Enzymatic C-F Bond Formation and Fluorinated Natural Product Biosynthesis

Reported by Richard W. Pierce December 2, 2004

INTRODUCTION

Natural products are both a major source of pharmaceutically relevant compounds and an inspiration for pharmaceutical development. Over 25% of all drugs originated from natural products, and more than 80% of the world's total population rely on natural extracts for primary healthcare.[1] A potent subset of natural products is halogenated compounds, which are diverse and have attracted great interest from manufacturers and researchers.[2] The relative distribution of the halogens in these natural products does not coincide with the natural availability of the halogens themselves. Fluorine is the most prevalent halogen comprising 0.065 percent of the Earth's crust, but it is incorporated into only 30 known natural products.[3] The less naturally abundant halogens, chlorine and bromine are incorporated into more than 4,500 compounds, with more than 100 more that contain iodine.

The incorporation of halogens into organic molecules, especially fluorine, has a profound effect on biological activity. In contrast to the rarity of fluorinated natural products, the number of fluorine containing pharmaceutical agents is enormous. The increasing prevalence and success of fluorine-containing pharmaceuticals has instigated further interest into the metabolism of such compounds. Furthermore, the studies on halogenated drugs have led to the development many novel biological scaffolds and targets.

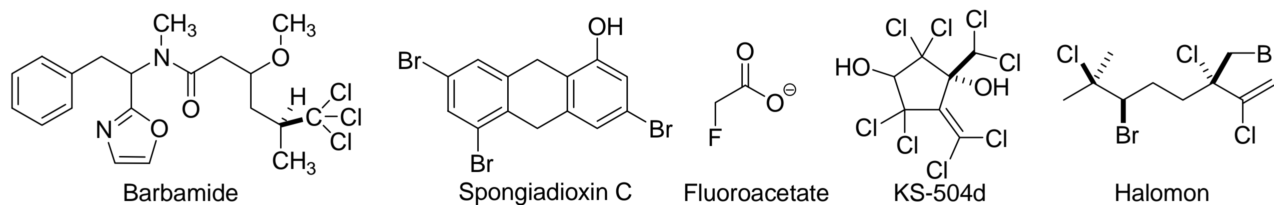

Figure 1. Selected halogenated natural products

This review will detail the uniqueness of fluorine in natural products and the use of engineered enzymes to catalyze C-F bond formation. Enzymes that are capable of substrate level fluorination and how fluorinated substrates are assembled into observed fluorinated natural products as well as the stereochemical course of the key metabolic enzyme will also be discussed. Fluorinated natural products are unique because many of the biosynthetic mechanisms for other halogenated products are not mechanistically plausible with fluorine.

PROPERTIES OF FLUORINE

The special characteristics of fluorine drastically affect its availability and mechanistic utility in biological systems. Fluorine is more electronegative (χ), smaller, has higher oxidation potential, and is

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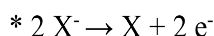
less polarizable (α) than the other halogens as shown in Table 1. In nature, fluoride is most prevalent in the form of insoluble salts such as fluorspar (CaF_2) and cryolite (Na_3AlF_6).[4] When dissolved in water, fluoride would be a potent nucleophile except for the tightly coordinated solvent sphere accounting for the high energy of solvation of fluoride. The high oxidation potential to form F^+ is a third factor prohibiting fluorine incorporation into natural products.

Table 1. Atomic properties of the halogens

	χ	Atomic Radius (Å)	Ionic Radius (Å)	α ($Å^3$)	Oxidation Potential (V)*	Hydration Energy (kcal/mol)
-F	4.0	1.47	1.33	0.5	-3.06	117
-Cl	3.2	1.75	1.81	2.2	-1.36	84
-Br	3.0	1.85	1.96	3.1	-1.07	78
-I	2.7	1.98	2.20	4.7	-0.54	68


