Recent Advances in the Substrate Selectivity of Aldolases

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ABSTRACT. Aldolases are powerful C-C bond-forming enzymes in biocatalysis due to their unparalleled stereoselectivity, the ease with which reactions that do not require cofactor recycling can be set up, the large number of different types and families available, and reaction feasibility under mild operating conditions. Since 2016, major discoveries have been made that broaden the scope of both nucleophile and electrophile substrates. For instance, more hydrophobic, sterically

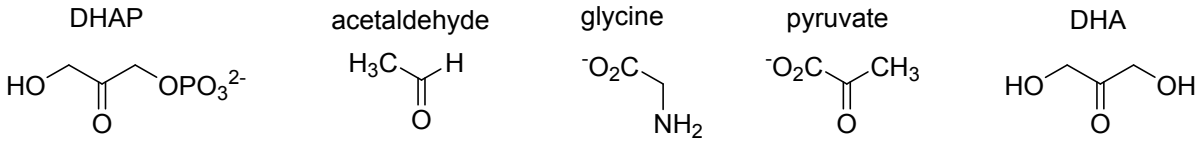
hindered nucleophile components have led to structures that are difficult to synthetize with purely chemical procedures. Likewise, the use of structurally diverse ketones as electrophiles has allowed the stereoselective synthesis of tertiary alcohols. These major advances will be presented and discussed in this review.

1 Introduction

Carbon–carbon bond formation is a cornerstone reaction and a challenging transformation in organic synthesis. The use of biocatalysts makes it possible to combine a more environmentally friendly chemistry with access to optically pure complex compounds. Aldolases are enzymes that have been widely described in the literature for many years[1–6] as being able to synthesize molecules of interest with a high chemo-, regio- and stereo-selectivity. Indeed, the chiral hydroxyketone motif they create can be found in the structure of many natural products.[7]

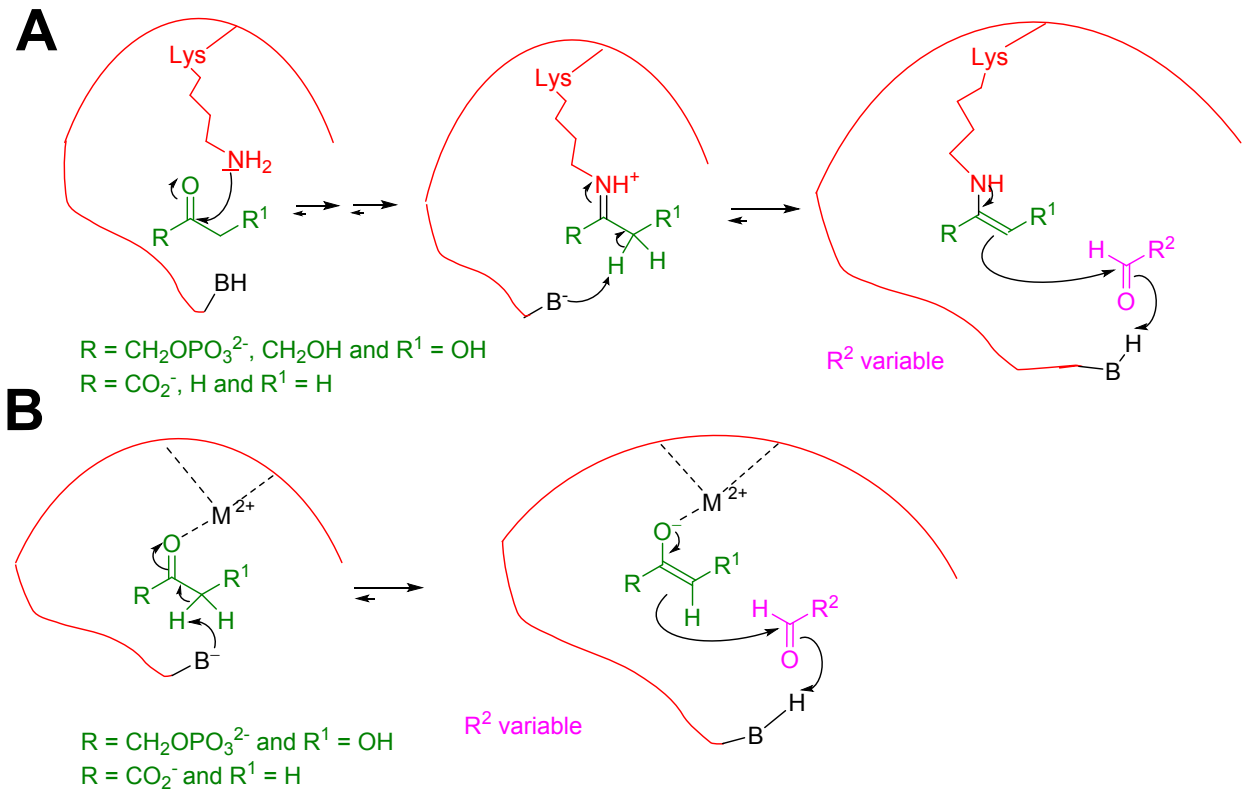
Aldolases are a class of lyases (EC-4.1.2.) that are classified into five families based on the substrate that plays the role of nucleophile component in the metabolism: dihydroxyacetone phosphate (DHAP)-, acetaldehyde-, glycine-, pyruvate- and dihydroxyacetone (DHA)-aldolases (Scheme 1). Exceptions are the DHA and analogues utilizing aldolases. For this particular class,

the natural substrate remains unknown, so its name derives from the first active nucleophile discovered in this family.[8]



Scheme 1. Nucleophile substrates of the five aldolase families.

From a mechanistic point of view, aldolases operate according to two distinct types of mechanism. In the first (class I, Scheme 2A), the nucleophile substrate is activated as an enamine through a Schiff base between the carbonyl group of the substrate and the ε-amino group of a conserved lysine residue in the active site to initiate the aldol or retroaldol reaction. In the second (class II, Scheme 2B), an essential transition metal ion acting as a Lewis acid cofactor promotes the deprotonation of the nucleophile with subsequent formation of an enediolate.



Scheme 2. Summarized mechanisms of C-C bond formation for **A**: Class I, and **B**: Class II aldolases.

In 2019, J. P. Adams et al[9] described the aldolase family as being underused in the pharmaceutical industry due to their strict requirement for a unique nucleophile. Challenging the dogma on their stringent nucleophilic substrate specificity, major advances have been made over the last five years to produce structurally diverse products using nucleophiles that are distinct from natural ones. Moreover, ketones were proved to be tolerated on the electrophile site, leading to optically pure tertiary alcohols, which is a highly sought-after motif.

This review will highlight recent work on the scope of nucleophile/electrophile substrates in four families of aldolases: DHAP, DHA and analogues, acetaldehyde (i.e., 2-deoxy-D-ribose-5-phosphate aldolase: DERA) and pyruvate aldolases. The pyridoxal 5'-phosphate (PLP) dependent glycine aldolases will not be discussed here because of i) their significant mechanistic difference (neither class I, nor class II, but formation of an external aldimine (i.e., PLP+glycine) that facilitates deprotonation of the nucleophile) compared to the four other families, and ii) their nucleophile is neither an aldehyde nor a ketone but an alpha-amino acid which does not produce an aldol or ketol adduct. In addition, this review will not include promiscuous aldolase activities concerning other classes of enzymes or non-catalytic proteins (e.g., 4-oxalocrotonate tautomerase, transferases, lipases, antibodies and formolase). Furthermore, we do not intend to present a comprehensive survey of the literature, but rather to illustrate the advance in tolerance of both nucleophile and electrophile substrate scope of aldolases developed for the construction of complex molecules.

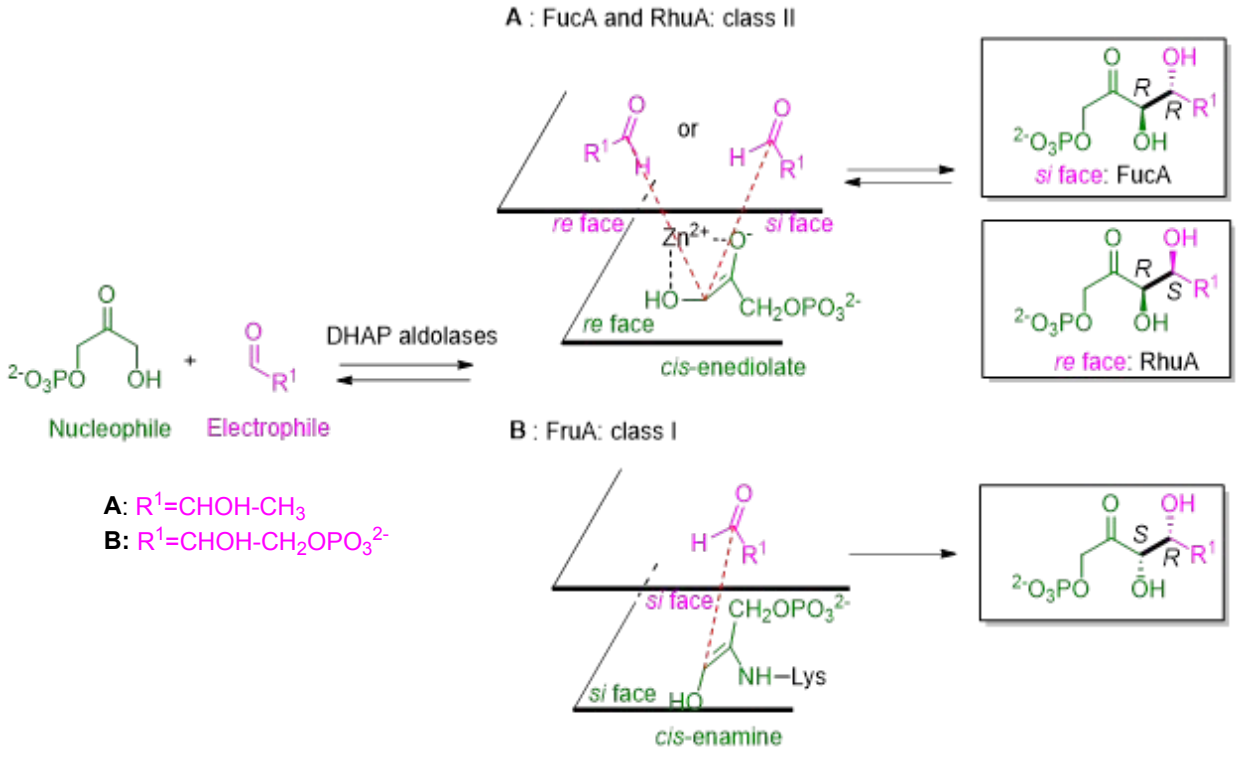
2 Overview of the different aldolases

In this section, general information will be given on DHAP, DHA, acetaldehyde and pyruvate aldolase families, for which significant discoveries have been made in the last five years.

2.1 Dihydroxyacetone phosphate-dependent aldolases (DHAP-aldolases)

DHAP aldolases were the first to be studied for organic asymmetric aldol reactions. They allow the creation of two new asymmetric carbons with the concomitant functionalization and, most importantly, three out of the four possible stereoisomers can be accessed with an excellent enantio- and diastereoselectivity. D-Fructose-1,6-*bis*phosphate aldolases (FruA) give adducts having (3*S*,4*R*) configuration, L-fuculose-1-phosphate aldolases (FucA) produce (3*R*,4*R*) aldols while L-rhamnulose-1-phosphate aldolases (RhuA) give (3*R*,4*S*) products.[1,3,10] D-Tagatose-1,6-*bis*phosphate aldolases (TagA) have limited applications in organic synthesis due to their lack of stereoselectivity. Only one example has been described using TagA for the stereoselective synthesis of D-tagatose from in situ generation of DHAP and D-glyceraldehyde-3-phosphate (D-G3P).[11] This limits the production of the four possible stereo-adducts from DHAP and an electrophile.[12] Although both class I and II co-exist in the FruA aldolase family, class I is more highly studied, with class II only reported for FucA and RhuA. For the latter two families, the DHAP C3 *pro-R* proton is abstracted leading to a *cis*-enediolate whose *re* face is exposed to the electrophile, leading to a strict 3*R* stereochemistry. The nucleophilic *cis*-enediolate then attacks the *si* face of the aldehyde, resulting in a 4*R* configuration for FucA, whereas the electrophile *re*

face is only available within the RhuA active site, giving a 4*S* configuration (Scheme 3A). It should be noted that, for both enzymes, occasional inverse binding of the aldehyde can be observed depending on its structure, leading to a lack of stereoselectivity in C4.[13] In terms of class I FruA (Scheme 3B), the DHAP proton removed is the C3 *pro-S,* giving a *cis*-enamine with an accessible *si* face for the electrophile, which leads to a strict 3*S* stereochemistry. After a *si* face attack of the aldehyde, a resulting 4*R* configuration is obtained.[14]



Scheme 3. Aldolisation mechanisms for **A**: class II FucA and RhuA, and for **B**: class I FruA, explaining DHAP aldolase stereocomplementarity.

