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Chemoenzymatic platform for synthesis of chiral organofluorines based on type II aldolases

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Abstract

Aldolases are C-C bond forming enzymes that have become prominent tools for sustainable synthesis of complex synthons. However, enzymatic methods of fluorine incorporation into such compounds are lacking due to the rarity of fluorine in nature. Recently, the use of fluoropyruvate as a non-native aldolase substrate has arisen as a solution. Here, we report that the type II HpcH aldolases efficiently catalyze fluoropyruvate addition to diverse aldehydes, with exclusive (3*S*)-selectivity at fluorine that is rationalized by DFT calculations on a mechanistic model. We also measure the kinetic parameters of aldol addition and demonstrate engineering of the hydroxyl group stereoselectivity. Our aldolase collection is then employed in the chemoenzymatic synthesis of novel fluoroacids and ester derivatives in high stereopurity (d.r. 80–98%). The compounds made available by this method serve as precursors to fluorinated analogs of sugars, amino acids, and other valuable chiral building blocks.

Fluorine has unique elemental characteristics that have served as the basis for its rising use in the design of synthetic compounds with a breadth of functions ranging from materials to pharmaceuticals.[1] Due to its ability to enhance metabolic stability and alter pharmacokinetic profiles without adding significant steric bulk, fluorine is now found in greater than 20% of drugs.[1a] As such, expanding the scope of accessible organofluorine structures would greatly enhance our ability to discover and develop new functional compounds. While many synthetic methodologies are known for the site-selective introduction of fluorine onto various scaffolds,[2] fluorine biocatalysis is less mature yet also offers many potential advantages with respect to chemo-, regio-, and stereoselectivity of enzymatic approaches.[3] Our group has focused on biological synthesis of fluorinated polyketide natural products and fluorinated polymers,[4] but we also seek to expand biocatalytic methods for the preparation of broader classes of organofluorine compounds. One versatile approach to this problem entails the use of simple fluorinated building blocks in carbon-carbon bond forming reactions to generate complexity.

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The aldol addition represents a key reaction in the rapid assembly of building blocks with the concomitant generation of two stereocenters. Controlling the stereoselectivity at both sites is possible with chiral auxiliaries, precious metal catalysts, or organocatalysts.[5] Although numerous and well-developed, these methods still lack in efficiency and universality. The challenge is magnified for fluorine stereocenters, such that asymmetric fluoro-aldol reactions have been reported only recently.[5c] For these reasons, aldolase enzymes that catalyze the addition of a carbonyl donor to an aldehyde acceptor have been explored as an alternative to chemical methods.[6] Pyruvate aldolases in particular furnish a useful, densely-functionalized α-ketoacid motif. However, the donor specificity is usually strict and fluorine substitution is tolerated in only select cases. In this work, we set out to investigate fluoropyruvate aldolases, and to explore downstream transformations of the resultant ß-fluoro-α-keto acids into fluorosugars and other important building blocks for pharmaceuticals.

There exist four main structural families of pyruvate aldolases, two belonging to each the type I (KDPG [PF01801] and DHDPS [PF00701]) and type II (HpcH [PF03328] and DmpG [PF07836/00782]) mechanistic types. Type I aldolases utilize a catalytic lysine residue to form a Schiff base with the donor substrate and have been well-characterized. For example, KDPG aldolases are powerful biocatalysts with wide acceptor scope for pyruvate addition, but the ability to utilize fluoropyruvate is either limited to the native acceptor or non-existent.[7a,b] DHDPS family aldolases have successfully been used for fluoropyruvate addition with resulting (3*R*)-configuration of fluorine, but have specialized acceptor requirements such as long-chain aldose or heteroaromatic aldehydes.[7c–e] Furthermore, type I aldolases are sensitive to high aldehyde concentrations via inhibition of the catalytic lysine.[8] Thus, we turned our attention to the type II pyruvate aldolases, which use only a divalent metal cation for donor binding and enolization.