* $2\,X^- \rightarrow X + 2\,e^-$

BIOSYNTHESIS OF NON-FLUORINATED HALOGENATED PRODUCTS

Several major classes of enzymes, termed halogenases, have been identified that incorporate a halogen into a variety of different molecules.[5] These halogenases catalyze the incorporation of either a bromine or chlorine atom, with varying degrees of regio- and stereospecificity. The heme- and vanadium-dependent halogenases use H_2O_2 and an active site metal to form a hypohalous species from the halide anion to install a halogen at a point of unsaturation in an enzyme substrate.[6] Metal-free halogenases require H_2O_2 to catalyze the formation of a peroxy acid which subsequently forms the hypohalous species to react with an unsaturated substrate.[7] The extraordinarily high oxidation potential of fluorine precludes this pathway for fluorination in biological systems. $FADH_2$-dependent halogenases require molecular oxygen to form an epoxide intermediate which is opened via nucleophilic attack with halide anion.[8] Biosynthesis of barbamide may proceed via a radical mechanism for the polychlorination of a methyl group.[9] The properties of fluorine do not exclude incorporation via the flavin-dependent or radical mechanisms.

BIOSYNTHESIS OF FLUORINATED NATURAL PRODUCTS

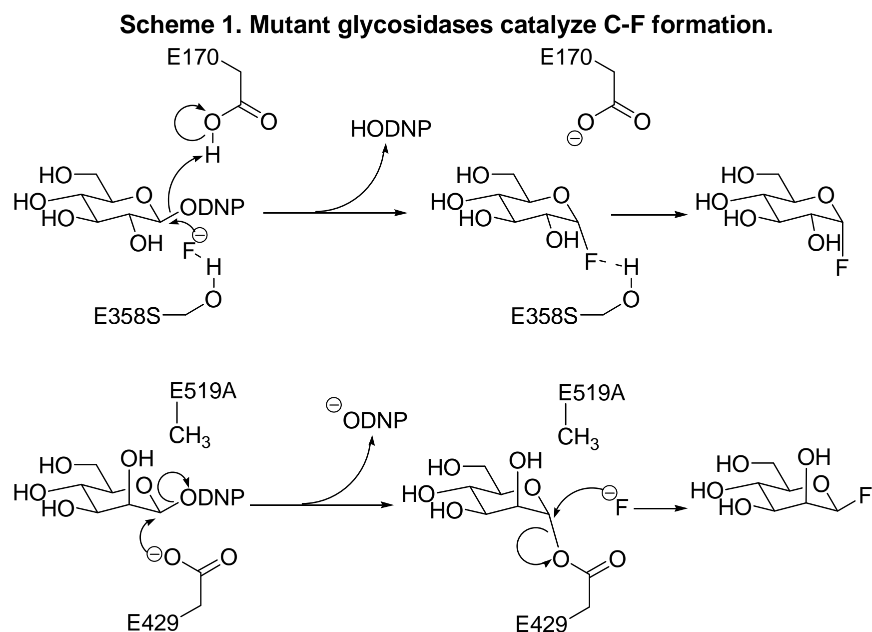
The low solubility of naturally occurring fluoride salts, low nucleophilicty of fluoride due to tight solvation, and extremely high oxidation potential of fluorine are prohibitive factors for biosynthesis of fluorinated compounds. Enzymes are well-suited to overcome the high solvation energy of fluoride by use of networks of hydrogen bonding. However, enzymes are not suited to generate an F^+ species.

Nonetheless, enzymes have evolved fluorinase function and have been rationally designed for fluoride incorporation into natural products.

Engineered C-F Enzymatic Bond Formation

Glycosidases have been designed to incorporate fluoride ion into gluco- and mannosaccharides via nucleophilic displacement of 2,4-dinitrophenol (DNP) at the C(1) position of β-glucopyranosyl and β-manopyranosyl substrates as shown in Schemes 1.[10] Such systems require excellent leaving groups such as DNP and concentrations of fluoride as high as 2 M. Mutations of active site carboxylic acid residues are essential to inhibit glycosiadase activity. Mutation of the active site acid/base residue to alanine to allow more room (Man2A E429A, Abg E358A) results in enzyme that is inactive until addition of NaF. Likewise, mutation of the active site nucleophile to a serine to provide greater opportunity for hydrogen bonding of solvated fluoride (Man2A E519S, Abg E358S) also results in activity that must be rescued by NaF. Such mutations have generated enzymes with limited but significant specificity for fluoride as the nucleophile over other halogens. Kinetic parameters (Table 2) indicate that reaction rate and enzyme specificity are low.