DHAP-dependent aldolases can accept a great diversity of electrophilic aldehydes. This flexibility in terms of their active sites has led to the synthesis of complex polyhydroxylated monosaccharides being used as food additives, cosmetics, flavors and building blocks for drug synthesis.[1,3,4,10,15,16] Over the past five years, research on DHAP-dependent aldolases has focused on two main areas that will be discussed below in more detail. The first concerns in vitro or in vivo generation of the nucleophilic substrate DHAP by multienzymatic cascades using simple and abundant compounds as starting materials. The second is based on the conversion of previously unused electrophiles, such as ketones, providing access into the challenging sector of tertiary alcohols.

2.2 Dihydroxyacetone-dependent aldolases (DHA aldolases)

Class I D-fructose-6-phosphate aldolase from *Escherichia coli* (FSA_{Ecoli}), discovered in 2001, brings about the reversible catalysis of aldol addition from DHA to D-G3P to produce D-fructose-6-phosphate (D-F6P) ((3*S*,4*R*) stereospecificity).[8] Its physiological role in *E. coli* as well as its natural substrate remains unknown.[17] The crystal structure showed a decameric quaternary framework, built up of two pentamers packed face to face. This confers an extraordinary thermostability, resulting in persistently good activity after treatment at 70 °C for 40 min. The

subunit of FSA_{Ecoli} consists of a single domain that folds into an α/β barrel.[18] The great advantage of FSA_{Ecoli} in organic synthesis is its ability to catalyze aldol additions of non-phosphorylated nucleophilic components to aldehydes, instead of needing DHAP as in the case of DHAP-aldolases. The use of simple DHA analogues greatly simplifies and streamlines the synthetic process. Interestingly, wild type FSA_{Ecoli}, and variants thereof, were able to tolerate DHA nucleophile analogues such as hydroxyacetone (HA), hydroxybutanone (HB) and even the aldehyde hydroxyethanal (HE), catalyzing the reactions with high activity and uncompromised stereoselectivity.[19–21] This makes FSA_{Ecoli} an unprecedented aldolase with tremendous synthetic potential.

Since the discovery of FSA_{Ecoli} in 2001, in contrast to most other aldolases, no other natural, efficient DHA aldolases were identified from other sources until 2015, where a sequence-driven approach was applied to explore a large microbial genome for the DHA aldolase families.[22] Most of the newly discovered DHA aldolases had a conserved Tyr residue, which, like Tyr131 in FSA_{Ecoli}, contributes to the aldolase activity. Its involvement has given rise to some contradictory results. It has been suggested that Tyr131 acts as a general acid/base via a catalytic water molecule, which forms hydrogen bonds with Gln59 and Thr109 residues (Figure 1).[23,24]

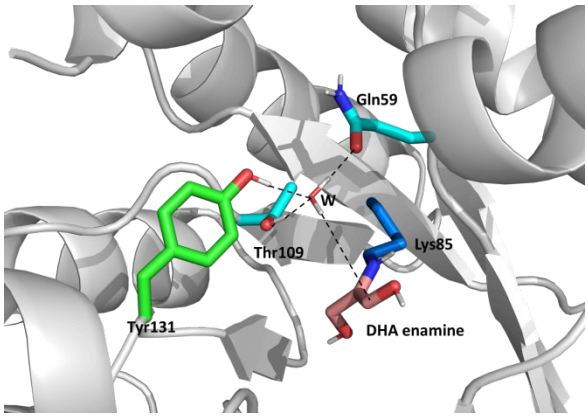


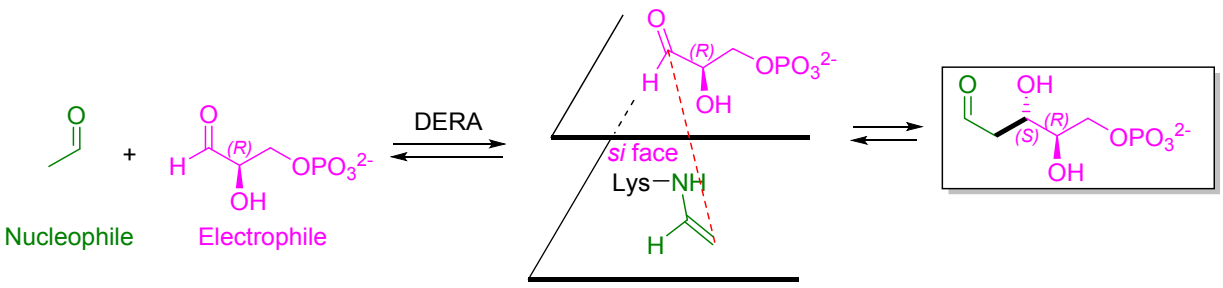
Figure 1. PyMOL model (http://www.pymol.org) of the active site of the wild-type FSA_{Ecoli} (PDB ID:1l6w) in a complex with DHA in enamine form (unpublished results). Tyr131 acts as a general acid/base via a catalytic water molecule (W), which forms hydrogen bonds with Gln59 and Thr109 residues.

Substitution of Phe for Tyr at position 131, however, while conserving partial activity in catalyzing the aldol addition of HA to cinnamaldehyde derivatives, prevented activity involving the reaction of DHA with D-G3P as well as the retro aldol cleavage of D-F6P.[23,25] Further knowledge of the active site has made possible to design variants that considerably increase the activity spectrum of DHA-aldolases, as discussed later.

2.3 Acetaldehyde-dependent aldolases: deoxy-D-ribose-5-phosphate aldolase (DERA)

2-Deoxy-D-ribose-5-phosphate class I aldolase (DERA) catalyzes the reversible conversion of acetaldehyde and D-G3P to 2-deoxy-D-ribose-5-phosphate (D-R5P), with a strict 3*S*

stereoselectivity.[26] This can be explained by the enamine nucleophilic attack of the *si* face of the natural D-G3P electrophile (Scheme 4).



Scheme 4: DERA mechanism exhibiting its stereoselectivity.

In vivo, DERA provides key intermediates that are essential for different metabolic pathways, e.g., glycolysis, the Krebs cycle, the pentose-phosphate pathway, and nucleotide catabolism[27] in bacterial, archaeal, and mammalian cells.[28] DERA from *E. coli* ($DERA_{Ecoli}$) was the most studied aldolase, existing as a dimer, with one active site per monomer.[29] Recent advances in the elucidation of the mechanism have been published.[30,31] In particular, a relatively high flexibility of the last eight C-terminal residues has been demonstrated by the absence of electron density in this region, indicating their possible participation in the activation of the nucleophile component via proton abstraction.[30] This explains why Ni-NTA purification of C-terminal His-tagged DERA had limited success. The main phosphate binding residues directed to the native acceptor substrate (D-G3P) are: Lys172 via a water molecule as well as direct side-chain interactions with Ser238,

direct peptide backbone interactions with Ser238 and Gly205, and via a water bridge with the backbone of residues Gly171, Val206, Gly236 and Ser239.[31] Amino acid positions responsible for DERA enantioselectivity have been identified to be Thr18 and Leu20 (both in β1-sheet) as well as Ala203 (β7-sheet). This was substantiated by comparison with enantio-complementary pyruvate-dependent 2-keto-3-deoxy-6-phosphogluconate aldolase and 2-keto-3-deoxy-6-phosphogalactonate aldolase using a homologous grafting approach, MD simulations, geometric, and phylogenetic analyses.[32] Among the mutants generated, T18S led to a decreased enantiomeric excess (ee 20%), but none of them were able to lead to inversion of stereoselectivity, suggesting that the DERA mechanism was more complex than anticipated.

The most attractive feature of the DERA aldolase family is that both substrate and product are aldehydes. However, it also highlights a problem of substrate selectivity, i.e., the unspecific role of nucleophile and electrophile, potentially resulting in two self-aldol reactions and two cross-aldol reactions. Part of the answer lies in the intrinsic behavior of the enzyme: it is an acetaldehyde-dependent aldolase, hence it displays an excellent selectivity for the nucleophile, like other aldolases, even though few other nucleophiles such as propionaldehyde,[33] acetone or fluoroacetone are also accepted, but at much lower rates.[34] It is unique among aldolases in converting an aldehyde

rather than a ketone as the natural nucleophile, the only other exception being the promiscuous activity observed for DHA aldolases.[20] On the contrary, it accepts a wide variety of aldehydes as electrophile substrates, with long chains of up to four carbon atoms.[4] This enables the synthesis of pyranoid building blocks, as well as the preparation of different types of deoxysugars, such as deoxy-, dideoxy-, trideoxy-, aza- and thio-sugars,[35] pyrimidine nucleosides,[36,37] 5-deoxy ketoses and nine carbon sialic acid-type sugar derivatives.[38,39] It is noteworthy that when polar groups are present at the electrophile C2 carbon, the D isomers are preferred over the L isomers, whereas in the case of a hydrophobic group at the same position, a reverse enantiopreference is observed.[40]

Cascade reactions were also envisaged since the aldol adduct is an aldehyde that can be used as an electrophile in an ensuing aldol reaction. For instance, when the first aldol product is an (*R*)-configured aldehyde, it can be accepted by DERA as an electrophile,[41] and the sequential cross-aldol reaction stops when a stable intra-molecular hemiketal is formed. This promising double aldolisation, involving three molecules of acetaldehyde or two plus one chloroacetaldehyde substrates, has been widely used to prepare statin side chains and epothilones, creating both stereocenters with excellent stereoselectivity (Figure 2).[42–45]

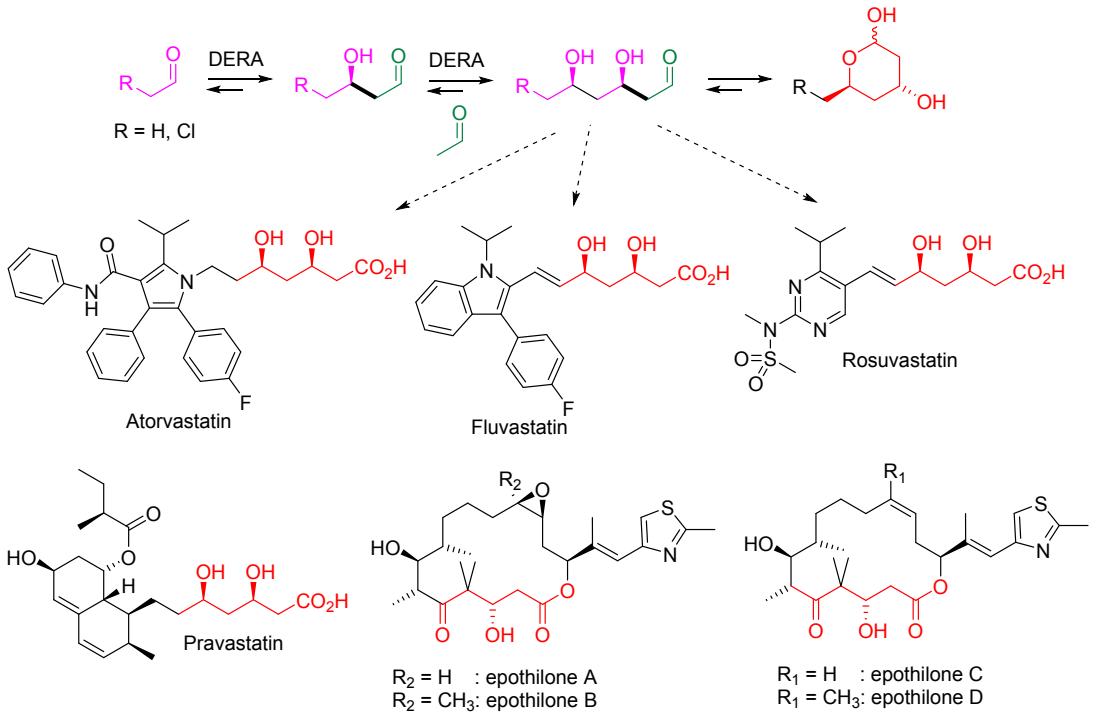


Figure 2. Industrially relevant molecules containing fragments (in red) that can be obtained with DERA.