The type II aldolases have received comparatively little attention from the biocatalysis community until recently. The DmpG aldolases typically require complex formation with dehydrogenases that drive the retro-aldol direction, and thus appeared less tractable for our synthetic purposes.[9] Therefore, we initiated investigations into the remaining HpcH aldolase family, utilizing the five members EcGarL[10], EcRhmA[11], and EcHpcH[12] from *Escherichia coli* and SwHpcH1 and SwHpcH2 from *Sphingomonas wittichi*.[13] Fluoropyruvate addition assays were conducted by incubation of substrates with enzyme, buffer, and $MgCl_2$, followed by oxidative decarboxylation with H_2O_2 and analysis of the α-fluoroacids by ^{19}F NMR (Figure 1). We initially screened the addition to glycolaldehyde **1e**, dimethoxyacetaldehyde **1f**, and glyoxylate **1g** in either phosphate or HEPES buffer, and saw promising overnight conversions with most enzyme-substrate-buffer combinations (Figure S2). Using the optimal buffer for each enzyme, the analysis was then extended to twelve aldehydes **1a-1l** comprising chiral polar, achiral polar, and nonpolar groups. High to quantitative conversions (80–100%) were achieved for nearly all combinations of an aldolase with a polar aldehyde (Figure 1). Nonpolar aldehydes were converted less efficiently (10–70%), with a preference for propionaldehyde over other alkyl chain lengths. Steady-state kinetic parameters were measured by a discontinuous assay with mass spectrometry detection, and were compared for the EcHpcH-catalyzed addition of either

pyruvate or fluoropyruvate to glycolaldehyde. We found that the fluorine substitution results in a ~500-fold decrease in k_{cat} and the appearance of a substrate inhibition phenomenon, but little change in K_m. The k_{cat} defect is largely responsible for the requirement that fluoropyruvate additions be conducted overnight, whereas otherwise identical progress assays with pyruvate reached completion in a few minutes. With this promising system for fluoropyruvate addition in hand, we sought to clarify the stereochemical course of the reaction.

The new fluorine and hydroxyl stereocenters set by fluoropyruvate addition led us to establish both relative and absolute stereochemistry by indirect methods. The relative stereochemistry was preliminarily assigned on the basis of chemical shift and vicinal coupling constants ($^3J_{F\text{-}H}$).[7] For further confirmation, we synthesized a standard of (2*S*,3*S*)-2-fluoro-3-hydroxysuccinate (*anti*-**4g**) and compared it to enzymatically-derived fluoroacids **4efg** using a nitric acid oxidation strategy (Figure S3). Regarding absolute configuration, we observed that only one stereogenic center could be variable, since reactions with chiral aldehydes **1a-1d** gave no more than two peaks in ^{19}F NMR. Also, the aldol adducts with achiral aldehydes **3efgj** could be reduced enzymatically by lactate dehydrogenase to the corresponding ß-fluoro-α-hydroxyacids. From this reaction, only two products were observed of the four possible diastereomers, with $^3J_{F\text{-}H}$ values indicating a (3*S*)-configuration of fluorine (Figure S4). Thus the *syn*-*anti* distributions arise from the hydroxyl group, consistent with reports of HpcH enzymes lacking facial discrimination at the aldehyde.[11a,12c] Interestingly, these ratios could be dictated by either the aldehyde or the enzyme in different cases (Figure 1). For example, the L-configured **1b** and **1d** led to a strong preference for the *anti* isomer regardless of enzyme. In contrast, EcGarL and EcHpcH were strikingly stereocomplementary with **1g** (99% *syn* and 92% *anti*, respectively). The effect of catalyst loading on stereochemistry was also investigated in reactions with **1e** and **1f** (Figure S2). These aldehydes provided mixtures of 39–59% *anti* composition using the standard 0.05 mol% enzyme, but with 0.01 mol% of enzyme the diastereoselectivity improved to 71–93% *anti* at the expense of quantitative conversions. These data show that careful selection of homologs and conditions can tune the reaction towards the desired configuration of the hydroxyl group.

We then turned to density functional theory calculations in order to explore the origin of the stereochemical preference, using the proposed mechanism for EcHpcH[12b-d] as a basis for modelling the metal-enolate complex (Figure 2). The (3*S*)-configuration of fluorine in the aldol adduct is consistent with a bidentate (*Z*)-fluoroenolate intermediate. Density functional theory calculations on a $[Mg(H_2O)_2(OAc)_2(Fpyr\text{-}enolate)]^{2-}$ model complex (Figure S5) indicate that the (*Z*)-isomer is indeed favored by ~4.6 kcal/mol. The instability of the (*E*)-isomer likely partially arises from an unfavorable 1,3-interaction between the fluorine and carboxylate oxygens, since the free (*E*)-ligand at equilibrium features the carboxylate twisted out of plane. Of note, a (*Z*)-fluoroenamine has been observed in a type I DHDPS family aldolase crystal structure,[7c] but this enzyme leads to the (3*R*)-configuration of fluorine since the aldehyde binding site is located on the opposite face of the bound fluoropyruvate. This supports our working hypothesis that consistent fluorine-determined electronic features provide a driving force for high enantioselectivity. However, the absolute

configuration of fluorine that arises depends on which face of the fluoroenolate or fluoroenamine is exposed to an incoming aldehyde. This is ultimately dictated by the enzymatic architecture, such that the fluorine stereocenter may reliably be set as *R* or *S* by selecting an aldolase from the appropriate structural family.