Scheme 1. Mutant glycosidases catalyze C-F formation.



Fluorinated Natural Products

Although more than thirty fluorinated natural products are known, many have the same general structure such as ω-fluorostearic and ω-fluorooleic acids (Figure 2).[11,12] Fluoroacetate is the first biosynthetic fluorine-containing compound discovered and is a progenitor to other fluorinated products. Fluoroacetate enters the Krebs cycle as the coenzyme A thioester and may be converted into the (2*R*,3*R*)-2-fluorocitrate or used to synthesize ω-fluoro fatty acids.

Table 2. Kinetic parameters for glycosiadase mutants*

	k_{cat} (s^{-1})	K_m (M)	k_{cat}/K_m ($s^{-1}M^{-1}$)
Agb W.T.	60	21	2.9
Agb E358S	0.48	16	0.03
Agb E358A	0.36	13	2.7 x 10-2
Man2A W.T.	132	220	0.60
Man2A E519S	0.23	36	6.4 x 10-3
Man2A E519A	0.34	36	9.6 x 10-3

* All reactions run at 2 M KF, pH 6, 25°C

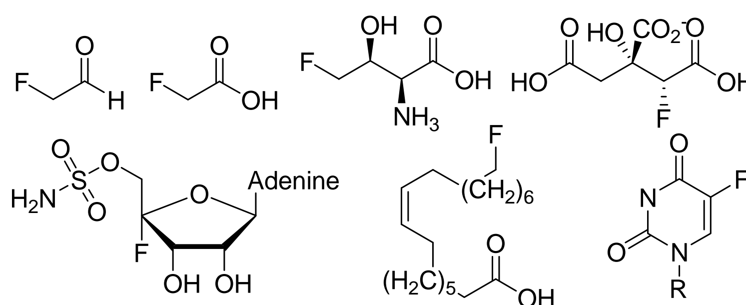
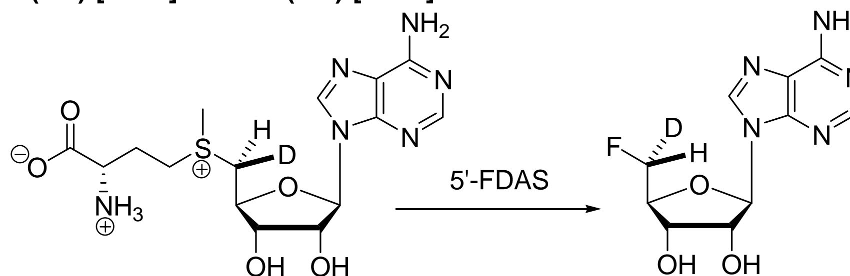


Figure 2. Naturally occuring fluorinated compounds

Biosynthetic C-F Bond Formation

An enzyme capable of nucleophilic fluorination has been identified from *Streptomyces cattleya*. *S cattleya* is known to excrete significant amounts of fluoroacetate and fluorothreonine after several days growth in medium enriched with NaF.[13] Partially purified *S. cattleya* cell lysate fractions exhibit the ability to convert inorganic fluoride to fluoroacetate.[14] The isolated enzyme that is able to catalyze the transformation of *S*-adenosylmethionine (SAM) to 5'-fluoro-5'-deoxyfluoroadenosine (5'-FDA) is named 5'-FDA synthase.[15] This reaction proceeds with inversion of configuration at the 5' center. A 1.9 Å resolution X-ray crystal structure of the enzyme-product complex indicates correct geometry for inorganic fluoride to displace the methionine via an S_N2 mechanism, as indicated by a cartoon representation (Figure 3).[16]

Scheme 2. Reaction and stereochemical outcome of fluorination of (5'S)-[5'-2H]-SAM to (5'R)-[5'-2H]- 5'-FDA.