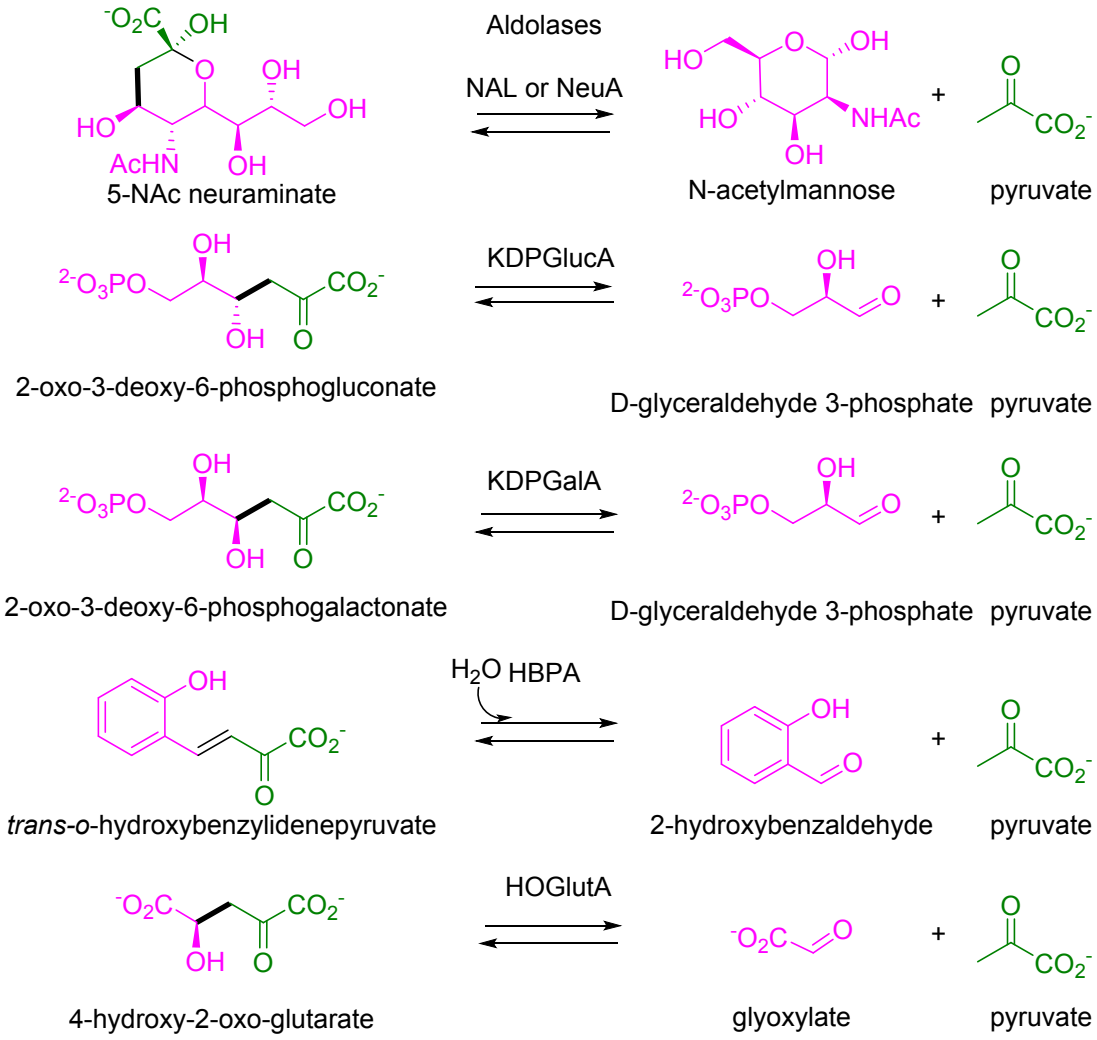
Epothilones prevent cancer cells from dividing by interfering with tubulin[40], whereas statins are cholesterol-lowering drugs inhibiting the 3-hydroxy-3-methylglutarylCoA reductase; the latter in particular is in huge demand in the pharmaceutical market.[46–48] As seen later, the attractiveness of such molecules of biological interest, which can be easily prepared by an eco-compatible method involving DERA, has led many researchers to focus on the optimization of this enzyme with a view to developing industrial processes.

Since acetaldehyde is harmful to the cell, a natural protective mechanism might exist where acetaldehyde inhibits DERA at high concentrations by terminating the D-R5P cleavage. This feature has been a major hindrance to the development of laboratory and industrial processes, and is still relevant, as mentioned later. Other recent advances will be discussed, such as nucleophile substrate promiscuity and the amazing discovery that it is possible to carry out four successive aldolisations when using one particular electrophile.

2.4 Pyruvate-dependent aldolases (PyrAL)

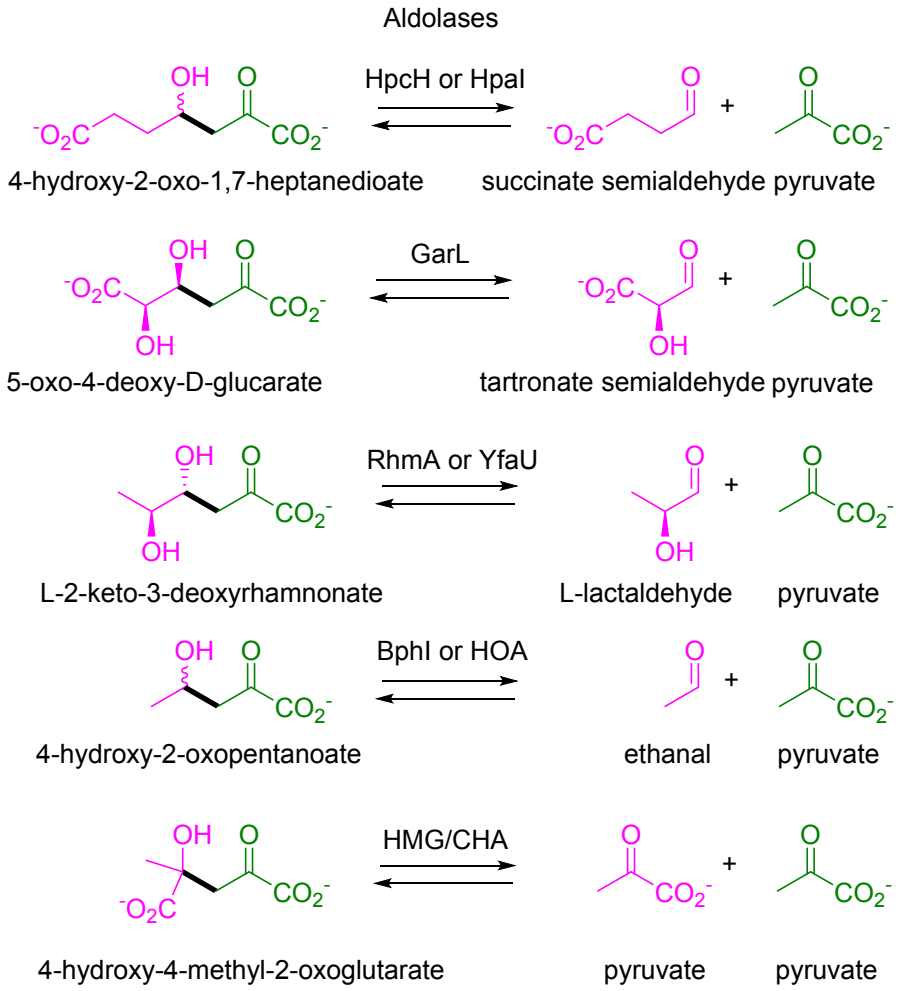
Pyruvate-dependent aldolases (PyrAL) reversibly catalyze the aldol addition of pyruvate to different aldehydes or even, in a few cases, to other ketoacids, yielding γ-hydroxy-α-oxoacids. PyrAL are involved in different biological functions and thus they exist in a great variety of families[49]. For example, they are involved in aromatic compound degradation pathways and in the metabolism of sugars through Entner-Doudoroff or modified Entner-Doudoroff pathways. Thus, given that they all share pyruvate as the nucleophile substrate, a large number of electrophiles can be involved, from aromatic to polyhydroxylated aldehydes.[1–3] In mechanistic terms they exist in classes I and II, and in structural terms they are present in four Pfam (https://pfam.xfam.org)[50]. *N*-Acetylneuraminic aldolase (NeuA or NAL), 2-dehydro-3-deoxy-6-phosphogalactonate aldolase

(KDPGalA), 2-dehydro-3-deoxy-6-phosphogluconate aldolase (KDPGlucA), *trans-o*-hydroxybenzylidenepyruvate hydratase aldolase (HBPA) and 4-hydroxy-2-oxoglutarate aldolase (HOGlutA), all from class I, belong to the same PF00701/01801/07071 dihydrodipicolinate synthetase family (DHDPS) (Scheme 5). Until recently, these class I PyrALs were mostly involved in organic synthesis.[5] To our knowledge, HOGlutA has never been used for synthetic applications.



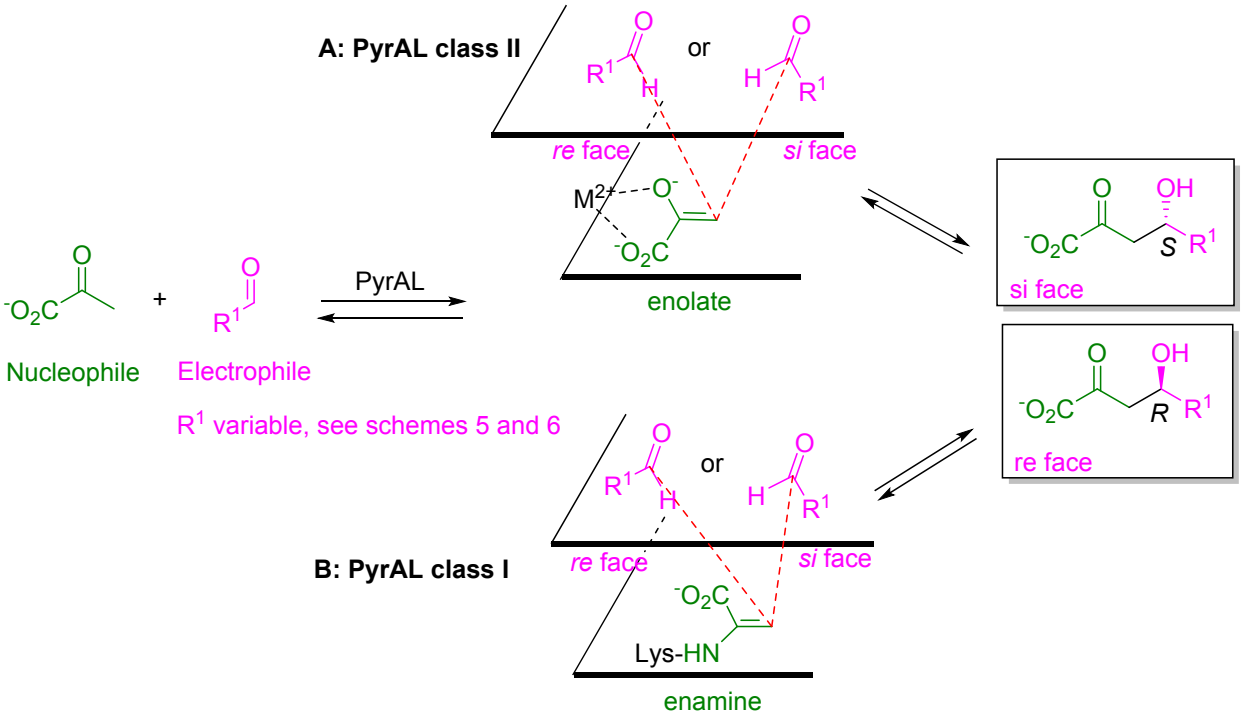
Scheme 5: Natural reactions catalyzed by class I PyrALs.

The PF03328 hpcH/citrate lyase group comprises the class II 4-hydroxy-2-oxo-heptane-1,7-dioate (HpcH), 5-keto-4-deoxy-D-glucarate (GarL) and 2-keto-3-deoxy-L-rhamnonate (RhmA or YfaU) aldolases. They will be described below, demonstrating their synthetic complementarity to the class I representatives. The PF07836 DmpG-like communication domain, containing 4-hydroxy-2-oxovalerate aldolase (HOA or BphI), is less used in synthesis despite its high stereoselectivity.[51] Finally, 4-hydroxy-4-methyl-2-oxoglutarate (HMG/CHA) aldolase, a member of the PF03737 regulators of ribonuclease E activity A family (RraA-like),[52] is able to catalyze the self-addition of pyruvate, leading to a tertiary alcohol pattern. Their catalyzed natural reactions are illustrated in Scheme 6.