Given our model for the origin of fluorine stereochemistry, we next explored how the stereochemistry of the hydroxyl group could be controlled. Although SwHpcH1 and SwHpcH2 were anticipated to be stereocomplementary based on a high-throughput screening study,[13] this was not seen under our conditions with fluoropyruvate substrate. However, SwHpcH1 was unique in its high *anti* selectivity with **1f**. Sequence alignment of 459 EcHpcH homologs indicated that variations in non-catalytic active site residues are fairly common (Figure S7). In particular, SwHpcH1 features phenylalanine at positions 117 and 210 in place of valine and leucine, respectively, found in the other four tested homologs. It is possible that the bulkier Phe residue could modulate the hydroxyl group stereoselectivity by restricting the poses available to an aldehyde. The V118F, A174F, and L212F variants of EcHpcH were thus purified for screening against our aldehyde panel. Notably, EcHpcH A174F and L212F were stereocomplementary in reactions with D-glyceraldehyde **1a** and D-lactaldehyde **1c** (Figure 3). Meanwhile, the V118F variant had high *anti* selectivity in reactions with **1e** and **1f** even at high conversions; the wild-type enzymes achieved this selectivity only with reduced enzyme loading and sacrificed conversion. In the course of our study, others showed with EcRhmA that elimination of bulk in the active site by mutation of non-conserved residues could broaden the substrate scope.[11e] Our results show that the opposite strategy of increasing bulk also has value for improvement or even reversal of stereoselectivity.

With a collection of wild-type and engineered aldolases in hand, we desired to demonstrate their applicability by scaling up from the assay conditions to synthesize fluorinated compounds in high stereopurity (Figure 4, Table S4). Due to the expensive nature of chiral aldehydes **1a-d**, the corresponding reactions were conducted on milligram scale with direct purification by preparative HPLC. This provided the six α-fluoroacids *anti*-**4a-d** and *syn*-**4ac** (d.r. 87–98%), corresponding to three stereoisomers each of 2-fluoro-3,4,5-trihydroxypentanoate and 2-fluoro-3,4-dihydroxypentanoate. For the inexpensive aldehydes **1efg** we carried out gram scale aldol-esterification sequences and obtained α-fluoroesters **5–8** (d.r. 80–97%) after extractive workup. Purified compounds were characterized by ^{1}H, ^{13}C, and ^{19}F NMR and confirmed by high-resolution mass spectrometry.

The structures accessed by fluoropyruvate addition can serve as versatile substrates for downstream processing via both enzymatic and chemical means, thus providing a range of compounds such as β-fluoro-α-hydroxyacids, β-fluoro amino acids, and fluorosugars (Figure 5). From the initial ß-fluoro-α-ketoacid fluoropyruvate adducts, we demonstrate the feasibility of two enzymatic transformations inspired by the metabolism of pyruvate in nature: dehydrogenation and transamination. In fact, commercial lactate dehydrogenase, which we used earlier in stereochemical experiments, accepted all of **3c-3l** as substrates to afford ß-fluoro-α-hydroxyacids **9c-9l**. We further show that the transaminase from *Vibrio fluvialis* (VfAT)[14] can convert **3h** and **3j** into the corresponding ß-fluoro amino acids **10h** and **10j**. Finally, to produce 2′- or 3′-fluorosugars, we subjected the previously purified

acids or esters *syn*-**4a**, *anti*-**4a**, *anti*-**4b**, **6**, and **7** to synthetic routes involving chemical reduction. This process was successful in producing fluorosugars **11**-**15**, with the notable pentose fragment **11** being a component of the anticancer drug Clofarabine which has been targeted for chemoenzymatic synthesis.[15] These efforts show that these aldolases can serve as the basis of a platform for stereoselective synthesis of important motifs that may be difficult to access by other means.

