Biocatalytic asymmetric construction of secondary and tertiary fluorides from β-fluoro-α-ketoacids

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Abstract: Fluorine is a critical element for the design of bioactive compounds, but its incorporation with high regio- and stereoselectivity using environmentally friendly reagents and catalysts remains an area of development. Stereogenic tertiary fluorides pose a particular synthetic challenge and are thus present in only a few approved pharmaceuticals such as fluticasone, solithromycin, and sofosbuvir. The aldol reaction of fluorinated donors provides an atom-economical approach to asymmetric C-F motifs via C-C bond formation. Here we report that the type II pyruvate aldolase HpcH and engineered mutants thereof are biocatalysts for carboligation of ß-fluoro-α-ketoacids (including fluoropyruvate, ß-fluoro-α-ketobutyrate, and ß-fluoro-α-ketovalerate) with many diverse aldehydes. The reaction proceeds with kinetic resolution in the case of racemic donors. The reactivity of HpcH towards these new donors, which are non-native in both steric and electronic properties, grants access to enantiopure fragments with secondary or tertiary fluoride stereocenters. In addition to representing the first asymmetric synthesis of tertiary fluorides via biocatalytic carboligation, the afforded products could improve the diversity of fluorinated building blocks and enable the synthesis of fluorinated drug analogs.

Main text:

The design of safe and effective pharmaceuticals has benefitted greatly from advancements in organofluorine chemistry over recent decades. Because the properties of fluorine, such as its high electronegativity, unique lipophilicity, small size, and metabolic inertness, allow for the fine tuning of drug characteristics, ~25% of all drugs now contain at least one fluorine atom.[1-2] This metric increases to 35% for newer compounds in late-stage clinical trials and further to 50% for the most successful blockbuster drugs.[3] Because regio- and stereoselectivity of fluorine are critical for bioactivity, fluorine chemocatalysis has blossomed with asymmetric methods that can be largely divided into three strategies: electrophilic fluorination,[4-7] nucleophilic fluorination,[8-11] and elaboration of achiral or racemic organofluorines.[12-17] However, one challenge that has only been specifically addressed more recently is the construction of the stereogenic tertiary fluoride moiety (also referred to in literature as a carbon-fluorine quaternary stereocenter[3]), exemplified by the fact that only three major approved drugs bear this motif (Figure 1A).

Biocatalysis offers a complementary approach with the potential for an exceptional selectivity and safety profile, but must contend with the rarity of fluorine in the biosphere. No enzymes are known to perform electrophilic fluorination, and only one has been found to perform nucleophilic fluorination on a highly activated substrate.[18] Thus, the strategy of using promiscuous enzymes to elaborate upon simple optically-inactive organofluorine building blocks has been adopted to construct C-F stereocenters. For example, stereogenic secondary fluorides (R_1-CFH-R_2) have been prepared by our group and others, exploiting pyruvate aldolases that utilize fluoropyruvate as a non-native donor substrate (Figure 1B).[19-21] Fluorine can be rendered in either stereochemistry depending on the specific structural family of the aldolase, but the switch to a fluoro-donor typically restricts the acceptor scope to a small set of highly polar aldehydes due to the origin of these enzymes in carbohydrate metabolism. Enzymatic preparation of stereogenic tertiary fluorides (R_1-CFR_2-R_3) has been accomplished with lipase-catalyzed kinetic resolution and desymmetrization (Figure 1C),[22-23] but provided modest stereoselectivity and reaction diversity. In this work, we investigated the type II pyruvate aldolase HpcH and its engineered variants and assessed their ability to catalyze aldol addition of ß-fluoro-α-ketoacids to diverse aldehydes (Figure 1D). As a result, we now report the first example to our knowledge of asymmetric synthesis of tertiary fluorides by enzymatic C-C bond formation.

