Enantiodivergent *α*-Amino C–H Fluoroalkylation Catalyzed by Engineered Cytochrome P450s

Juner Zhang†, Xiongyi Huang†, Ruijie K. Zhang‡, and Frances H. Arnold*

Division of Chemistry and Chemical Engineering, California Institute of Technology, 1200 East California Boulevard, MC 210-41, Pasadena, California 91125, United States

Supporting Information Placeholder

ABSTRACT: The introduction of fluoroalkyl groups into organic compounds can significantly alter pharmacological characteristics. One enabling but underexplored approach for the installation of fluoroalkyl groups is selective C(*sp³*)–H functionalization due to the ubiquity of C–H bonds in organic molecules. We have engineered heme enzymes that can insert fluoroalkyl carbene intermediates into *α*-amino C(*sp³*)–H bonds and enable enantiodivergent synthesis of fluoroalkyl-containing molecules. Using directed evolution, we engineered cytochrome P450 enzymes to catalyze this abiological reaction under mild conditions with total turnovers (TTN) up to 4,070 and enantiomeric excess (ee) up to 99%. The iron-heme catalyst is fully genetically-encoded and configurable by directed evolution so that just a few mutations to the enzyme completely inverted product enantioselectivity. These catalysts provide a powerful method for synthesis of chiral organofluorine molecules that is currently not possible with small-molecule catalysts.

Fluoroalkyl groups are important bioisosteres in medicinal chemistry that can enhance the metabolic stability, lipophilicity, and bioavailability of drug molecules.[1] Conversion of C–H bonds into carbon-fluoroalkyl bonds represents one of the most appealing strategies for fluoroalkyl group incorporation.[2] Such methods are of high atom economy and provide efficient ways to obtain new organofluorine molecules via late-stage functionalization of complex bioactive molecules.[3] Despite the synthetic appeal of this strategy, however, enantioselective C(*sp³*)–H fluoroalkylation reactions are noticeably lacking. Major obstacles to development of transition-metal catalyzed C–H fluoroalkylation reactions are the inherent challenges associated with carbon-fluoroalkyl bond cross-coupling pathways, such as slow oxidative addition of fluoroalkyl nucleophiles[4] and facile fluoride elimination of organometallic species.[4b,5]

A strategy involving insertion of fluoroalkylcarbene intermediates into C(*sp³*)–H bonds could potentially circumvent these challenges. Although metal fluoroalkylcarbene intermediates have been utilized for a number of carbene transfer reactions,[5b,6] their applications for C–H functionalization have rarely been explored.[7] Transition metal catalysts, including those based on rhodium[8], iridium,[9] copper,[10] iron,[11] and other metals,[12] have been shown to catalyze carbene insertion into C(*sp³*)–H bonds. Intermolecular stereoselective reactions, however, are typically constrained to dirhodium-based catalysts with carbene precursors bearing both electron-donating and electron-withdrawing substituents at the carbene carbon (referred to as donor-acceptor carbene reagents).[8a,12a] The electron-donating group is required to attenuate the high reactivity of dirhodium-carbene intermediates and offers better stereo-control of the C–H functionalization/C–C bond forming step.[13] Catalysts that can use acceptor-only-type perfluorodiazoalkanes as carbene precursors for direct, enantioselective C(*sp³*)–H fluoroalkylation have not been reported.

Our group recently disclosed iron-heme enzymes derived from cytochromes P450 that catalyze abiological carbene C–H bond insertion reactions using several acceptor-only diazo compounds.[14] Building on this effort, we now show that engineered cytochrome P450 enzymes can adopt C–H fluoroalkylation activity with high efficiency and enantioselectivity, achieving direct C–H fluoroalkylation of substrates that contain α-amino C–H bonds. Given the high prevalence of amines in pharmaceuticals, this simple biocatalytic method provides an efficient route to molecular diversification through selective C–H functionalization.

To identify a suitable starting point for directed evolution of a C–H fluoroalkylation enzyme, we first challenged a panel of 14 heme proteins in clarified *Escherichia coli* lysate with *N*-phenylpyrrolidine (**1a**) and 2,2,2-trifluoro-1-diazoethane (**2**) as model substrates under anaerobic conditions (Table S1). Several proteins, including *Rhodothermus marinus* cyt *c* (*Rma* cyt *c*), engineered *Rma* NOD, and wild-type $P450_{BM3}$ from *Bacillus megaterium*, exhibited trace catalytic activities. Reactions with only the heme cofactor (iron protoporphyrin IX) as the catalyst also delivered trace amounts of product **3a**. Several serine-ligated cytochromes P450 (P411s),[15] however, exhibited promising initial activity for the target trifluoroethylation reaction. The highest activity (1,250 TTN) was obtained with P411-CH-C8. This P411ΔFAD variant, which comprises the heme and FMN but not the FAD domain of $P450_{BM3}$, was originally engineered for carbene C–H insertion with ethyl diazoacetate (EDA).[14]