In summary, we have described a type II aldolase family capable of efficient fluoropyruvate addition to aldehydes with a high fluorine stereoselectivity. Our quantum mechanical modelling of the enzyme active site describes the thermodynamic factors that yield a large driving force for formation of the (*Z*)-fluoroenolate and subsequently the (3*S*)-fluoro product. HpcH aldolases have recently emerged as suitable engineering targets, and our work on modulating hydroxyl group stereoselectivity expands this knowledge base. Taken together, this work offers new approaches towards the preparation of a range of organofluorine building blocks, including fluorinated analogs of amino acids, sugars, and other valuable compounds.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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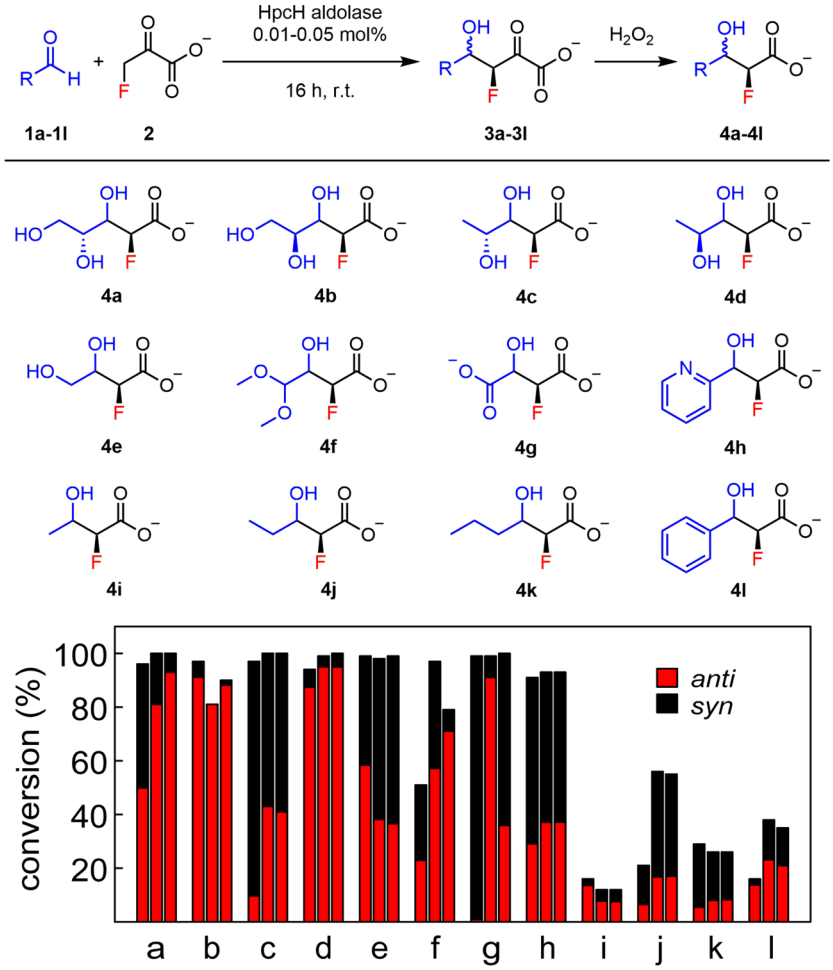

Figure 1.

Scheme and results of fluoropyruvate addition assay with a panel of twelve aldehydes. Reactions (50 mM substrates, 0.05 mol% enzyme, 1 mM $MgCl_2$) were quenched by decarboxylation after 16 h. From left to right in each group: EcGarL, EcHpcH, SwHpcH1.

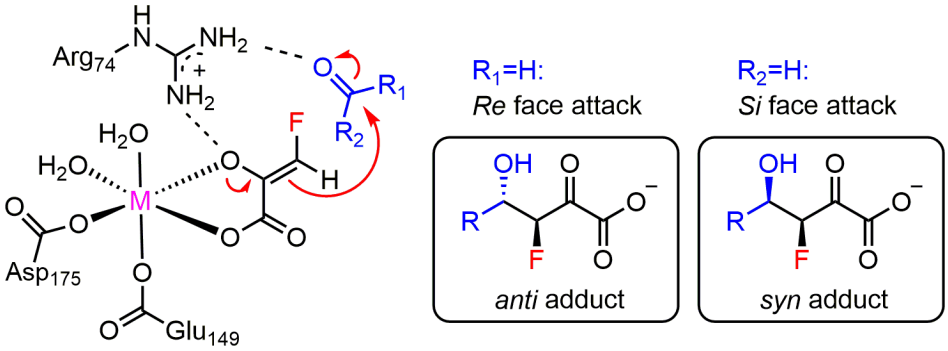

Figure 2.