The HpcH/HpaI family of type II pyruvate aldolases (PF03328) has recently emerged as a source of useful biocatalysts.[24-26] In contrast to the type I mechanism where a catalytic lysine residue must form a Schiff base with the donor, the type II mechanism requires only a divalent cation such as Mg^{2+} to activate the donor as an enolate. We previously found that various homologs of this family can tolerate the aldol addition of fluoropyruvate to polar aldehydes, affording value-added products like fluorinated sugar acids with 0.01-0.1 mol% enzyme catalyst.[21] Seeking to probe the limits of substrate scope in both donor and acceptor, we chose HpcH from *Escherichia coli C* for this study due to its excellent heterologous expression yield.[27-29]

We tested a panel of ß-fluoro-α-ketoacids bearing different C-3 substitutions to investigate as new aldolase donors (Figure 2, Figure S1). Along with the commercially available sodium fluoropyruvate (FP, **1**, R = H), we also screened ß-fluoro-α-ketobutyrate (FKB, **2a**, R = Me), ß-fluoro-α-ketovalerate (FKV, **2b**, R = Et), ß-fluoro-α-keto-γ-methylvalerate (FKMV, **2c**, R = *i*-Pr), and ß-fluoro-α-keto-γ-phenylbutyrate (FKPB, **2d**, R = Bn). Donors **2b-2d** were prepared from the corresponding α-ketoesters through a three-step sequence of silylation with TMS-Cl, fluorination with Selectfluor reagent, and saponification with LiOH.[30] The substrates were purified as free acids before neutralization to sodium salts for enzymatic use. Separately, racemic sodium fluoro(phenyl)pyruvate (FPP, **2e**, R = Ph) was prepared by the established glycidic ester route.[31]

Formaldehyde (FA) was used initially as a model acceptor to simplify the products to one nascent stereocenter. Next, a collection of HpcH mutants with size reduction of varying degrees at the Trp-19, Phe-170, and Leu-212 residues were prepared in hopes of steric alleviation towards bulky fluoro-donors (Table S1, Figure S2-S3). Assays were conducted by incubation of substrates with 0.1 mol% enzyme and Mg^{2+}-containing HEPES pH 7.5 buffer, followed by H_2O_2 decarboxylation of aldol adducts and analysis of resulting α-fluoroacids by ^{19}F NMR (Figure 2, Table S2-S4). In the reactions of FP (**1**) or FKB (**2a**) with FA, the best conversions (>50%) were observed with wild-type enzyme (wt-HpcH), showing that mutations decreased the intrinsic enzyme activity and were detrimental with respect to sterically non-challenging donors. However, in the case of FKV (**2b**), wt-HpcH was ineffective (<2%), while single mutations at Phe-170 or Leu-212 gave increased conversion (6-12%). Thus, steric relief becomes important upon introducing a C-3 ethyl group. Furthermore, the necessary incubation time to collect reliable screening data increased corresponding to the C-3 substituent size (4 h for FP, 16 h for FKB, 48 h for larger substrates). We also observed that the consumption of total FKB never exceeded 50%, and showed with a separate experiment that the racemic donor undergoes kinetic resolution (Figure S4). The bulkiest donors FKMV (**2c**) and FKPB (**2d**) were not tolerated by any enzyme variant even in forcing conditions with glycolaldehyde, an extremely good acceptor. Lastly, FPP (**2e**) which bears a C-3 aryl group was unstable in water and reacted non-enzymatically with FA. These results show that HpcH or its point mutants can utilize extended ß-fluoro-α-ketoacids such as FKB and FKV to produce alkyl-branched fluorine chiral centers.

All C-F stereocenters generated by HpcH are (3*S*)-configured, with no detection of (3*R*)-products, as we had previously established with NMR studies of fluoropyruvate adducts.[21] The origins of this exquisite stereoselectivity, alongside with the kinetic resolution phenomenon and the beneficial nature of Phe-170 and Leu-212 mutations, can altogether be rationalized by two factors: (1) the electronics-determined requirement for cis-geometry of the metal-bound fluoroenolate intermediate, and (2) the enzyme-determined Si-facial consistency (relative to C-3 of the fluoroenolate) for proton abstraction and attack onto an aldehyde. For the racemic fluoro-donors, only one substrate enantiomer has an acidic proton orientable such that H-abstraction leads to a cis-fluoroenolate. The alkyl group is thus forced into the trans position of the fluoroenolate and directed towards the space occupied by Phe-170 and Leu-212 (Figure 3).

