Biocatalytic Asymmetric Construction of Secondary and Tertiary Fluorides from \( \beta \)-Fluoro-\( \alpha \)-Ketoacids**

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**Abstract:** Fluorine is a critical element for the design of bioactive compounds, driving advances in selective and sustainable fluorination. However, stereogenic tertiary fluorides pose a synthetic challenge and are thus present in only a few approved drugs (fluticasone, solithromycin, and sofosbuvir). The aldol reaction of fluorinated donors provides an atom-economic approach to asymmetric C–F motifs via C–C bond formation. We report that the type II pyruvate aldolase HpcH and engineered variants perform addition of \( \beta \)-fluoro-\( \alpha \)-ketoacids (including fluoropyruvate, \( \beta \)-fluoro-\( \alpha \)-ketobutyrate, and \( \beta \)-fluoro-\( \alpha \)-ketovalerate) to diverse aldehydes. The reactivity of HpcH towards these fluoro-donors grants access to enantiopure secondary or tertiary fluorides. In addition to representing the first 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.

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 electronegativity, lipophilicity, small size, and metabolic inertness, allow for fine tuning of drug characteristics, 20–25% of all drugs based on organic small molecules now contain at least one fluorine atom. This metric increases to 30–35% for newer drugs and compounds in late-stage clinical trials. Because regio- and stereoselectivity of fluorine are critical for bioactivity, fluorine chemocatalysis has blossomed with asymmetric methods that can be divided into three strategies: electrophilic fluorination, nucleophilic fluorination, and elaboration of achiral or racemic organofluorines. 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), exemplified by the fact that only three major approved drugs bear this motif (Figure 1A).

Biocatalysis offers a complementary approach with the potential for exceptional selectivity and safety profiles, and was widely adopted in the last two decades for the synthesis of commodity chemicals and pharmaceuticals. However, interfacing biocatalysis with fluorine chemistry must contend with the rarity of fluorine in the biosphere. No natural or engineered enzymes are known to perform direct electrophilic fluorination. Only one enzyme, the fluorinase, has been found to perform nucleophilic fluorination on a highly activated substrate in the first step of organofluorine biosynthesis pathways. Developments of the fluorinase have allowed for its successful exploitation in biotechnological applications especially with regards to \( \text{^{18}F} \) radiolabelling. With native fluorine-manipulating enzymes thus limited, especially for C–F stereocenters, a strategy of using promiscuous enzymes to elaborate upon simple optically-inactive organofluorine building blocks has been adopted. For example, stereogenic secondary fluorides have been prepared by our group and others, with pyruvate aldolases that use fluoropyruvate as a non-native donor substrate (Figure 1B). 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 polar aldehydes due to the origin of these enzymes in carbohydrate metabolism. Enzymatic preparation of stereogenic tertiary fluorides has been accomplished with lipase-catalyzed kinetic resolution and desymmetrization (Figure 1C), 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 \( \beta \)-fluoro-\( \alpha \)-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/Hpal family of type II pyruvate aldolases (PF03328) has recently emerged as a source of useful biocatalysts. In contrast to the type I mechanism where a catalytic lysine residue forms 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...
reported that homologs of this family perform aldol addition of fluoropyruvate to polar aldehydes to generate (S)-fluoro products with >99% e.e., affording value-added products like fluorinated sugar acids with 0.01–0.1 mol % enzyme catalyst. Given its high stereoselectivity and broad acceptor scope, we reasoned that HpcH from *Escherichia coli* C would be a good candidate to explore the use of substituted pyruvate donors that would generate tertiary fluoride stereocenters. We tested a panel of β-fluoro-α-ketoacids bearing different C-3 substitutions to investigate as new aldolase donors (Figure 2, Figure S1). Along with commercially available sodium fluoropyruvate (1, R<sub>1</sub> = H), we also screened β-fluoro-α-ketobutyrate (2a, R<sub>1</sub> = Me), β-fluoro-α-ketovalerate (2b, R<sub>1</sub> = Et), β-fluoro-α-keto-γ-methylvalerate (2c, R<sub>1</sub> = i-Pr), and β-fluoro-α-keto-γ-phenylbutyrate (2d, R<sub>1</sub> = Bn). Racemic donors 2b–d 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. The substrates were purified as free acids before neutralization to sodium salts for enzymatic use. Separately, racemic sodium fluoro(phenyl)pyruvate (2e, R<sub>1</sub> = Ph) was prepared by the established glycidic ester route.