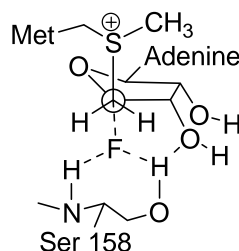


Figure 3. Active site geometry of 5'-FDA synthase.

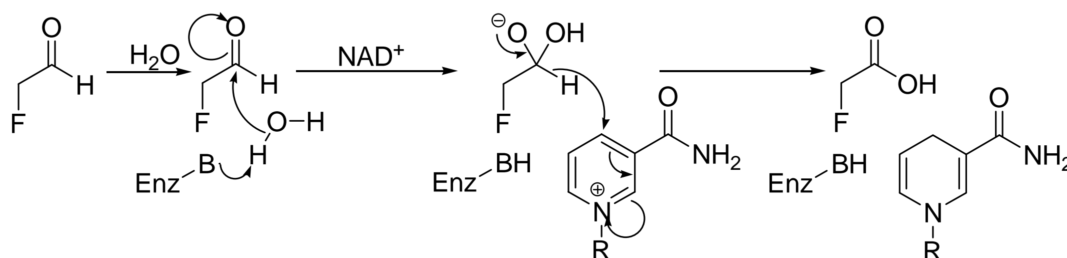
5'-FDA synthase is not a chemically efficient enzyme as indicated by the low k_{cat}= 0.00069 s^{-1}. The binding constants for fluoride $K_m(F^-)$= 2 mM, and SAM $K_m(SAM)$= 74 μM are also high. The specificity constant, k_{cat}/K_m=1.64 $M^{-1}s^{-1}$ is very far from the diffusion controlled limit of 10^8 $M^{-1}s^{-1}$ indicating that 5'-FDA synthase is a very inefficient enzyme. *S*-Adenosyl-l-homocysteine is a competitive inhibitor of 5'-FDA synthase with K_I = 29μM however sinfungin, another common inhibitor of enzymes that require SAM, was not a significant inhibitor. Computation studies indicate that fluoride must bind first, followed by SAM.

Discovery of a natural fluorinase is very significant; however there is a missing link between 5'-FDA synthase and the well studied fluoro-metabolites. The biological processes converting 5'-FDA to fluoracetaldehyde are not known. All fluoro-metabolites, with the exception of the recently discovered 5-fluorouracil derivatives, are derived from fluoroacetaldehyde.[17] Atomic labels, both deuterium and carbon, indicate that the carbon-fluorine bond formed by 5'-FDA is conserved in fluoracetaldehyde and other fluoro-metabolites. Despite the lack of understanding of the mechanistic fate of 5'-FDA, much is known about the biosynthetic processes converting fluoracetaldehyde into more complex fluoro-metabolites such as fluoroacetate, 4-fluorothreonine, ω-fluorofatty acids, and (2*R*,3*R*)-2-fluorocitrate.

Biosynthesis of fluoroacetate

The first enzyme identified that is directly involved in fluoro-metabolite biosynthesis is fluoracetaldehyde dehydrogenase.[18] Fluoracetaldehyde dehydrogenase is able to catalyze the NAD^+ dependent oxidation or fluoracetaldehyde to fluoroacetate. Although no mechanism has been proposed, significant sequence identity (>50%) to better-studied NAD^+-dependent aldehyde dehydorogenases suggests a nucleophilic attack of water to the carbonyl, followed by loss of a hydride as shown in Scheme 3.

Scheme 3. NAD+ Dependent oxidation of fluoroacetaldehyde to fluoroacetic acid.



