The ORF ScaIso was subcloned into the pHISTev plasmid (Liu et al., 2007) using the following two primers, ScaIso-F and ScaIso-R, with restriction sites of NcoI and XhoI, respectively. PCRs were performed using *S. cattleya* genomic DNA as the template. The resultant plasmid pHIS-ScaIso was transfected into BL21 (DE3) Gold (Stratagene) competent cells. The same expression and purification procedures for the SCO3014 gene were used (Supplemental Data).

Expression and Purification of FTase

The ORF ScaFTase was subcloned into the pET28a (+) (Novagen) vector using the following two primers: ScaFTase-F and ScaFTase-R, with restriction sites of NdeI and XhoI, respectively. The resultant plasmid pET-ScaFTase was transfected into BL21 (DE3) Gold (Stratagene) competent cells. After induction, the cell lysate was subject to SDS-page analysis. Inclusion bodies were observed. Upon the incubation of cell-free extract (500 μL) with fluoroacetaldehyde (1mM), PLP (20 μM), and threonine (1 mM) at 37°C, up to 16 hr, there was no enzymatic activity monitoring by ^{19}F NMR. The FTase gene was then subcloned into an *E. coli*/*Streptomyces* shuttle vector pXY2000 with NdeI and EcoRI restriction sites. The resultant plasmid pXY-ScaFTase was transfected into the protoplast of *S lividans* TK24, followed by standard procedures (Kieser et al., 2000). The transfected *S. lividans* protoplast was then plated in SFM medium with $MgCl_2$ (10 mM) at 30°C for 16 hr and flooded with apramycin (1 ml, 25 μg/ml). After 3–5 days of incubation at 30°C, the surviving spores were picked up and grown in 10 ml YEME medium supplemented with apramycin (50 μg/ml) at 28°C until the spores were observed. The medium was then incubated with 100 ml YEME supplemented with apramycin (50 μg/ml) at 28°C for 60 hr, and the protein was induced by adding thiostrepton (10 μg/ml) for another 24 hr. The cells were harvested and subjected to sonication. The cell-free extract was partially purified by Ni-affinity column (QIAGEN), and the eluent was subject to overnight dialysis in PBS buffer (50 mM phosphate and 150 mM NaCl [pH 7.5]). The enzyme activity was monitored by the incubation of aliquots of protein fraction (500 μl) and fluoroacetaldehyde (1 mM), PLP (20 μM), and threonine (1 mM) at 37°C for 16 hr (^{19}F NMR analysis).

The expression of FTase was confirmed by in-gel tryptic digest and analysis of the resultant peptides by nanoLC-ESI MSMS (UltiMate (Dionex)) and Q-Star Pulsar XL (Applied Biosystems). The MS/MS data file generated was analyzed using the Mascot 2.1 search engine (Matrix Science, London, UK) against an internal database consisting of a bacterial genome background to which the FTase sequence (among others) had been added. The data were searched with tolerances of 0.2 Da for the precursor and fragment ions, trypsin as the cleavage enzyme, up to one missed cleavage assumed, carbamidomethyl modification of cysteines as a fixed modification, and L-methionine oxidation selected as a variable modification. The identity of the FTase gel band was confirmed with a Mascot Score of 888, with 29 unique peptides observed (15 with Ion Scores above the identify threshold and a further 3 between the homology and identity thresholds) and with a sequence coverage of 37%.

Overexpression of the Putative Fuculose Aldolase from *S. coelicolor*

The putative fuculose aldolase SCO1844 from *S. coelicolor* was subcloned into the pHISTev vector (Liu et al., 2007) using two primers, ScoFalD-F and ScoFalD-R (Supplemental Data). The ORF SCO1844 was ligated into the pHISTev vector using the above primers containing restriction sites of EcoRI and HindIII, respectively. The resultant plasmid pHISTev-FALD was introduced into *E. coli* BL21 (DE3) Gold (Stratagene) competent cells and grown in Luria broth containing kanamycin (50 μg/ml) at 37°C until an absorbance of 0.6 at 600 nm was reached. The SCO1844 protein was overexpressed by adding IPTG (1 mM), and cells were left to grow at 16°C for 16 hr. Cells were then harvested by centrifugation and were subject to sonication for lysis. The cell-free extract with PBS and imidazole (10 mM) was then centrifuged (2×, 20,000 g) at 4°C for 15 min. The supernatant was collected by passing through a Ni-affinity column (QIAGEN), and the active fractions were eluted by adding PBS buffer with imidazole (100 mM). The eluent was dialyzed for 16 hr at 25°C by adding thrombin (0.5 unit; Sigma Aldrich Co. Ltd.). The dialysate was then subjected to size exclusion chromatography (Column, Amersham Co). The active fractions gave a monomeric mass of 25 kDa by SDS-PAGE, confirmed by MS-MS. Size exclusion chromatography revealed the active protein as a tetramer, purified to 1.3 mg/ml, and then subjected to enzymatic assay. FAd **3** was prepared as described previously (Moss et al., 2000). DHAP was attained from Sigma Chemical Co. Incubation for 3 hr with DHAP (1 mM) and FAd **3** (1 mM) resulted in the production of 5-FDRulP and 5-fluororhamnulose-P, identified by ^{19}F{^{1}H} NMR (Onega et al., 2007). Incubation with EDTA (1 mM) resulted in the abolition of enzymatic activity.

In Vitro Reconstitution of 4-FT from Inorganic Fluoride Ion

The fluorinase, PNP, isomerase, and fuculose aldolase were all overexpressed in *E. coli* and purified to homogeneity by Ni-affinity and size exclusion chromatography to ~1 mg/ml in PBS buffer. The PLP transaldolase was purified as previously described to approximately 0.25 mg/ml in PBS buffer. All of the pathway enzymes were added into an eppendorf tube (1.5 ml) to a final concentration of 0.1 mg/ml. They were incubated with SAM (1.4 mM, Sigma), KF (35 mM, Sigma), PLP (0.7 mM, Sigma), and L-threonine (35 mM, Sigma) for 24 hr at 37°C. The reaction was stopped by heat inactivation at 95°C for 5 min followed by centrifugation of 2 min at 14,000 g. The supernatant was then subjected to ^{19}F{^{1}H} NMR analysis. Control experiments were performed by removing a single component of the reaction (enzymes and substrates/cofactors) in turn and replacing it with an equivalent volume of PBS and subjecting it to the same conditions and analysis (see Figure 3). 4-FT production was confirmed by ^{19}F{^{1}H} NMR, ^{19}F NMR, and GC-MS.

For GC-MS analysis, the lyophilized samples were per-trimethylsilylated by adding N-Methyl-N-(trimethylsilyl) trifluoroacetamide and heating for 60 min at 100°C. GC-MS analysis was performed on an Agilent 5890 GC, which was directly attached to an Agilent 5973A mass selective detector (MSD). The GC was equipped with an Ultra 1 fused-silica capillary column (Agilent Technologies; 12 m × 0.25mm × 0.17 μm). The oven temperature was programmed to hold for 1 min at 100°C and then ramp at 10°C/min to 300°C. The injector and transfer line temperatures were set at 250°C, and the per-trimethylsilylated sample (1 μl) automatically injected in the splitless mode. The MSD was operated in the full scan mode measuring ion currents between *m/z* 30 and 500 amu.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, five figures, and Supplemental References and can be found with this article online at http://www.cell.com/chemistry-biology/supplemental/S1074-5521(08)00415-8.

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