Mechanistic model of fluoropyruvate aldol addition by HpcH family pyruvate aldolases for rationalization of the observed stereochemistry. The (*Z*)-fluoroenolate geometry, found through DFT calculations to be ~4.6 kcal/mol favored over the (*E*)-fluoroenolate, gives rise to the (3*S*)-configuration of fluorine. The configuration of the hydroxyl group varies depending on the orientation of the aldehyde.

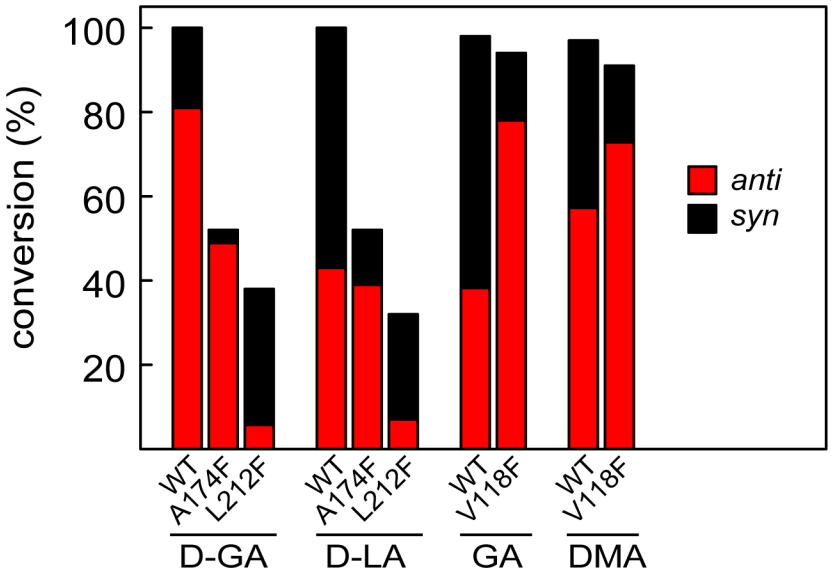

Figure 3.

EcHpcH variants with phenylalanine point mutations, displaying complementary and altered stereoselectivity of the hydroxyl group compared to wild-type. D-GA: D-glyceraldehyde **1a**; D-LA: D-lactaldehyde **1c**; GA: glycolaldehyde **1e**; DMA: dimethoxyacetaldehyde **1f**.