During the course of our studies, the Clapés group reported that reduction of Trp-19 to a small residue (W19V or W19A) was the key change that unlocked activity with bulky non-fluorinated α-ketoacids.[32-33] The product stereochemistry with these donors indicates that the C-3 bulk occupied the cis position of the enolate, which is directed towards Trp-19. In contrast, the W19A mutation was ineffective with our fluoro-donors, corroborating our theory that fluorine defeats an alkyl group in the competition of both groups for the cis position. To rule out an electronic explanation of reactivity differences between fluoro-donors, we note that in the hypothetical absence of steric factors, the inductively releasing effect of an alkyl group would increase the nucleophilicity of the fluoroenolate, and thus accelerate the rate-determining step of C-C bond formation.[34] However, the experimental addition rates of alkyl-substituted FKB (**2a)** and FKV (**2b)** were slower, not faster than FP (**1**) itself. Inhibition of the aldolase by our bulky fluoro-donors is also unlikely, as their co-presence had no effect on the conversion of FP. These findings overall reinforce that the fluoro-donor scope of HpcH is dominated by steric factors that appear to be different from those of non-fluorinated donors.

Proceeding towards studying the acceptor scope of the aldol reaction with ß-fluoro-α-ketoacid donors, we first optimized the individual reactions of FP (**1**), FKB (**2a**), and FKV (**2b**) with formaldehyde (FA, **3a**) based on time and buffer pH (Table S3-S5). For the difficult donor FKV, the promising single mutants of HpcH were re-screened at a higher enzyme loading of (0.3 mol%), which identified HpcH F170V to be optimal. Next, the optimized conditions were used to expand the acceptor scope to a panel of 17 aldehydes (**3a-3q**) with a diverse spectrum of size and polarity (Figure 4). Good conversions of FP were observed with every aldehyde. Furthermore, very hydrophobic aldehydes (**4f, 4g, 4n**) were used successfully by using a maltose-binding protein fusion (MBP-HpcH) to stabilize the enzyme. Aliphatic and polar heteroaromatic aldehydes were also well-tolerated when using FKB and FKV as donors. In most cases, reactions are not diastereoselective, because a lack of aldehyde-interacting residues leads to attack at both aldehyde faces, giving access to both configurations of the hydroxy stereocenter. All products were characterized by ^{19}F NMR (Table S2) and high-resolution LC-MS (Figure S5).

To demonstrate the synthetic utility of ß-fluoro-α-ketoacids as aldolase donors, selected reactions relevant to bioactive compounds were conducted on a larger scale and the products isolated (Figure 5, Figure S6). All products were confirmed by ^{1}H, ^{13}C, and ^{19}F NMR (Figure S7-S13). Compounds **7a**, **7b**, and **7c** derived from formaldehyde are fluorine-bearing versions of the Roche ester (methyl 3-hydroxy-2-methylpropionate). This versatile building block has been employed in classic total syntheses of complex natural products, such as the anticancer discodermolide.[35-36] The aromatic products **8a** and **8b** resemble known arylbutyryl intermediates of nikkomycins, a family of antifungal compounds also targeted for total synthesis.[37-38] The above five compounds were prepared by a sequence of enzymatic reaction (0.6 mmol), decarboxylation with H_2O_2, removal of water and enzymes, and esterification in MeOH/HCl or EtOH/$SOCl_2$ before extraction and chromatography. Additionally, the enantioselectivity was confirmed by derivatization with Mosher's acid and comparison with chemically synthesized racemic standards (Table S5, Figure S6,

Figure S14). Lastly, the α-fluoro-α-methyl branched sugar acids **5j** and **5k** were enzymatically synthesized (0.15 mmol) and directly purified by semi-preparative HPLC. Similar intermediates are used to synthesize the fluorinated pentose moiety of the blockbuster antiviral sofosbuvir,[39] which could be potentially accessed by using aldolase families with opposing stereochemical preference. These examples demonstrate that the combination of aldolases with novel fluoro-donor substrates provides flexible access to fragments bearing valuable C-F stereocenters, and potentially enables new fluorinated drugs or fluorinated analogs of known drugs.