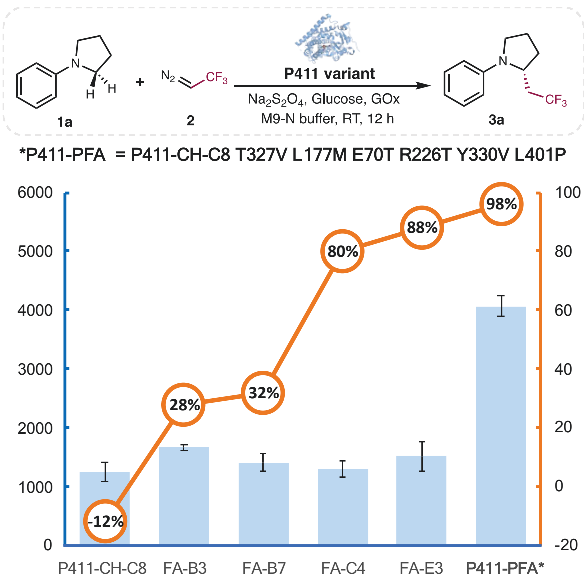

Figure 1. Directed evolution of P411 catalysts for C(sp^3)–H fluoroalkylation reaction. Experiments were performed using clarified *E. coli* lysate overexpressing the P411 variants, 10 mM **1a**, 20 mM **2**, 25 mM D-glucose, 5 mg/mL sodium dithionite, GOx and 5 vol% EtOH in M9-N buffer at room temperature under anaerobic condition for 12 h.

Although P411-CH-C8 exhibited high activity for the fluoroalkylation reaction between **1a** and **2**, the enantioselectivity of the resulting product **3a** was poor (12% ee, (*S*)-enantiomer, Table S1). We therefore used directed evolution to increase enantioselectivity (Figure 1). We first targeted several amino acid residues in the distal heme pocket for site-saturation mutagenesis and screened for variants with improved enantioselectivity. Many of the sites selected for mutagenesis were previously shown to affect activity and selectivity in abiological carbene- and nitrene-transfer reactions (Table S2). Although none of the mutants tested showed improvement in forming (*S*)-**3a**, we discovered that a T327V mutation inverted enantioselectivity and yielded (*R*)-**3a** with 28% ee. With the T327V mutant (P411-FA-B3) as the new parent, further rounds of site-saturation mutagenesis and recombination of beneficial mutations yielded variant FA-E3 with five mutations (T327V, E70T, L177M, R226T, and Y330V) compared to P411-CH-C8. This variant exhibited 88% ee for (*R*)-**3a**.

We next surveyed residues in the enzyme's proximal loop; residues in this region play an important role in regulating the oxidation activity of cytochromes P450.[16] Our lab and others have shown that mutations in this region also affect abiological carbene and nitrene transfer reactivities, mainly by tuning the electron-donating properties of the heme proximal axial ligand.[15,17] With FA-E3 as the parent, site-saturation mutagenesis on proximal loop residues and screening revealed the L401P mutation that further improved activity to 4,070 TTN and enantioselectivity to 98% ee (Figure 1). This final variant, named P411-PFA, contains six mutations from P411-CH-C8 (T327V, E70T, L177M, R226T, Y330V, and L401P, Figure S1).

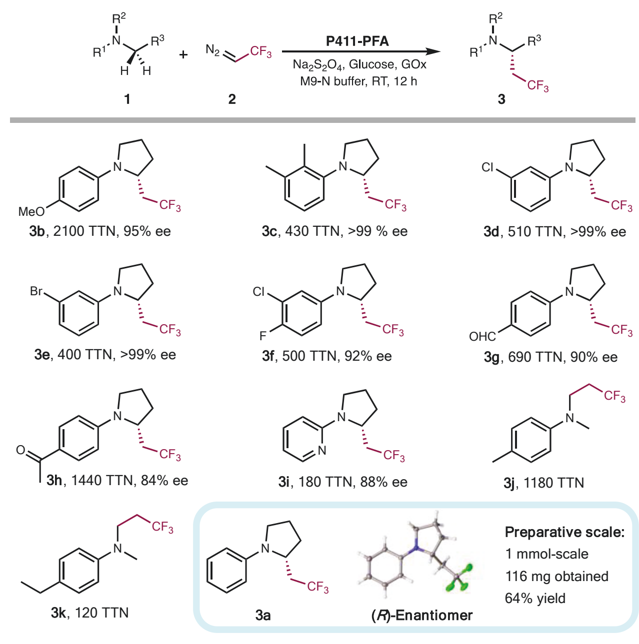

Figure 2. Substrate scope of P411-PFA-catalyzed C–H trifluoroethylation reaction. Absolute configuration of **3a** was determined by X-ray crystallography.