Scheme 6: Natural reactions catalyzed by class II PyrALs.

In this aldolisation reaction, the pyruvate enzymes create stereocenter *R* or *S*, depending on the position of the aldehyde in their active site. Scheme 7 illustrates the two possibilities for classes I and II. *S* and *R* configurations are selected on the basis that R^1 gives priority to the methylene.



Scheme 7: PyrAL stereoselectivity depending on the *re* or *si* face of the aldehyde presented to the nucleophile.

Pyruvate aldolases and enzymes displaying promiscuous aldolase activities using pyruvate and analogs as nucleophiles have been reviewed recently, and, as mentioned in the introduction, will not be covered in detail in this review.[53] Nevertheless, we have devoted sections 3.5 and 4.5 to this family due to the innovative results found in the literature over the past five years.

3 The breakthrough of nucleophile substrates

3.1 Introduction

It is often noted in the literature that aldolases of all families are very specific to their nucleophilic substrate. This remains true for DHAP aldolases, for which no nucleophiles other than DHAP (or DHAP mimics) have been discovered in recent years. The latest publications focus on the development of new pathways for DHAP production. In contrast, new enzymes with outstanding performance, and with a capacity to transform other nucleophiles, have been found for the other three aldolase families. These are all reviewed below.

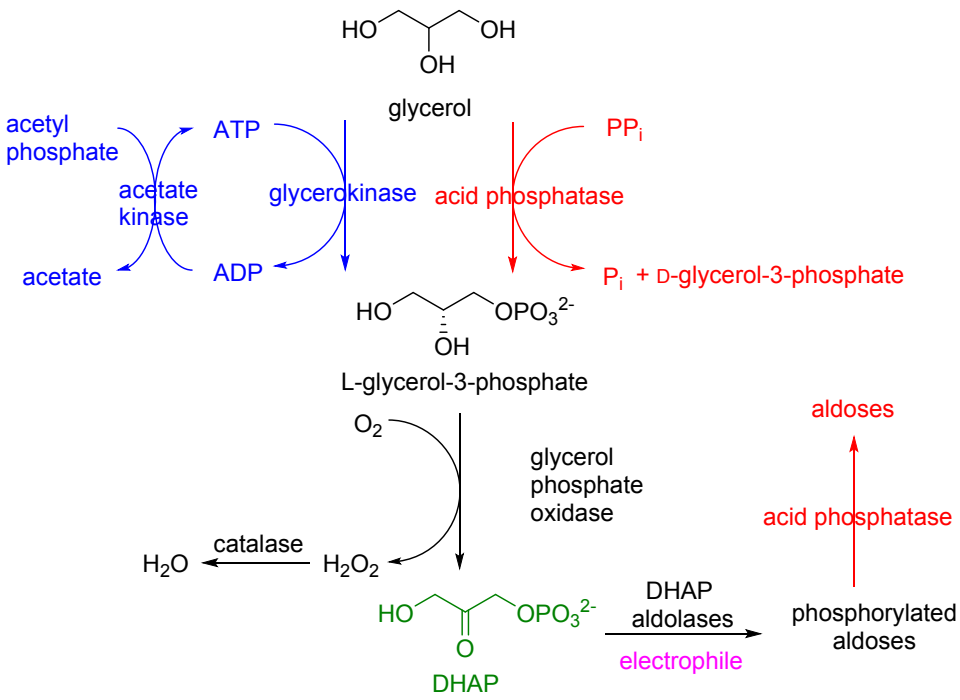
3.2 DHAP aldolases

One of the main disadvantages of DHAP aldolases is that they are only able to use DHAP as the nucleophile. Remarkably, RhuA was discovered to accept DHA, as such for the variant N29D, or with high concentrations of borate buffer for the wild-type.[54–56] It is well known that DHAP is a fragile substrate that is rather unstable under alkaline conditions, and this pH-sensitivity during synthesis results in its being commercially expensive.[4,57] Recent ecocompatible reactions, bioinspired or artificial, in vivo or in vitro, involving DHAP aldolases in multi-enzymatic cascades, have demonstrated well-proven benefits: i) minimizing the degradation of fragile

intermediates such as DHAP, ii) avoidance of any potential enzyme substrate inhibition, and iii) improvement in production yields coupled with reduced costs.

3.2.1 In vitro methods for DHAP synthesis

Since glycerol is a byproduct of biodiesel production, its conversion into high added value molecules is relevant.[58] In 2002, a two-step multienzymatic synthesis of DHAP from glycerol was developed.[59] This synthesis was improved in 2017 by incorporating a multienzymatic one-pot cascade, combining five enzymes at once (Scheme 8, blue and black parts).[60] Thereby, L-glycerol-3-phosphate was first generated by glycerokinase, using ATP as phosphate donor, and subsequently oxidized with glycerol phosphate oxidase. ATP regeneration was provided by acetylphosphate as phosphate donor in the presence of acetate kinase, while the inactivation of the oxidase by hydrogen peroxide was prevented by the addition of catalase.[61]



Scheme 8. Multienzymatic cascade for DHAP production and consumption. P_i: inorganic phosphate, PP_i: inorganic pyrophosphate.[60,64]

The original process was improved by searching for new sources of enzymes with enhanced activity, and by avoiding putative inhibitions. Thus, two new enzymes were implemented: an acetate kinase from *Mycobacterium smegmatis* and a flavin dependent glycerol phosphate oxidase from *Mycoplasma gallisepicum.* The catalase from *Micrococcus lysodeikticus* as well as the *Thermococcus kodakarensis* glycerokinase were already characterized.[62] This combination of enzymes led to an 88% glycerol conversion. The coupling of this cascade with a class I FruA from *Staphylococcus carnosus* ($FruA_{Scarn}$),[63] using acetaldehyde or D-G3P as electrophile, led to the

formation of ca 40% of aldol adducts at an analytical scale. According to the authors, this relatively low yield could be due to both product inhibition and equilibrium constraints. In 2020, Gao's team[64] proposed the synthesis of DHAP using a similar technique, but the phosphorylation step was catalyzed by an acidic phosphatase from *Shigella flexneri*, using the inexpensive pyrophosphate (PP_i) as phosphate donor instead of ATP (Scheme 8, shown in red). This phosphatase was previously developed by Wever's group[65,66] for the direct phosphorylation of DHA and then hydrolysis of the ester phosphate group of the aldol adduct that was created. The reaction system generated a racemic mixture of glycerol-3-phosphate, but only the L-enantiomer was transformed into DHAP, as had already been noted in 2000 by Sheldon's group.[67] The multienzymatic cascade enabled rare sugars of interest[15] to be generated through the use of D- and L-glyceraldehyde electrophiles, yielding D-psicose, D- and L-sorbose, L-tagatose and L-fructose at a hundred milligram scale with FucA from *Thermus thermophilus* ($FucA_{Ttherm}$), $FruA_{Scarn}$ or $RhuA_{Ecoli}$ as summarized in Table 1. Yields were calculated with respect to the limiting reagent, i.e., glyceraldehyde, while glycerol was present in excess (8.3 eq).

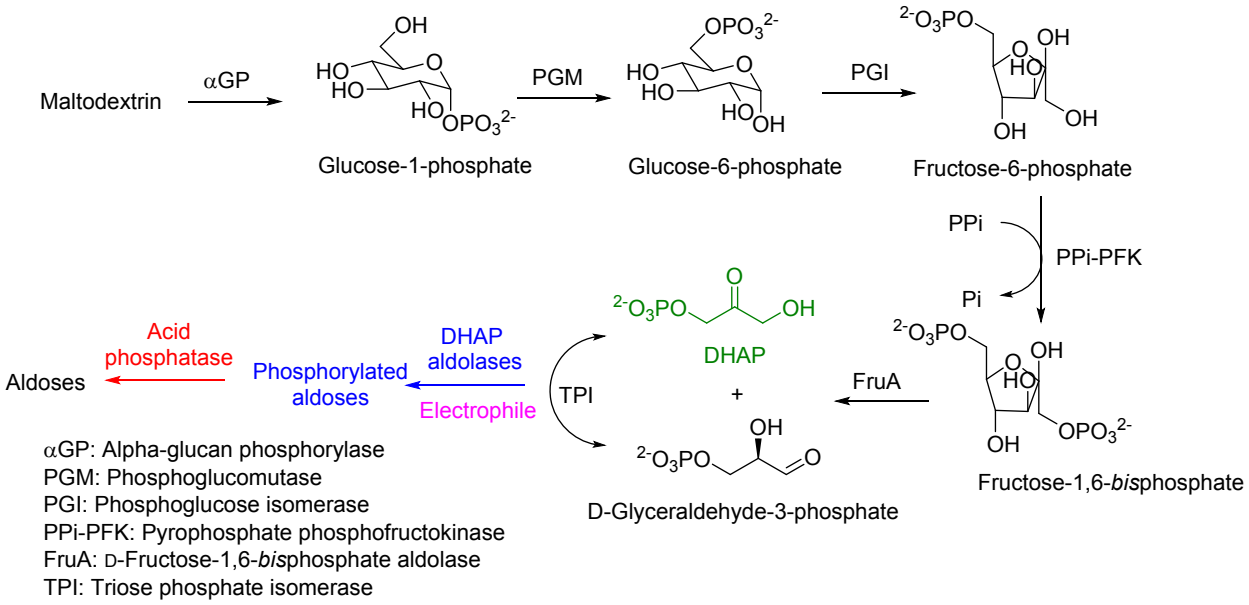
Table 1. Performance of aldolase-mediated cascade reactions based on the rare sugar obtained.[64]

Aldolase	Aldehyde	Product	Yield (%)

$RhuA_{Ecoli}$	D-glyceraldehyde	D-sorbose	24
$FucA_{Ttherm}$	D-glyceraldehyde	D-psicose	60
$RhuA_{Ecoli}$	L-glyceraldehyde	L-fructose	54
$FucA_{Ttherm}$	L-glyceraldehyde	L-tagatose	36
$FruA_{Scarn}$	L-glyceraldehyde	L-sorbose	56

Artificial ATP-free in vitro enzymatic biosystems have been generated from maltodextrin, a derivative of starch.[68] The process was inspired by a similar multienzymatic system, named "artificial metabolism" and developed by Fessner's group,[69] where DHAP was obtained from fructose-1,6-*bis*phosphate (FBP) starting from sucrose. In this new approach, four enzymes were involved to produce FBP starting from maltodextrin: alpha-glucan phosphorylase (αGP), phosphoglucomutase (PGM), phosphoglucose isomerase (PGI) and pyrophosphate phosphofructokinase (PP_i-PFK) (Scheme 9). This latter enzyme uses PP_i as the phosphate donor, thus avoiding ATP and its regeneration system. FBP was then retroaldolized into DHAP and D-G3P using FruA. Next, D-G3P and DHAP were interconverted by triose phosphate isomerase (TPI). Thanks to the thermostability of these enzymes, the process rate could be enhanced by increasing the temperature up to 70°C. Subsequently, $FucA_{Ecoli}$ or RhuA from *Bacteroides*

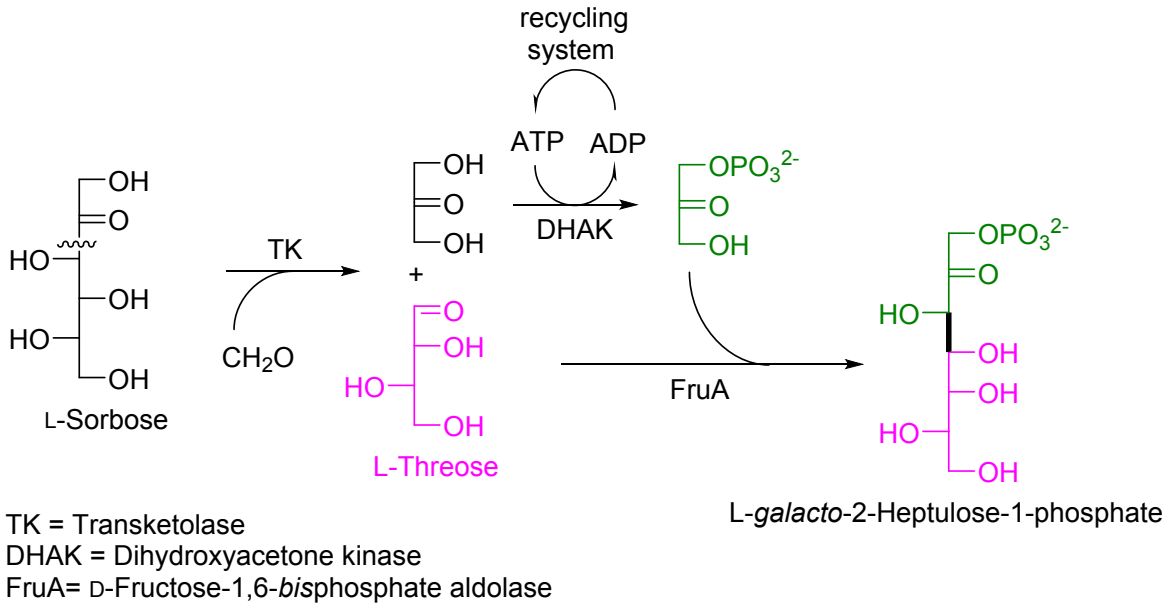
thetaiotaomicron ($RhuA_{Bthet}$) was used with different aldehydes but at room temperature due to the non-thermostability of these enzymes. Finally, dephosphorylation of the resulting aldol adduct was achieved by an acidic phosphatase. This last step could not be coupled in a one-pot fashion due to the lack of specificity of the phosphatase towards the aldose, and therefore it was performed as a one-pot three-step process. Aldol reactions with D-, L-glyceraldehyde, and HE were investigated on an analytical scale, but unfortunately none of them were scaled up.