Although the rarity of organofluorines in nature has long driven a large gap between the fields of fluorine chemistry and biocatalysis, the repurposing of promiscuous enzymes to elaborate simple fluorinated precursors has proven to be an attractive strategy to bridge this gap. We have shown that a pyruvate aldolase from the type II HpcH/HpaI family, which evolved to cleave pyruvate off from highly polar carbohydrate metabolites *in vivo*, can be engineered for remarkable substrate scope in the synthetic aldol direction. Secondary fluoride stereocenters are obtained from the addition of fluoropyruvate to a wide variety of polar, nonpolar, aliphatic, aromatic, and heteroaromatic aldehydes. Tertiary fluoride stereocenters, which have hitherto not been synthesized with biocatalytic carboligation, are obtained with readily synthesized ß-fluoro-α-ketoacid precursors and HpcH aldolase variants engineered for steric relief. After gaining a mechanistic understanding of reaction outcomes and optimizing the reaction conditions, we furthermore demonstrated the robustness of this system with examples pertinent to bioactive compounds of interest. In conclusion, this biocatalytic platform for construction of chiral organofluorines not only supplements the repertoire of methodology for functional units that are difficult to synthesize chemically, but also provides a safe and sustainable route towards fluorinated analogs of natural products and pharmaceuticals.

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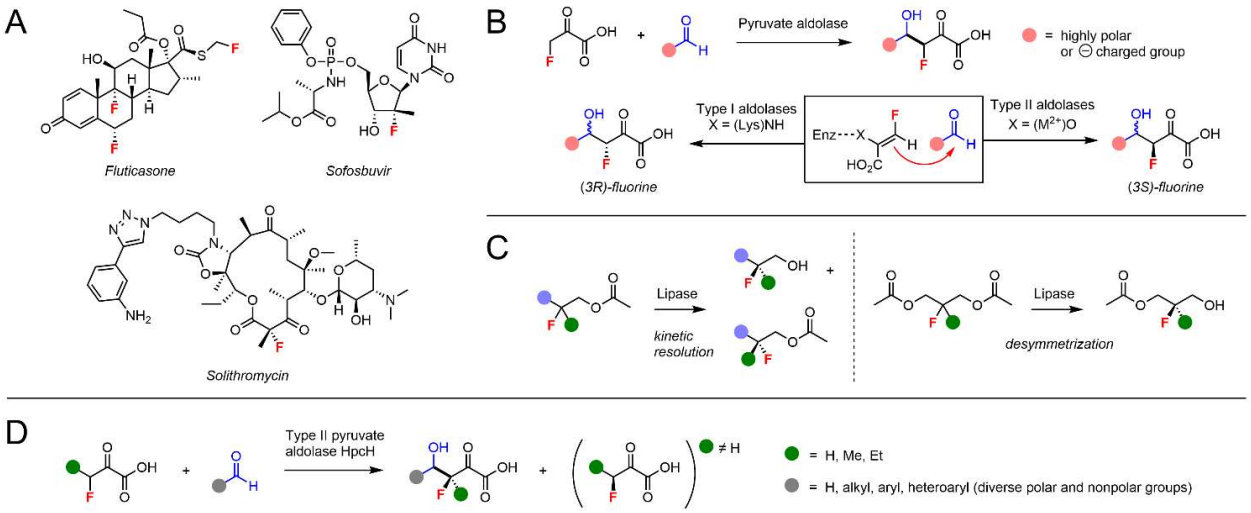
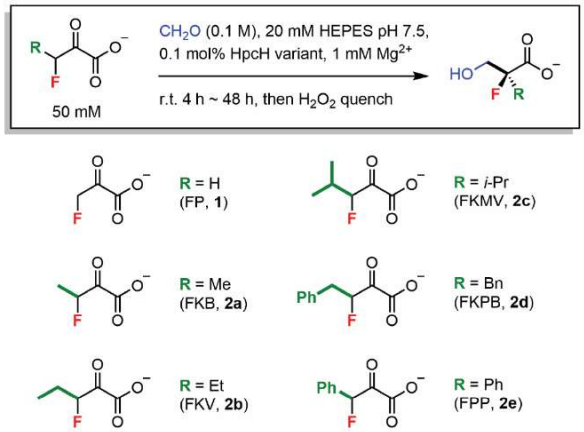