With this laboratory-evolved C–H fluoroalkylation enzyme in hand, we then explored its performance on a diverse set of substrates. As shown in Figure 2, P411-PFA could install a trifluoroethyl group onto various *N*-aryl pyrrolidine substrates by directly activating the *α*-amino C–H bonds. High activity and enantioselectivity were achieved for pyrrolidines containing a variety of *N*-aryl and *N*-heteroaryl substituents. A range of functional groups including methoxy, halogen, ketone, and aldehyde were well tolerated. The tolerance to the *p*-methoxylphenyl group (PMP) would enable the facile synthesis of other *N*-substituted pyrrolidines bearing a trifluoroethyl stereogenic center, as PMP is a well-established protecting group for the nitrogen atom and can be removed under mild conditions.[18] Furthermore, given the compatibility of our method with reactive functional groups like halogens and aldehydes, the application could be broadened further by harnessing these functionalities as reaction handles to access a diverse range of structural motifs through well-established cross-coupling and condensation reactions. This enzymatic approach opens possibilities to access a broad range of chiral trifluoroethylated pyrrolidines, whose current construction methods require stepwise, successive radical cross coupling chemistry that is time-consuming and not enantioselective.[19]

In addition to pyrrolidine-type substrates, this enzymatic method could also functionalize *N,N*-dialkyl anilines, which is another structural motif prevalent in pharmaceuticals. The enzyme is highly selective toward *α*-amino C−H bonds. For instance, in compound **3k**, the *N*-methyl is activated exclusively in the presence of weaker benzylic C−H bonds. The preference for C−H bonds at *α*-amino positions over those at the benzylic and OMe (**3b**) positions might arise from the strong electron-donating properties of nitrogen, which makes *α*-amino C−H bonds react more favorably with the electrophilic iron-carbene intermediates.[8a,20] To further demonstrate synthetic utility, we performed this enzymatic reaction on preparative scale, where it proceeded smoothly and afforded the chiral trifluoroethylated compound **3a** with 64% isolated yield and 98% ee (116 mg). We obtained the crystal structure of compound **3a,** and the absolute configuration of the trifluoroethylated chiral center was determined to be *R*.

Alternate stereoisomers of a bioactive molecule can have drastically different biological effects and need to be evaluated individually during drug candidate screening.[21] This necessitates the synthesis of all possible stereoisomers of a given molecule, preferably via stereo-divergent asymmetric catalysis.[22] Thus we developed an enzyme catalyst that could perform the targeted C–H trifluoroethylation with enantioselectivity opposite to that of P411-PFA. To find a suitable starting point for evolving an enzyme that exhibits reversed enantioselectivity, we first evaluated the catalytic performance on various substrates of all the variants along the evolution of P411-PFA. Early variant FA-B7 exhibited moderate reversed enantioselectivity (24% ee for the (*S*) enantiomer) for functionalization of aldehyde-substituted *N*-aryl pyrrolidine substrate **1g**. Further examination of variants derived from FA-B7 led to the discovery of a quadruple mutant of FA-B7 (T70E, V327T, G74P, Q437L, termed P411-PFA-(*S*)) that catalyzes the formation of (*S*)-**3g** with 92% ee (Figure 3, Figure S4). P411-PFA-(*S*) is a general catalyst for synthesis of the (*S*)-enantiomer of trifluoroethylated pyrrolidines, as demonstrated by its high activity and moderate-to-high (*S*)-enantioselectivity toward a variety of *N*-aryl and *N*-heteroaryl pyrrolidine substrates (Figure 3). These results further highlight the facile configurability of the enzymatic system for delivering diverse chiral organofluorine molecules.

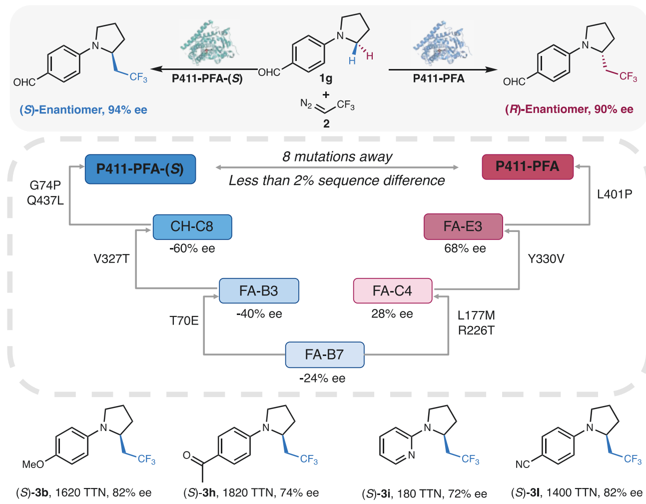

Figure 3. Enantiodivergent C(sp^3)–H trifluoroethylation catalyzed by P411-PFA and P411-PFA-(*S*) and substrate scope of P411-PFA-(*S*).