Scheme 9. Multi-enzymatic synthesis of aldoses from a bio-sourced substrate, by a one-pot three-step procedure. Each step is indicated in a different color: black, blue and red.[68]

Another strategy involved a cascade using a transketolase (TK) as biocatalyst, with a ketose as starting material. This reaction furnished DHA, the precursor of DHAP, and the electrophile

component for the subsequent aldol reaction.[70] As a proof of concept, L-sorbose and formaldehyde, an uncommon TK acceptor for the transfer reaction, were chosen as substrates, yielding DHA and L-threose (Scheme 10). Conversion of DHA into DHAP was then performed by a dihydroxyacetone kinase (DHAK) in the presence of ATP with the appropriate regeneration system. Under these conditions, the whole process was virtually irreversible, allowing only one equivalent of formaldehyde to be used. Finally, the addition of FruA from rabbit muscle enabled L-*galacto*-2-heptulose-1-phosphate to be obtained in a one-pot one-step process, providing 80% isolated yield, at a hundred milligram scale.

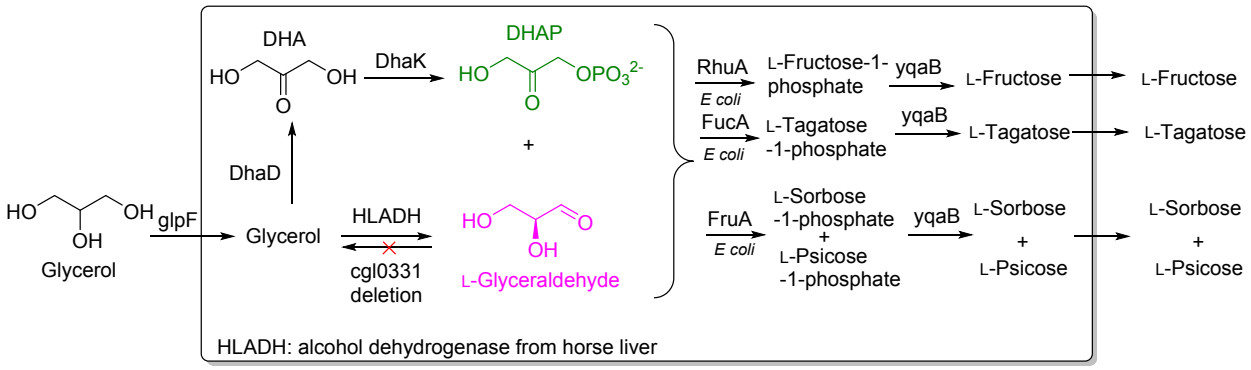


Scheme 10. TK mediated generation of both nucleophile and electrophile components in the aldolase biocatalyzed reaction.[70]

3.2.2 In vivo methods for DHAP synthesis

There are multiple benefits to whole cells overexpressing different enzymes: *i*) there is no need for external cofactor regeneration, *ii*) the enzyme of interest is produced within the cell, and *iii*) it is highly efficient, taking advantage of the cell machinery. Thus, DHAP could be generated from glycerol, based on the in vitro multienzymatic cascades described above. Sun's team[71] showed that a modified strain of *Corynebacterium glutamicum* was able to grow three times faster if the organism first converts glycerol to DHA before being phosphorylated into DHAP, rather than through the glycerolphosphate intermediate. This was because the cell growth was inhibited by glycerol phosphate. Thereby, genes encoding a glycerol facilitator (GlpF from *E. coli),* glycerol dehydrogenase (DhaD from *Klebsiella pneumoniae)* and ATP-dependent dihydroxyacetone kinase (DhaK from *Citrobacter freundii)* were chosen for their high enzyme activity. At the same time, a second pathway was constructed within the same strain to produce the electrophile component, L-glyceraldehyde, from glycerol, thanks to an alcohol dehydrogenase from horse liver. To avoid the glyceraldehyde reduction to glycerol, the gene of *C. glutamicum,* responsible for the expression of the corresponding oxidoreductase, was deleted (cgl0331). To produce the target aldoses, three strains were built containing three stereo-complementary DHAP aldolases: $FruA_{Ecoli}$, $RhuA_{Ecoli}$

and $FucA_{Ecoli}$, Finally, fructose-1-phosphatase was also introduced to allow the final dephosphorylation of the hexoses formed (i.e., $YqaB_{Ecoli}$). Using these whole-cell biocatalysts, L-fructose, from $RhuA_{Ecoli}$, L-tagatose from $FucA_{Ecoli}$, and a mixture of L-sorbose and L-psicose from $FruA_{Ecoli}$ were observed, though they were not isolated (Scheme 11).



Scheme 11. In vivo methodology to produce various L-monosaccharides.[71]

The strategy to produce DHAP developed by Scott's group[60], described in Scheme 8, was also implemented by Gao's team[72] within a whole-cell strategy, involving an overexpression of a glycerol kinase from *E. coli* for glycerol phosphorylation and a glycerophosphate oxidase from *Streptococcus pneumoniae* for DHAP production. The proof of concept was demonstrated with $RhuA_{Ecoli}$ and phosphatase $YqaB_{Ecoli}$. Two electrophiles, L- and D-glyceraldehyde, were introduced by portions producing L-fructose in ~50% yield and a mixture of D-sorbose and D-psicose (ratio

2.4/1) in 44% yield, both at a gram scale. Two equivalents of glycerol were used for one equivalent of aldehyde to create optimal conditions for glycerol (400 mM).

3.3 DHA aldolases

The nucleophile tolerance of FSA_{Ecoli} is not limited to the ones mentioned thus far, but also includes a series of higher DHA and HA homologues.[73] This is possible with minimal protein engineering at the nucleophile binding site. Two main residues, L107 and L163, form a hydrophobic wall, blocking any extension of the new sterically demanding nucleophile substrates in this direction (Figure 3). Thus, substituting Ala for Leu in these positions created sufficient space to accommodate the enlargement of the DHA and HA homologues.

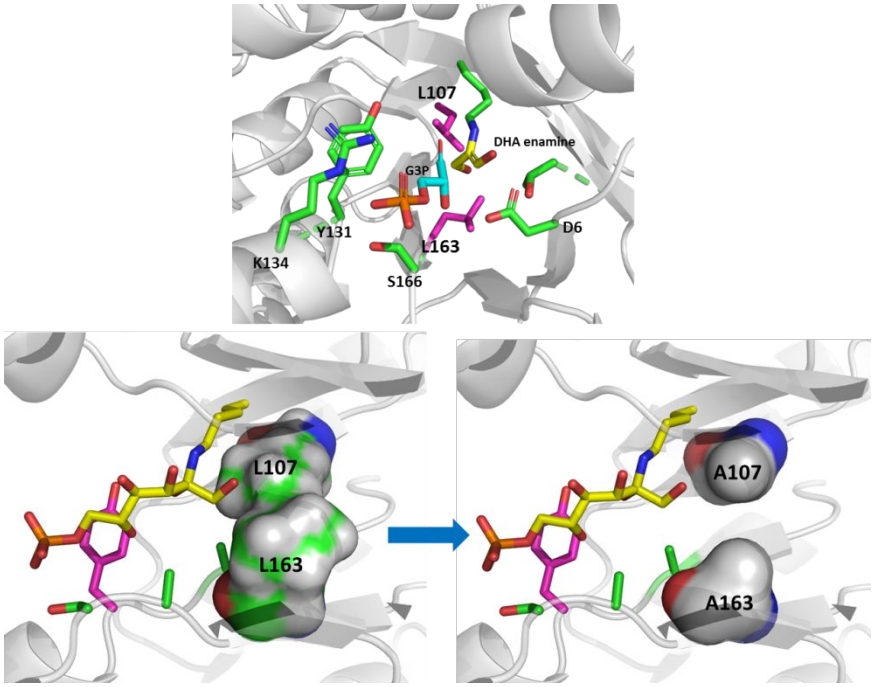
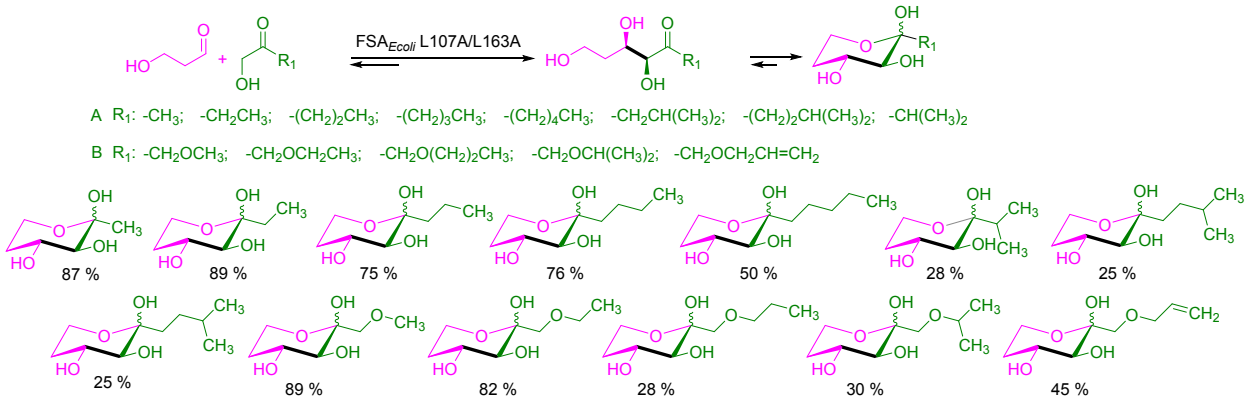


Figure 3. PyMOL model (http://www.pymol.org) of the active site of the wild-type FSA_{Ecoli} (PDB ID:1l6w) in complex with D-F6P.[74] Protein engineering at the nucleophile binding site. Substitution of Leu107 and Leu163 residues by Ala to accommodate larger nucleophile substrates.