Formaldehyde (FA) was used initially as a model acceptor to simplify the products to those with one nascent stereocenter. This electrophile had not been investigated in our previous work on reactions with 1. 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 and S3). All enzymes were expressed recombinantly in *E. coli* with N-terminal His<sub>10</sub> tags and purified by metal affinity chromatography. Assays were conducted by incubating fluoro-donors with 2 equivalents acceptor, 0.1 mol % purified enzyme, HEPES buffer pH 7.5, and catalytic MgCl<sub>2</sub> followed by H<sub>2</sub>O<sub>2</sub> decarboxylation of aldol adducts. The percent conversion was determined by integration of <sup>19</sup>F NMR peaks corresponding to α-fluoro-
cids arising from reactants and products (Figure 2, Table S2–S4). We were pleased to see that 1 and 2a reacted well 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, wt-HpcH was ineffective on 2b (< 2 %), while single mutations at Phe-170 or Leu-212 gave increased conversion (6–13 %). Thus, steric relief becomes important upon introducing a C-3 ethyl group. We observed that the total consumption of 2a never exceeded 50 %, and showed that the racemic donor undergoes kinetic resolution by analysis of samples subjected to stereoselective reduction at C-2 by 1-lactate dehydrogenase. Using this approach, the donor enantiomers of 2a were converted to NMR-distinguishable diastereomers, indicating that only (R)-2a was depleted by the aldolase (Figure S4). Although 2b was not a substrate for 1-lactate dehydrogenase, similar stalling of reactions at 50 % conversion suggests that kinetic resolution also occurs. The bulkiest donors 2c and 2d were not tolerated by any enzyme variant even in forcing conditions with glycolaldehyde, an extremely good acceptor. Lastly, 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 some extended β-fluoro-α-ketoacids such as 2a and 2b to produce alkyl-branched fluorine chiral centers.

The origin of the exquisite stereoelectivity of HpcH in generating (S)-fluoro stereocenters is thought to derive from two main factors: 1) the electronic 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.[9] This provides the framework to rationalize the kinetic resolution phenomenon and the beneficial nature of Phe-170 and Leu-212 mutations observed in the current study. For the racemic fluorono-donors, only one 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.[15] The product stereochemistry with these donors indicated 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.

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Proceeding towards studying the acceptor scope of the aldol reaction with β-fluoro-α-ketoacid donors, we first optimized the individual reactions of 1, 2a, and 2b with FA based on time and buffer pH (Table S3–S5). For the difficult donor 2b, F170L, F170V, F170A, and L212A were re-screened at a higher enzyme loading of 0.3 mol %. This identified F170V to be optimal despite F170L appearing slightly better in the initial data. (F170I was not included in re-screening due to protein solubility issues). Next, the optimized conditions were used to expand the acceptor scope to a panel of 17 aldehydes with a diverse spectrum of size and polarity (Figure 4). Good conversions of 2a and 2b were observed with a number of aldehydes, with aliphatic and polar heteroaromatic aldehydes especially well tolerated. Indeed, near quantitative formation of several products was observed even with the ethyl-substituted donor 2b (products 4c, 5c, 18c, 19c). However, there remained difficult acceptors that reacted only with donor 1 (products 6a, 8a, 9a, 10a, 11a, 16a). These bulky or branched aldehydes were rendered viable with our optimized conditions, which provided marked improvement in substrate scope and reaction rates compared to our previous report on this donor.[9] A maltose-binding protein fusion (MBP-HpcH) was used to stabilize the enzyme in the presence of the most hydrophobic aldehydes. 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 19F NMR (Table S2) and high-resolution LC-MS (Figure S5).

To demonstrate the synthetic utility of β-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 1H, 13C, and 19F NMR (Figure S7–S13). Compounds 3a–c derived from FA are fluoride-bearing analogs of the Roche ester (methyl 3-hydroxy-2-methylpropionate), a versatile building block employed in classic total synthesis of complex natural products such as the anticancer discodermolide.[17] The aromatic products 16a and 17b resemble known arylbutyryl intermediates of nikkomycins, a family of antifungal compounds also targeted for total synthesis.[18] The above five α-fluoroesters were prepared by enzymatic aldol reaction (0.6 mmol), decarboxylation with H2O2, removal of water and enzymes, and esterification in MeOH/HCl or EtOH/SCl2. Additionally, enantiopurity 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 12b and 13b 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,[19] which could be potentially accessed by using aldolase families with opposing stereochemical preference. These examples demonstrate that novel fluoro-donors providing access to C–F stereocenters

![Figure 5. Chemoenzymatic preparation of synthons containing C–F stereocenters. Examples of fluorinated compounds with relevance to building blocks of bioactive compounds were isolated in high enantiomeric or diastereomeric purity. The drawn structures correspond to the major stereoisomer. The fluoroester products were obtained by enzymatic aldol addition, decarboxylation, and esterification. Enantiopurity of fluoroesters was confirmed by Mosher analysis and the minor enantiomers were not detected (Figure S14). The branched fluorinated sugar acids were obtained as their ammonium salts after enzymatic aldol addition, decarboxylation, and semi-preparative HPLC. Abbreviations in middle panel: Bn = benzyl, TBS = tert-butyldimethylsilyl, TBDPS = tert-butyldiphenylsilyl.](image-url)
may potentially enable 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 fluoroacetate to a wide variety of polar, nonpolar, aliphatic, aromatic, and heteroaromatic aldehydes. Tertiary fluoride stereocenters, which have hitherto not been synthesized with biocatalytic carboglation, are obtained with readily synthesized β-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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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

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