Figure 1. Motivation and methodology for enzymatic construction of C-F stereocenters. A) Approved drugs with C-F stereocenters. B) Secondary fluoride stereocenters from enzymatic aldol addition of fluoropyruvate. B) Lipase-catalyzed formation of tertiary fluoride stereocenters. D) This work: secondary and tertiary fluoride stereocenters from enzymatic aldol addition of ß-fluoro-α-ketoacids to diverse aldehydes, accompanied by kinetic resolution of racemic substrates.



Donor substrate (time)

Variant of HpcH aldolase	FP (4 h)	FKB (16 h)	FKV (48 h)
No enzyme	tr	nd	nd
Wild-type	69	50	2
W19A	9	tr	tr
F170A	5	2	6
L212A	7	16	10
F170A/L212A	2	tr	2
F170V	6	tr	10
F170V/L212A	4	tr	2
F170L	30	24	13
F170I	7	2	13
L212I	57	tr	tr
L212V	33	nd	tr
Optimized condition	98	82	40

Conversion (%)

Figure 2. Screening for C-F stereocenter formation. New ß-fluoro-α-ketoacids that were synthesized and tested as potential donors with HpcH aldolase mutants. Results of % conversion, trace product detected (tr, <1%), or no product detected (nd). FKB and FKV undergo kinetic resolution so 100% conversion equals 50% donor consumption. FKMV, FKPB, and FPP were not substrates. Green-shaded conditions were optimized with the following changes: (FP) pH 8.5 (2:1 20 mM CHES/20 mM HEPES); (FKB) Reaction time 48 h; (FKV) Enzyme load 0.3 mol%, reaction time 1 week.

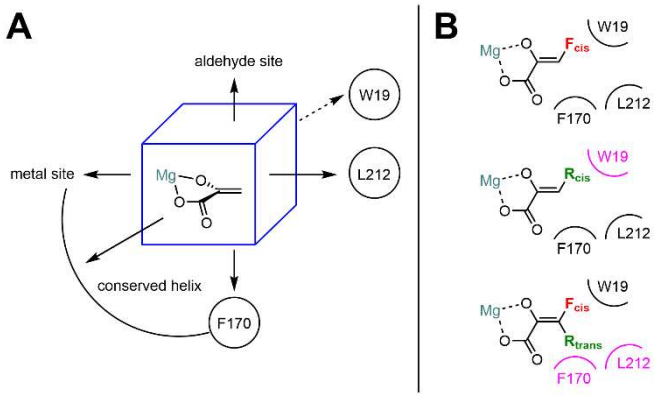


Figure 3. Stereochemical outcome of the HpcH aldol reaction with fluorinated donors. A) Schematic of the metal-bound enolate intermediate at the active site of HpcH aldolase. Non-catalytic residues W19, F170, and L212 form a pocket around the C-3 position. B) Geometric preference of C-3 fluorine or alkyl substituents on the enolate intermediate, in different cases resulting in steric clash of the alkyl group with W19 or with F170/L212.