DHA and HA homologues comprise those having long linear and branched aliphatic chains including some with functionalization (Scheme 12). The new nucleophiles were tested on the aldol addition to 3-hydroxypropanal. This reaction furnished unprecedented C1 substituted deoxyketopyranoses in 25−89% isolated yields from preparative reactions catalyzed by the most active FSA_{Ecoli} L107A/L163A variant. Furthermore, only a single stereoisomer (3*S*,4*R*) was formed in all cases, demonstrating that the stereochemistry was not compromised either by the nucleophile or by the structural changes in the active site. From the activity point of view, the reaction rates of DHA ether homologues were higher than the alkanones, whereas branched donor substrates had the lowest ones. A critical mutation was L163A, which created the additional space in which the substrate enlargement could be accommodated.[73]



Scheme 12. FSA$_{Ecoli}$ L107A/L163A variant catalyzed the synthesis of a series of C1 substituted deoxysugars. A: Alkanone homologues of HA; B: Ether homologues of DHA.[73]

This unique nucleophile tolerance of FSA$_{Ecoli}$ became even more useful when it was found that the hydroxymethyl structural functionality was not essential for the activation of the nucleophile component by the enzyme machinery. Experiments proved than simple ketones and aldehydes were also suitable substrates of FSA$_{Ecoli}$ in aldol addition reactions. This was first demonstrated in the FSA$_{Ecoli}$ catalyzed addition of ethanal, propanone, and butanone, structural analogs of HE, HA, and HB, respectively, to D,L-G3P.[75] D,L-G3P was selected because it has the lowest K_M value for FSA$_{Ecoli}$ (K_M 0.8 mM), giving it a kinetic advantage over other aldehydes.[21]

Models of wild-type FSA in complexes with the Lys85 D-fructose6-phosphate (Fru6P) imine (Figure 4) and the Lys85-dihydroxyacetone (DHA) enamine plus D-G3P,[76] indicated that residue Asp6 could interact via hydrogen-bonding with both the hydroxymethyl group of the DHA moiety

and the secondary hydroxyl group of D-G3P, presumably influencing the nucleophile and electrophile selectivity.[23,77]

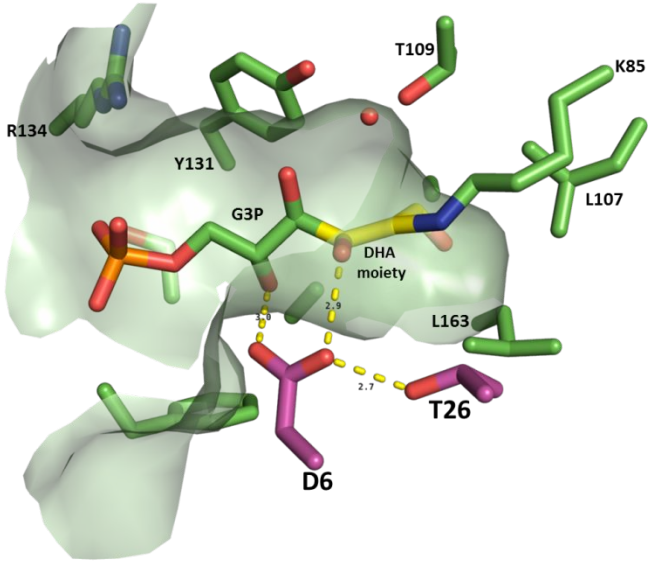
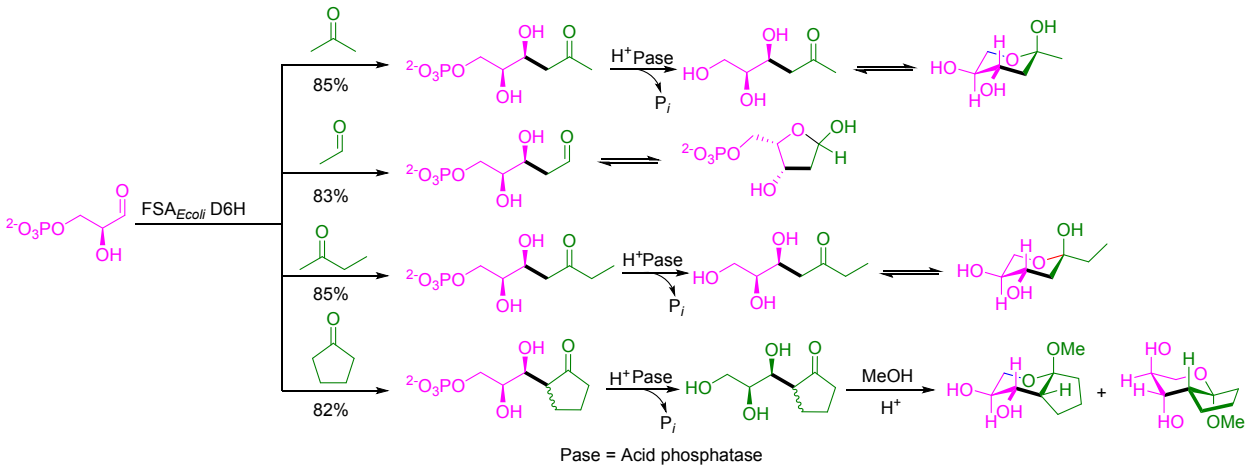


Figure 4. PyMOL model (http://www.pymol.org) of the active site of the wild-type FSA_{Ecoli} (PDB ID:1l6w) in a complex with D-F6P.[74] Residue Asp6 involved in nucleophile selectivity.

Hence, FSA_{Ecoli} D6X variants, where X can be a hydrophobic (Ala and Leu), polar charged (Glu and His), or polar uncharged (Asn, Gln, Ser, and Thr) residue, were assayed. FSA_{Ecoli} D6H variant gave excellent results (>80% yield) in aldol additions of propanone, ethanal, butanone and cyclopentanone to L-G3P, with excellent stereoselectivity (>95% diastereomeric ratio, dr) and delivering a collection of rare deoxysugars with alkyl and cycloalkyl substituents (Scheme 13).[75]

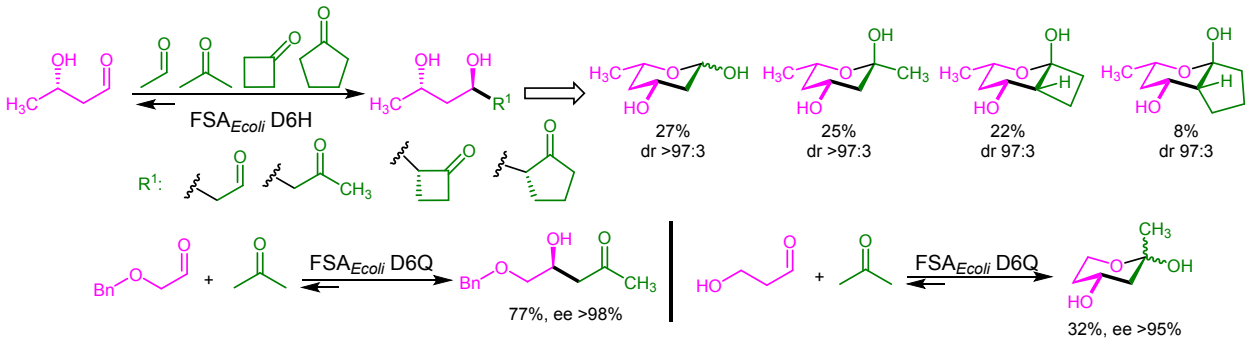
It was also shown that Asp6 was the key residue for the recognition of non-hydroxylated nucleophiles.



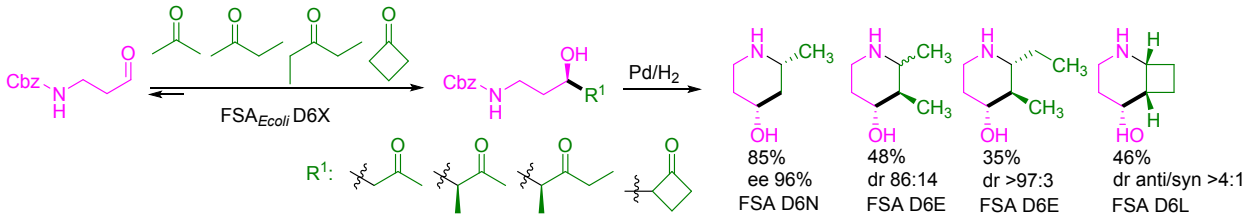
Scheme 13. FSA$_{Ecoli}$ D6H variant catalyzed aldol addition reaction of propanone, ethanal, butanone and cyclopentanone to L-G3P.[75]

The scope of this FSA$_{Ecoli}$ catalytic activity was probed in the aldol additions of ethanal, propanone, butanone, 3-pentanone, cyclobutanone and cyclopentanone to non-phosphorylated hydroxyaldehyde derivatives (Scheme 14)[76] and a benzyloxycarbonyl protected amino aldehyde (Scheme 15).[78] Interestingly, when using L-G3P, butanone reacted exclusively from the less substituted carbon atom (i.e., C1 attack). This is remarkable since organocatalytic aldol addition via enamine preferentially produced aldol adducts from the most substituted carbon atom (i.e., C3 attack) or resulted in regioisomeric mixtures depending on the catalyst.[79] However, when *N*-

benzyloxycarbonyl-3-aminopropanal was used, the aldol adduct from the C3 attack was preferentially formed (Scheme 15). This could be due, among other things, to the different FSA variant at the D6X position used or the nature of the electrophile G3P or *N*-benzyloxycarbonyl-3-aminopropanal. These reactions provide a strategy for the bottom-up synthesis of rare deoxysugars and chiral intermediates as well as of functionalized N-heterocycles.



Scheme 14. FSA$_{Ecoli}$ D6H and D6Q catalyzed aldol addition of ethanal, propanone, cyclobutanone and cyclopentanone to (*S*)-3-hydroxybutanal and benzyloxyacetaldehyde.[76]

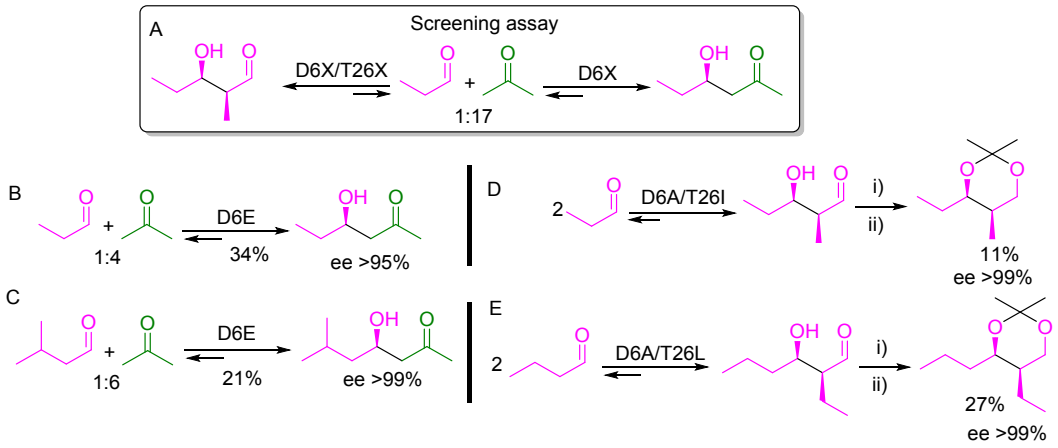


Scheme 15. Different FSA$_{Ecoli}$ D6X (where X=Asn, Glu, Leu) variants catalyzed aldol additions of ethanal, propanone, 3-pentanone, and cyclobutanone to *N*-Cbz-3-aminopropanal and

subsequent Cbz deprotection and intramolecular reductive amination, toward the N-heterocycle preparation.[78]

FSA$_{Ecoli}$ also made it possible to address targets from homo aldol products of aldehydes, which are the basis for naturally occurring products like polyketides and terpenoids, as well as bulk chemicals (e.g., Guerbet-type compounds) (Scheme 16).[80–82] By a structure-guided approach, a second important residue, Thr26, in the FSA$_{Ecoli}$ active site, was identified and targeted along with Asp6 in a dual way (Figure 4). The Asp6 residue was mutated using the SHH (i.e., Ala, Asp, Gln, Glu, His, Leu, Pro, and Val), and Thr26 using the VYT (i.e., Ala, Ile, Leu, Pro, Thr, and Val) codon degeneracies requiring the screening of 200 clones for 95% coverage. SHH and VYT are degenerated codons (i.e. S: is 1:1 mixture of G and C; H: 1:1:1 of A, C and T; V:1:1:1 A, C, G and Y: 1:1 of C and T; T: is T). Library screening was performed using an aqueous medium containing a mixture of propanone and propanal (17:1, v:v) as competitive substrates (Scheme 16A). It was found that the D6X replacement showed a significant preference for propanone as nucleophile, while the introduction of T26X mutations strongly shifted toward propanal. FSA$_{Ecoli}$ D6H and D6E variants gave the best results for the addition of propanone to propanal and to isobutanal, while

D6A/T26I and D6A/T26L gave the best rates for the homo aldol addition of propanal and butanal, respectively. Both reactions had a highly stereoselective outcome (Scheme 16).[83]

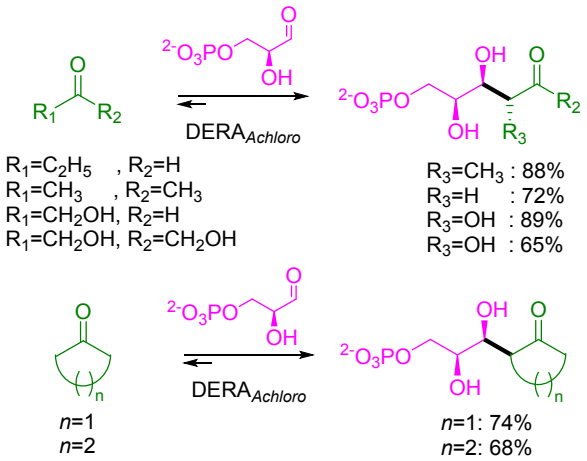


Scheme 16. (**A**) Screening assay for the library FSA$_{Ecoli}$ D6X/T26X. The screening led to variants with a preference for propanone as nucleophile (i.e., cross aldol addition of propanone to propanal) or toward propanal as nucleophile (i.e., homo aldol addition of propanal, since propanone could not act as electrophile). FSA$_{Ecoli}$ D6E variant catalyzed the addition of propanone to propanal (**B**) and propanone to isopentanal (**C**) whereas FSA$_{Ecoli}$ D6A/T26I catalyzed the homo aldol addition of propanal (**D**) and D6A/T26L the homo addition of butanal (**E**). i) $NaBH_4$, MeOH; ii) 2,2-dimethoxypropane, H^+.[83]