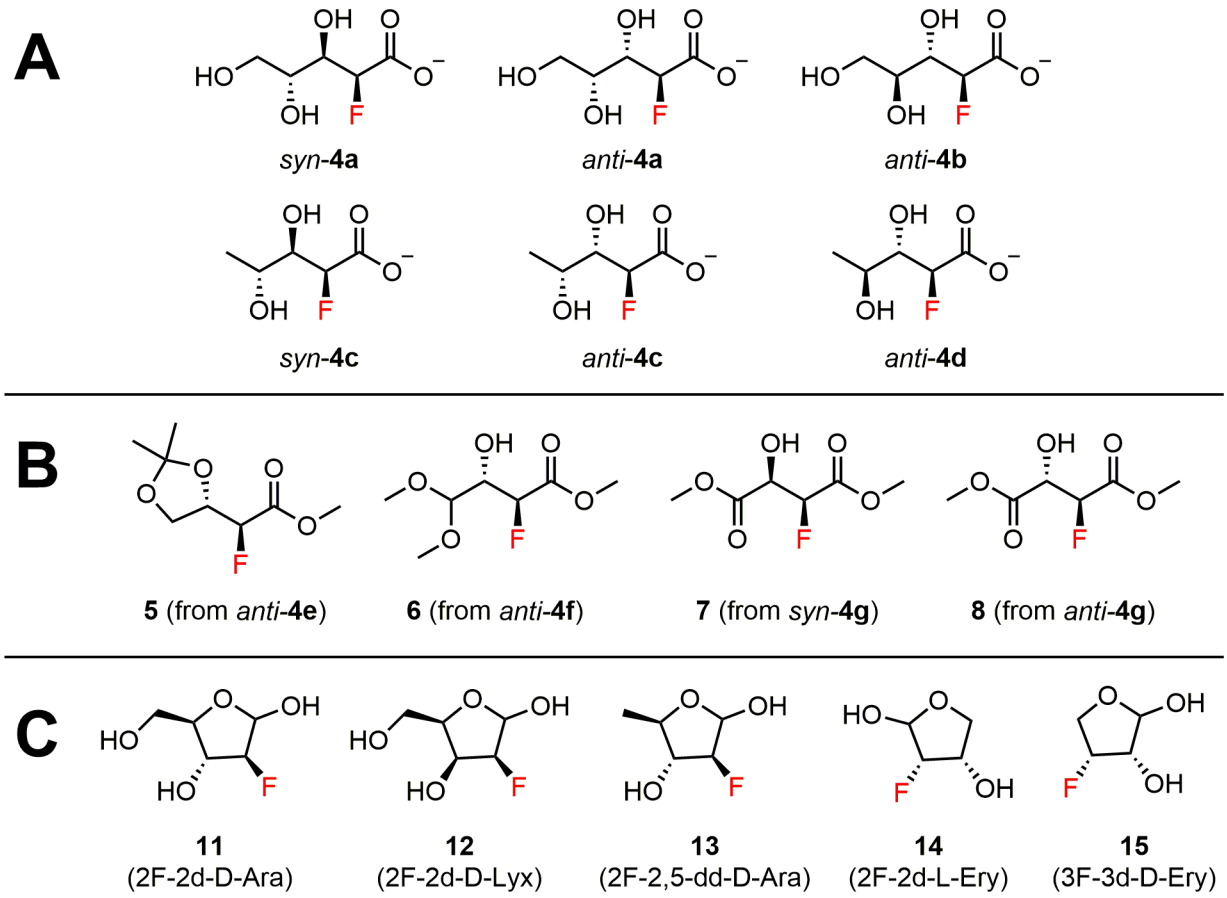

Figure 4.

Compounds produced by chemoenzymatic synthesis. A) Two sets of three isomers of polyhydroxy α-fluoroacids which were purified by HPLC on a semi-preparative HILIC column. B) α-Fluoroesters produced from large-scale biotransformations followed by protection chemistry and isolation by extraction. C) Fluorosugars derived from select compounds in groups A and B; characterized by high-resolution MS following oxime derivitization.

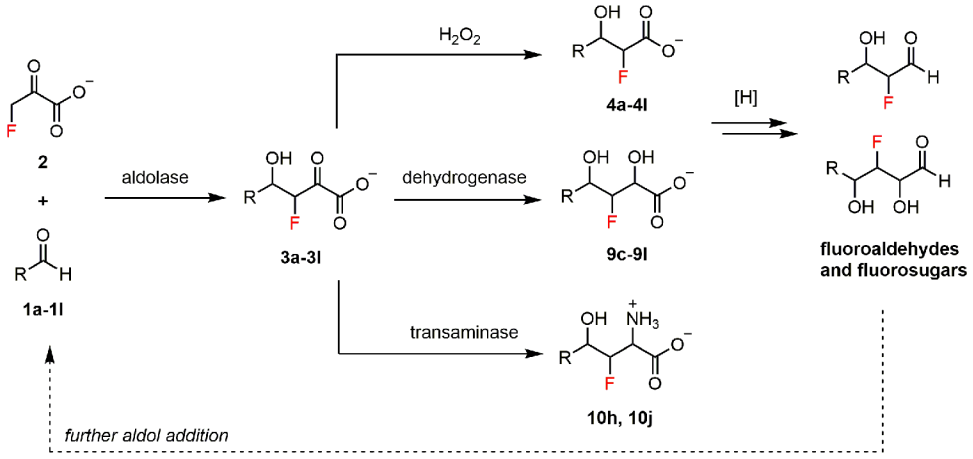

Figure 5.

Schematic of the pyruvate aldolase platform for organofluorine synthesis. The densely-functionalized fluoropyruvate adducts can be transformed into new functionalities through decarboxylation, ketoreduction, or transamination. Various fluorosugars can be accessed from fluoroacids by partial reduction of the carboxylate to an aldehyde (direct strategy) or by full reduction of the carboxylate to an alcohol when R contains a masked aldehyde (inversion strategy). Novel aldehydes synthesized in this manner could be used as the acceptor in further aldol reactions to incrementally build molecular complexity.