Kinetic parameters for a series of substrates are given in Table 3. Fluoroacetaldehyde dehydrogenase exhibits a tenfold higher affinity for fluoroacetaldehyde than for acetaldehyde as exhibited by the K_m values indicating that electronic factors are more significant than steric interactions in substrate recognition. Glycoaldehyde is the optimal substrate as indicated by a larger turnover number. Several other aldehyde dehydrogenases demonstrate fluoracetaldehyde dehydrogenase activity but much higher concentrations of substrate are required for similar activity.[18]

Table 3. Kinetic parameters for fluoracetaldehyde deydrogenase*

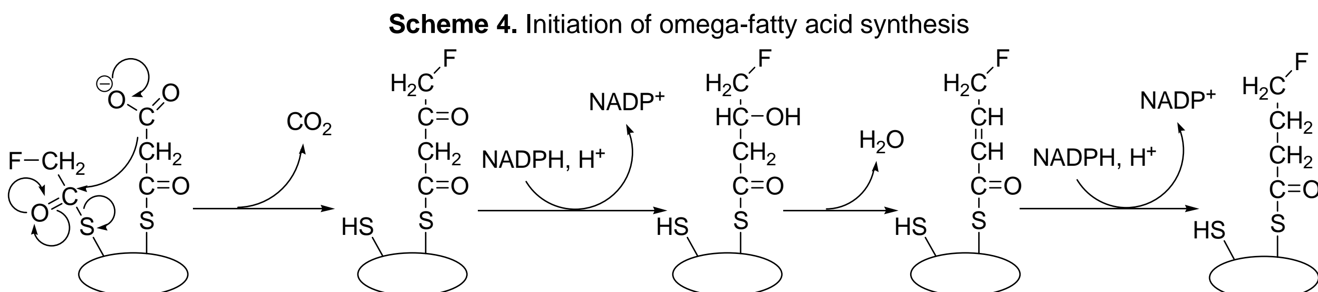
	k_{cat} (s^{-1})	K_m (M)	k_{cat}/K_m ($s^{-1}M^{-1}$)
2-fluoroacetalaldehyde	0.131	8.0 x 10-5	1.6 x 103
glycoaldehyde	0.189	15 x 10-5	1.3 x 103
acetaldehyde	0.118	81 x 10-5	0.15 x 103
benzaldehyde	0.091	32 x 10-5	0.28 x 103

* Assayed at 340 nm at pH 6.5, 25°C with 0.25 mM substrate

Biosynthesis of ω-Fluoro-Fatty Acids and (2*R*,3*R*) 2-Fluorocitrate

2-Fluoracetaldehyde is a biosynthetic precursor to several fluorinated metabolites.[11] This is because fluoroacetate may be converted to the fluoroacetyl-CoA ester. The fluoroacetyl-CoA ester may metabolized in the same manner as the non-fluorinated analog. Found mostly in the seeds of tropical plants, ω-fluorofatty acids are biosynthesized via the same mechanism as non-fluorinated analogs.[19] Fluoroacetate condenses with coenzyme A to form the thioester which is able to utilized by fatty acid synthase as the initiating module of fatty acid synthesis, as shown in Scheme 4. Because only terminally fluorinated fatty acids are found in nature, fluorinated malonyl-CoA units are not formed or incorporated into fatty acid bisynthesis. Only the fluoroacetate thioester is incorporated in the initiation stage.