3.4 DERA

Examination of the biodiversity in a sequence-driven approach has led to the discovery of two new DERAs from *Arthrobacter chlorophenolicus* (DERA$_{Achloro}$) and *Haemophilus influenzae*

(DERA$_{Hinfl}$), with significant activity for singular nucleophiles such as cyclobutanone, cyclopentanone, HE and DHA, in addition to the already known nucleophiles ethanal and propanone. By using L-G3P, the corresponding rare L-monosaccharide-phosphates were prepared with DERA$_{Achloro}$ in 68 to 89% isolated yields (Scheme 17).[84] After the discovery of FSA$_{Ecoli}$ as a unique aldolase with broad nucleophile tolerance, native DERA$_{Achloro}$ could be highlighted showing similar plasticity. Considering DHA as a nucleophile, the sole stereocomplementary tool to FSA (3*S*,4*R* selective) was the RhuA$_{Ecoli}$ variant N29D (3*R*,4*S* selective).[56] Interestingly, in this study, DERA$_{Achloro}$ gave aldol adducts with a (3*R*,4*R*) configuration, a stereoselectivity that had not previously been identified in the DHA utilizing aldolases. This is in agreement with Wong's preliminary work which showed the same (*R*) configuration alpha to the carbonyl of the aldol adduct when propanal was used as the nucleophile with DERA$_{Ecoli}$.[85]



Scheme 17. Synthesis of rare L-monosaccharides, involving unusual DERA$_{Achloro}$ nucleophiles.[84]

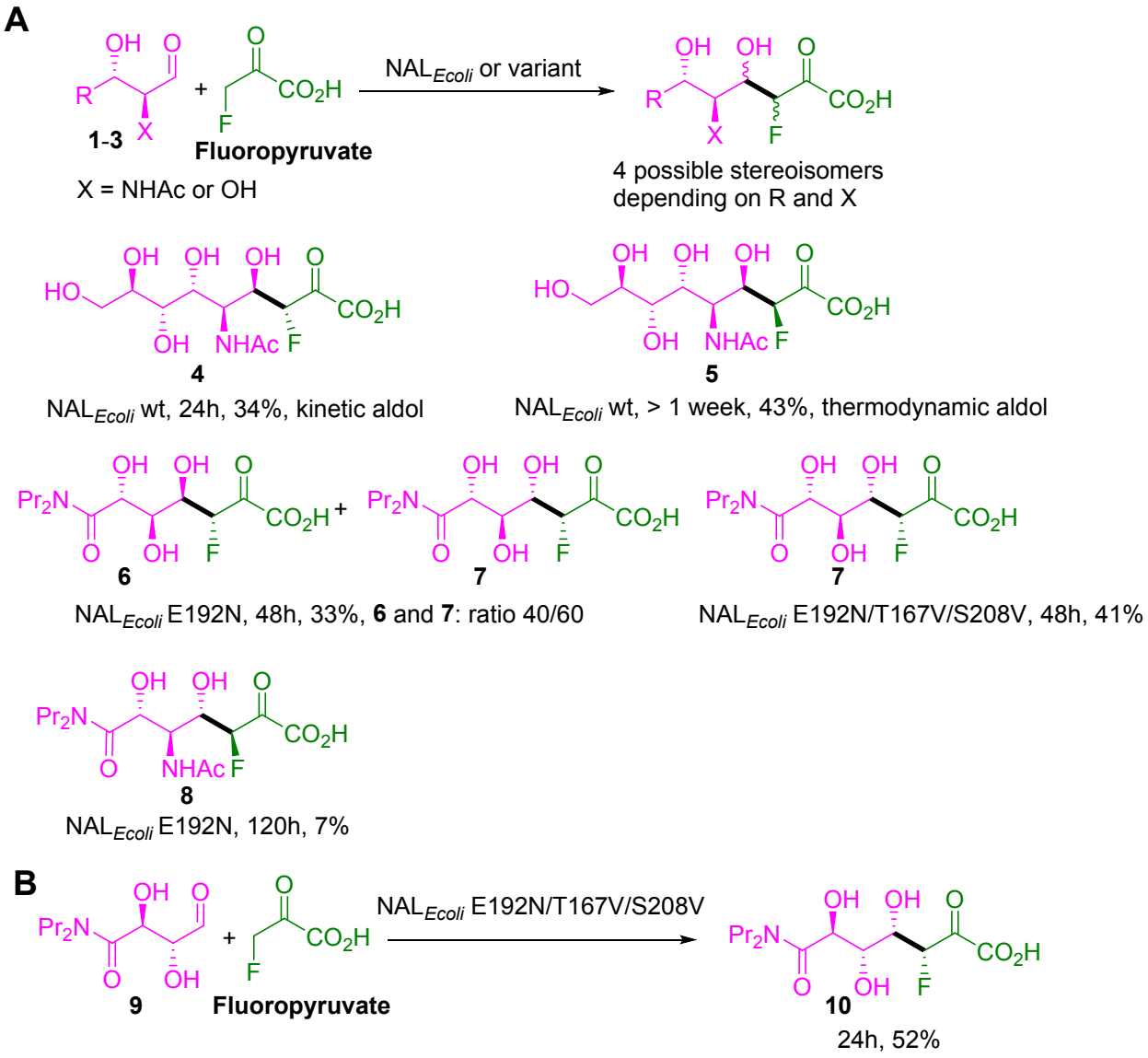
3.5 Pyruvate aldolases

3.5.1 Fluoropyruvate

Since 2002, it has been shown that class I pyruvate aldolases can accept fluoropyruvate[86–88] as the nucleophile allowing the synthesis of fluorosialic acid analogs. Recently, two class I pyruvate aldolases were used as catalysts for the synthesis of 3-fluoro-2-oxo acid derivatives using fluoropyruvate as the nucleophile,[89] namely *N*-acetyl neuraminic acid lyase from *E. coli*[90] (NAL_{Ecoli}), E192N and E192N/T167V/S208V variants, and *trans-o*-hydroxybenzylidenepyruvate hydratase aldolase from *Pseudomonas putida* ($HBPA_{Pput}$).[91] NAL_{Ecoli} E192N was previously generated to increase electrophile specificity and E192N/T167V/S208V for stereoselective complementarity.[90] Thus, using NAL_{Ecoli}, the aldol addition of fluoropyruvate to various α-*S* configured aldehydes (**1**-**3**, Scheme 18A) and an *R* configured aldehyde (**7**, Scheme 18B) were investigated, yielding four possible stereoisomers (Scheme 18A). Interestingly, the stereoselectivity outcome depended on the aldehyde structure and reaction time. Wild type NAL_{Ecoli} and electrophile **1** gave stereoisomer **4** under kinetic control, while under thermodynamic control **5** was formed. In the presence of an α-hydroxyl in the aldehyde, e.g. **2**, NAL_{Ecoli} E192N/T167V/S208V variant was always 3*R* stereoselective allowing the formation of **6** and **7**, while with the NHAc moiety only isomer **8**, of the four possible, was obtained. Still with NAL_{Ecoli}

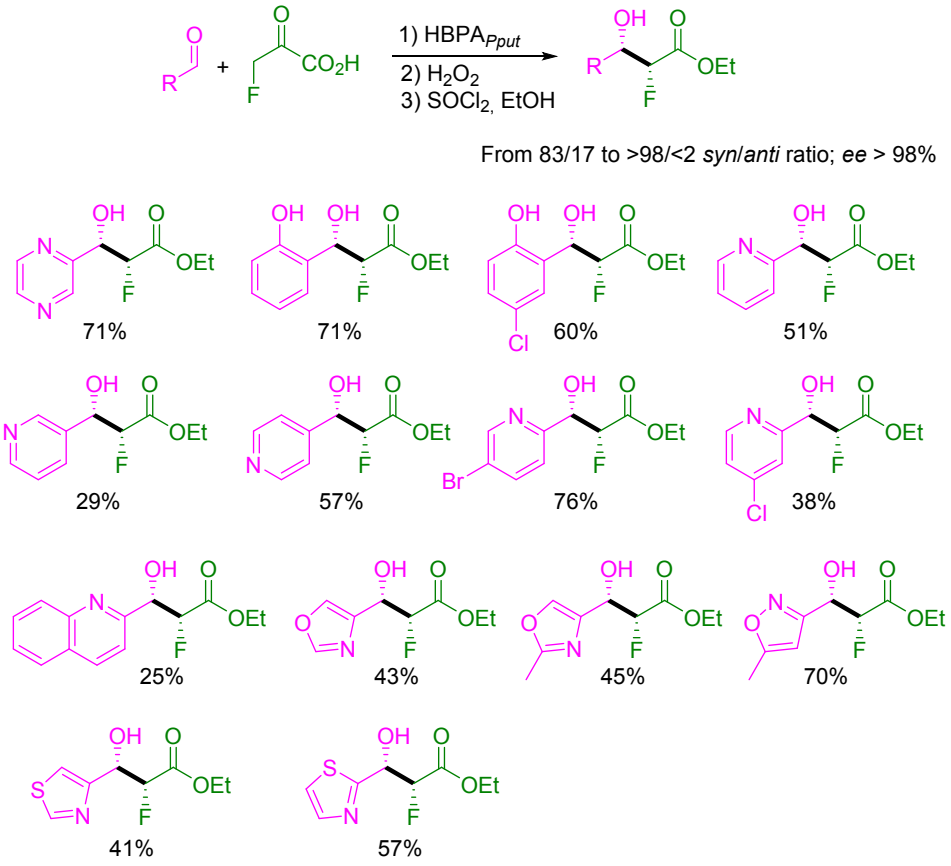
E192N/T167V/S208V as catalyst, changing the α-configuration of the aldehyde for *R* (**9**, Scheme 18B) also led to the formation of only one isomer (**10**, Scheme 18B).

HBPA$_{Pput}$ was chosen as the catalyst to prepare aldol adducts from fluoropyruvate and various aromatic or heteroaromatic aldehydes (Scheme 19). This aldolase has been described to catalyze pyruvate additions to aromatic aldehydes followed by dehydration of the aldol, leading to conjugated ketoalkenes.[92,93] However, probably due to the presence of the fluorine atom, dehydration was not observed using fluoropyruvate.[90] Thus, the 2-oxoacids were not isolated and directly converted into their corresponding ethyl esters after oxidative decarboxylation. Fluoro compounds were achieved in 25 to 76% isolated yields in high ee (>98%) and a *syn*/*anti* ratio that varied from 83:17 to 98:2 in favor of the (2*R*,3*S*) diastereomer which exceeds the results achieved using NAL$_{Ecoli}$ variants.



Scheme 18. NAL$_{Ecoli}$ catalyzed synthesis of fluoro ketoacids from fluoropyruvate and aldehydes.