Another advantage of this chemistry is its ability to install other fluoroalkyl groups via the same C–H functionalization process. As a proof of concept, we challenged the protein catalysts with 2,2,3,3,3-pentafluoro-1-diazopropane **4** as the carbene precursor. As shown in Figure 4, P411-PFA can use **4** to introduce pentafluoropropyl groups into the *α*-amino C–H bonds of both acyclic and cyclic amine substrates with excellent activity and enantioselectivity. We successfully obtained 59.1 mg of the enzymatic product **5e**. Intriguingly, subsequent X-ray crystallographic analysis showed that the pentafluoropropylation products obtained by P411-PFA exhibited an opposite absolute configuration to that of the trifluoroethylation ones. Although further investigation is needed to fully elucidate the origin of this inversion of absolute configuration, a potential cause is a conformational change of the corresponding fluoroalkylated heme-carbene intermediates, which alters the orientation of the fluoroalkyl groups and reverts the configuration of the prochiral face accessed by the substrates for C–H bond activation. This hypothesis is supported by the fact that carbene intermediates in heme proteins can adopt different conformations depending on their structural properties.[6f,6g]

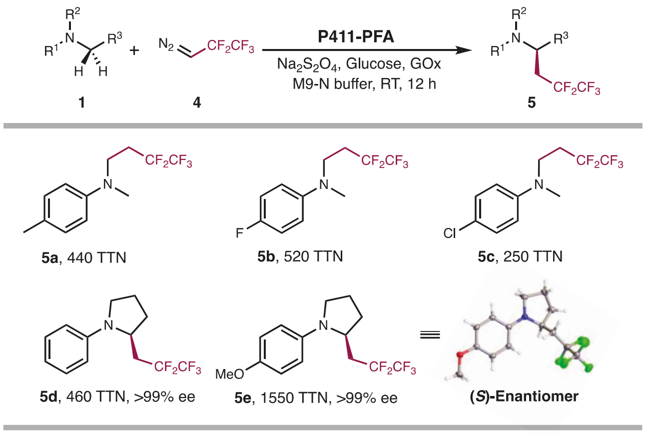

Figure 4. Substrate scope of P411-PFA-catalyzed C–H pentafluoropropylation. Absolute configuration of **5e** was determined by X-ray crystallography.

In summary, we have developed a catalytic platform for insertion of fluoroalkyl-substituted carbenes into C(sp^3)–H bonds with high activity and enantioselectivity under mild conditions. With directed evolution, the enantioselectivity of the enzymes can be tuned to achieve enantiodivergent synthesis of organofluorine compounds by this versatile carbene C–H insertion process. This work provides a powerful new approach for addition of fluorine-containing structural motifs prevalent in pharmaceuticals and further expands the reaction scope of new-to-nature enzymatic C–H alkylation. We envision that the enzymes developed in this research will open up new avenues for synthesis of fluorinated bioactive molecules.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Experimental details, and spectral data for all new compounds. (PDF)
X-ray crystallographic data for **3a** (CIF)
X-ray crystallographic data for **5e** (CIF)

AUTHOR INFORMATION

Corresponding Author

*frances@cheme.caltech.edu

Present Addresses

‡R.K.Z: Research and Development, Amyris Inc., 5885 Hollis Street, Suite 100, Emeryville, CA 94608 United States.

Author Contributions

†J.Z. and X.H. contributed equally.

Notes

The authors declare no competing financial interest.

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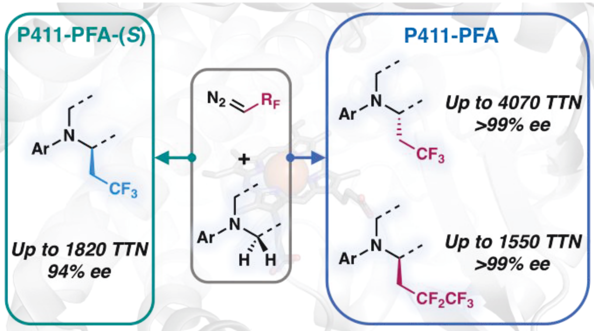

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