Scheme 4. Initiation of omega-fatty acid synthesis



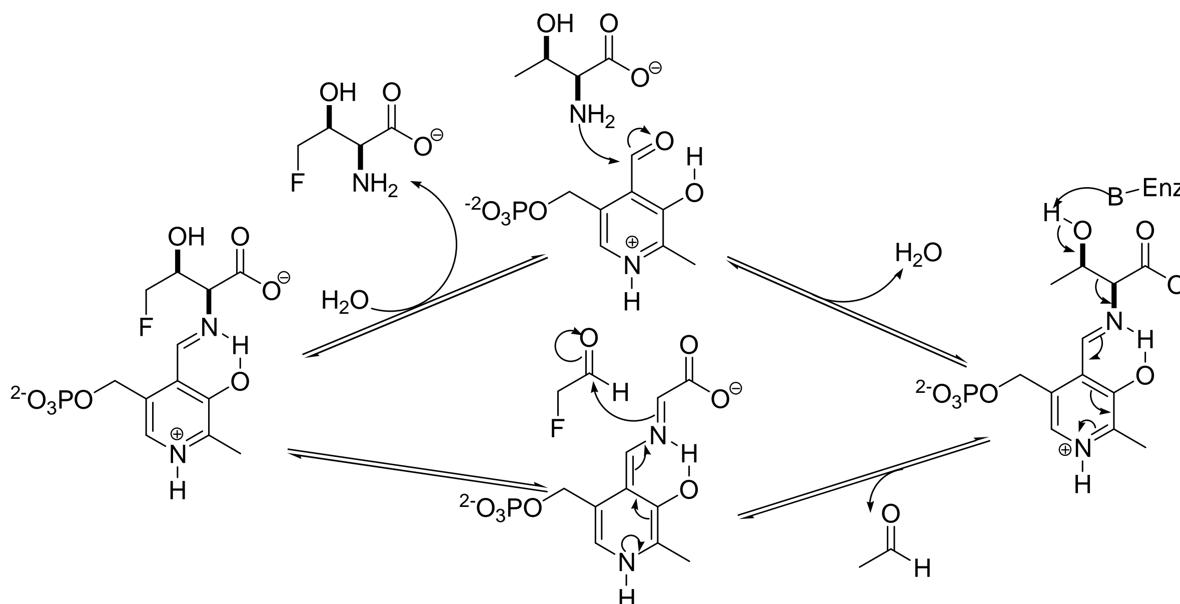
2-Fluorocitrate is also biosynthesized via the same mechanism as the non-fluorinated analog. Once incorporated into the citric acid cycle, the fluoroacetate-CoA thioester condenses with oxaloacetate. This reaction is catalyzed by citrate synthase and only the 2*R*, 3*R* diastereomer is formed.

Because only the 2*R*, 3*R* diastereomers is a potent inhibitor of aconitase, this specific process has been dubbed a 'lethal synthesis.'

Biosynthesis of fluorothreonine

Fluorothreonine is biosynthesized by a novel transaldolase, isolated from *S.cattleya*, via a known mechanism.[20] The transaldolase is novel because, unlike other such enzymes, glycine is not utilized as a substrate. A required cofactor is pyridoxal-5'-phosphate and a mechanism has been proposed (Scheme 5). When chloroacetate is incubated with the enzyme and threonine, chlororthreonine is produced. Threonine is essential because the transaldolase is not able to utilize glycine as a substrate.[20] It is not known if *S.cattleya* incorporates fluorothreonine into protein products or if the only purpose is to excrete it as a toxin.

Scheme 5. Mechanism of fluorothreonine transaldolase.



Applications of 5'-FDA Synthase

Despite its recent discovery, a biomedical application for 5'-FDA synthase has already emerged.[21] SAM derivatives with radioactive labels have been used to visualize metastasizing tumors in non-invasive PET scans. A major limitation of this technology is the half life of the nuclei used such as ^{11}C, ^{18}F, ^{15}O, ^{13}N with half lives of 20, 110, 2, and 10 minutes respectively. Longer half lives allow for the superior usefulness, and 110 minutes for ^{19}F affords the greatest synthetic utility. Even with a 110 minute half-life, the types of molecules that can be rapidly synthesized and purified for PET scanning are limited. 5'-FDA synthase has been shown to effectively catalyze incorporation of ^{19}F in 5'-fluoro-5'-deoxyfluoroadenosine in preparation for PET scanning.

CONCLUSION AND OUTLOOK

A natural fluorinase has been discovered that is able to catalyze the incorporation of fluorine at the (5') position of *S*-deoxyadenosine via nucleophilic displacement of a methionine group. Although much is known about the subsequent biosynthetic pathways, more work is needed to understand the immediate fate of 5'-FDA. With the ever-increasing proportion of fluorinated pharmaceuticals, understanding how fluorinated compound are metabolized has far reaching implications in the pharmaceutical industry. Glycosidases with engineered fluorinases activity have great potential to afford stereospecifc glycosides in high yield, but require more work to reach practicality.

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