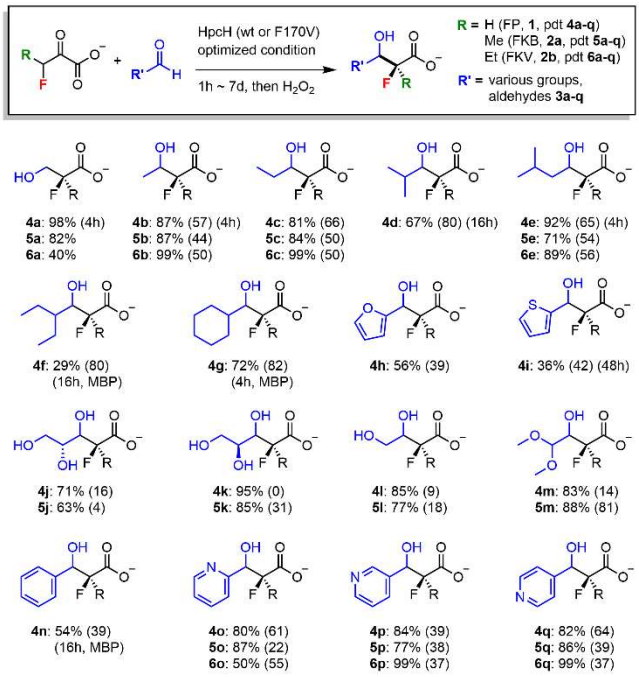


Figure 4. Substrate scope in the aldehyde for aldol additions of ß-fluoro-α-ketoacids. The % conversion is given along with the diastereomeric ratio expressed in parentheses as %*syn* (%*anti* = 100 - %*syn*). FKB and FKV undergo kinetic resolution so 100% conversion equals 50% donor consumption. Optimized conditions are the same as described in Figure 2, except that the reaction time for FP was only 1 h (unless otherwise indicated). MBP-fused HpcH was used where indicated.

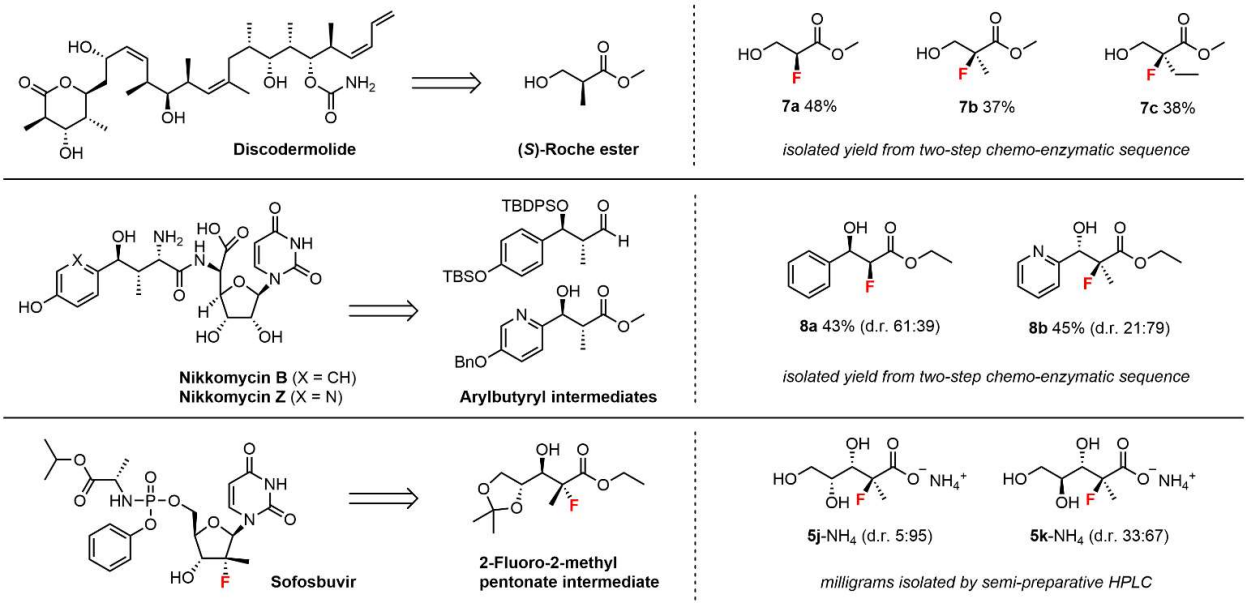


Figure 5. Chemoenzymatic preparation of synthons containing C-F stereocenters. Examples of fluorinated compounds with relevance to building blocks of bioactive compounds. All were isolated as single enantiomers, and the diastereomeric *syn/anti* ratio is given where applicable (structures correspond to the major diastereomer). The fluoroester products were obtained after enzymatic aldol addition, decarboxylative quench, esterification, and organic extraction. The branched fluorinated sugar acids were obtained as their ammonium salts after enzymatic aldol addition, decarboxylative quench, and semi-preparative HPLC.

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