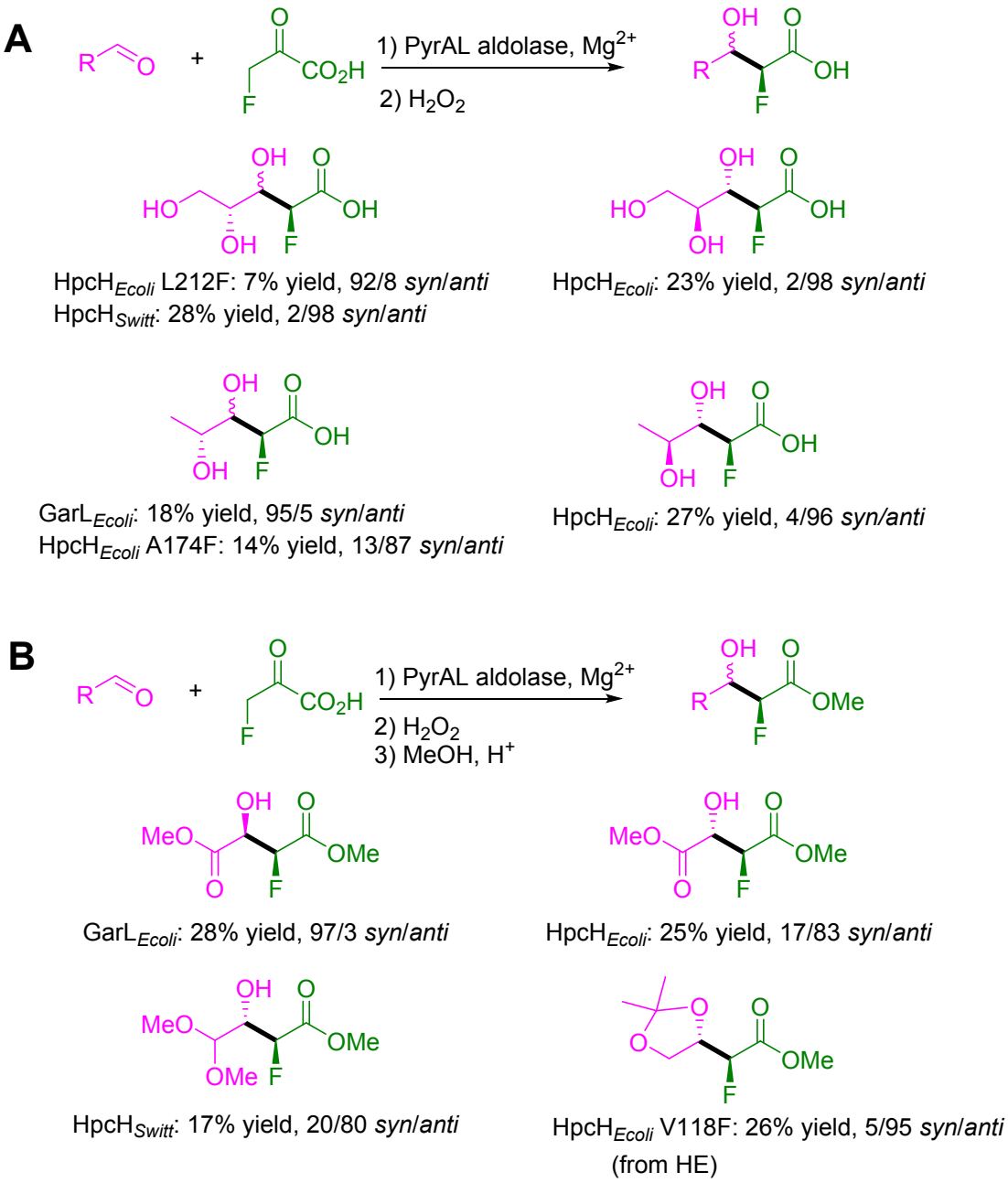
A: α-*S* configured aldehydes (**1**, **2** and **3**), **B**: α-*R* configured aldehyde (**9**).[89,90]



Scheme 19. HBPA$_{Pput}$ catalyzed synthesis of fluoro esters from fluoropyruvate, aromatic and heteroaromatic aldehydes.[91]

In 2019, Chang's group[94] studied several class II pyruvate aldolases belonging to the PF03328 family (Uniprot KB Id.: P23522, B1IS70, P76469, A5VH82; respectively GarL, HpcH and RhmA from *E. coli*, putative HpcH from *Sphingomonas wittichii*). They demonstrated that these aldolases also catalyzed the addition of fluoropyruvate to a variety of aldehydes. They were exclusively (3*S*)-selective, although non-stereoselective at C4 (4*R* or 4*S*). This configuration depended on the

enzyme and electrophile (Scheme 20). To improve the diastereoselectivity, $HpcH_{Ecoli}$ was engineered and three variants, L212F, A174F and V118F, have shown higher stereoselectivities. Using these enzymes and variants thereof, the authors prepared carboxylic acid and ester derivatives after oxidative decarboxylation and esterification, respectively, starting from either chiral aldehydes (Scheme 20A, D- or L-glyceraldehyde and D- or L-lactaldehyde) or achiral ones (Scheme 20B, 2,2-dimethoxyethanal, HE and glyoxylate). It is noteworthy that only *anti* stereoisomers were isolated as major adducts when using L-aldehydes. In terms of the achiral aldehydes, only glyoxylate allowed the isolation of major *syn* or *anti* isomers, depending on the enzyme used, whereas 2,2-dimethoxyethanal and HE gave major *anti* isomers.



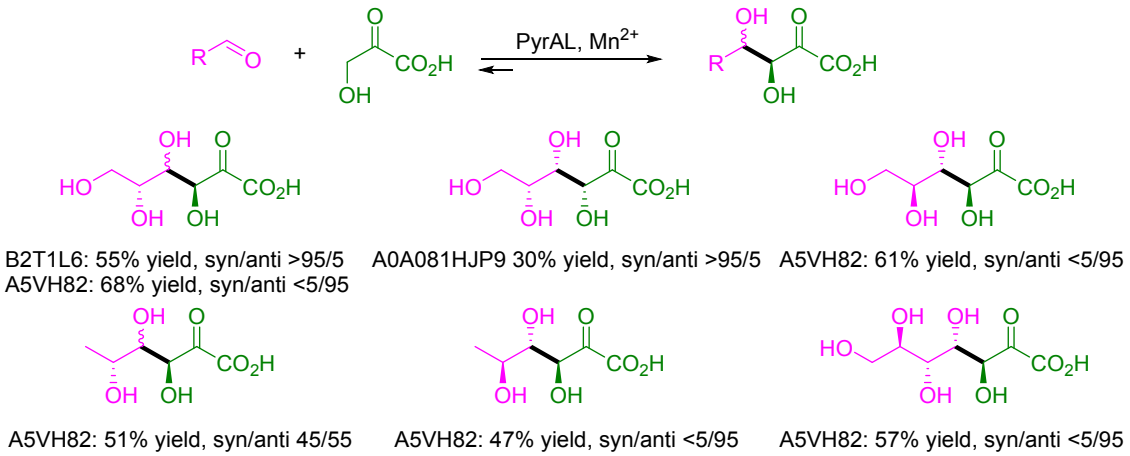
Scheme 20. PyrAL catalyzed synthesis of fluoro acids and esters from fluoropyruvate and various aldehydes. **A**: fluoro acids from D- or L-glyceraldehyde and D- or L-lactaldehyde (yields from HPLC analysis). **B**: fluoro esters from 2,2-dimethoxyethanal, hydroxyethanal and glyoxylate (isolated yields).[94]

3.5.2 Hydroxypyruvate (HPA)

Hydroxypyruvate (HPA) was described as being converted by PyrAL at the analytical scale in the 90s, but it was only recently that a study provided the proof of concept for this nucleophile at the preparative scale.[95–98] A collection of 571 enzymes, selected from various microorganisms and built through a sequence-driven approach, was screened using HPA as the nucleophile in the aldol reaction with D,L-glyceraldehyde as the electrophile.[99] From this study, twenty-one wild type enzymes of the PF03328 family, including known $YfaU_{Ecoli}$ (also named RhmA), $GarL_{Ecoli}$ and $HpcH_{Ecoli}$ aldolases, were found to tolerate HPA. Further, using D-glyceraldehyde as the model electrophile with these twenty-one aldolases, three stereoisomers of the four possible ketoacids (i.e., 3*S*,4*R*; 3*R*,4*S* and 3*S*,4*S*) were isolated, their relative proportion depending on the aldolase. PyrAL A5VH82 from *S. wittichii*, B2T1L6 from *B. phytofirmans* and A0A081HJP9 from *P. aeruginosa* (respectively putative HpcH, GarL and HpcH aldolases) proved to be excellent biocatalysts since they afforded good yields and high complementary stereoselectivities (Scheme 21, numbers are Uniprot KB Id., to link to the original paper, and also due to their still unknown biological functions). Moreover, to complement the synthetic applications, A5VH82, i.e., $hpcH_{Switt}$, tolerated other electrophiles, such as D- and L-lactaldehyde as well as D-erythrose,

providing the corresponding aldols in good yields and dr >95% (~50% from D-lactaldehyde). This enzyme was selected for its remarkable capacity to provide adducts with a (3*S*,4*S*) configuration, forming a complementary tool to the existing known aldolases.

An explanation of A5VH82 (HpcH$_{Switt}$) high efficiency towards HPA was proposed by Hanefeld's group.[100] A phenylalanine residue in the active site (Phe210) establishes CH-π interactions with the C-H bonds at the C3 of the HPA, thus improving the nucleophile binding to the active site. This Phe210 in A5VH82 (HpcH$_{Switt}$) occupies a generally conserved leucine residue found in other PyrAL. When this Phe residue was replaced by a tyrosine to further increase the electron density, a two-fold improvement of the catalytic rate constant was measured for the variant.



Scheme 21. PyrAL A5VH82 from *S. wittichii*, B2T1L6 from *B. phytofirmans* and A0A081HJP9 from *P. aeruginosa* catalyzed synthesis of polyhydroxylated ketoacids.[99]

3.5.3 Alkyl-substituted pyruvate analogs

a) 2-Oxobutyrate

Class II PyrAL have been described in the literature for many years as being able to use 2-oxobutyrate as a nucleophile. However, no product characterizations, purifications, nor yields had been demonstrated. Recently, Clapés' group published the isolation of ester derivatives prepared from the addition of 2-oxobutyrate to *N*-Cbz protected α-amino aldehydes catalyzed by 2-keto-3-deoxy-L-rhamnonate aldolase ($YfaU_{Ecoli}$, also named RhmA, Uniprot KB Id.: P76469), fused with a maltose binding protein from *E. coli* (MBP-$YfaU_{Ecoli}$).[101] $YfaU_{Ecoli}$ is a class II 2-oxoacid aldolase (EC 4.1.2.53) that was found mainly in an active soluble form when fused with MPB, thus avoiding the formation of inclusion bodies. Moreover, as observed for other class II aldolases, some non-physiological metal ion cofactors improved the new-to-nature reaction activity.[102,103] In this case, Ni^{2+} exhibited ~2-fold higher activity than that with the natural Mg^{2+} metal ion cofactor.[104] Selected synthetic examples illustrated the utility of MBP-$YfaU_{Ecoli}$ in aldol additions of 2-oxobutyrate to (*R*) and (*S*)-*N*-Cbz-alaninal (R^1 = **A** and **B**) and (*R*) and (*S*)-*N*-Cbz-prolinal (R^1 = **C** and **D**) electrophiles (Scheme 22). Two variants (W23V and/or W23V/L216A) helped to reach

more than 90/10 dr in favor of the (3*S*,4*S*)-*syn* diastereomer with (*R*)-*N*-Cbz aldehydes (R^1 = **A** and **C**). (Figure 5).

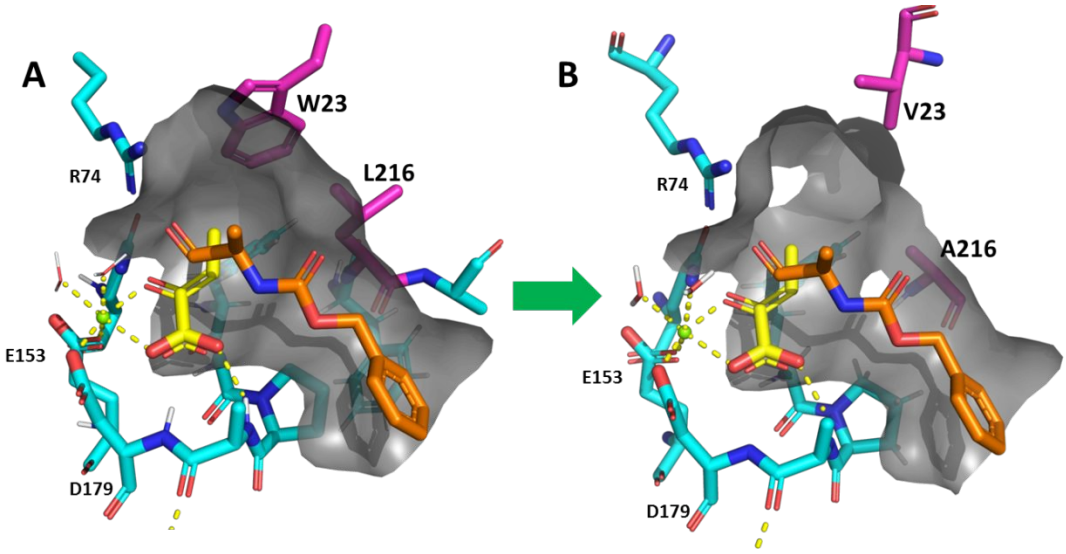
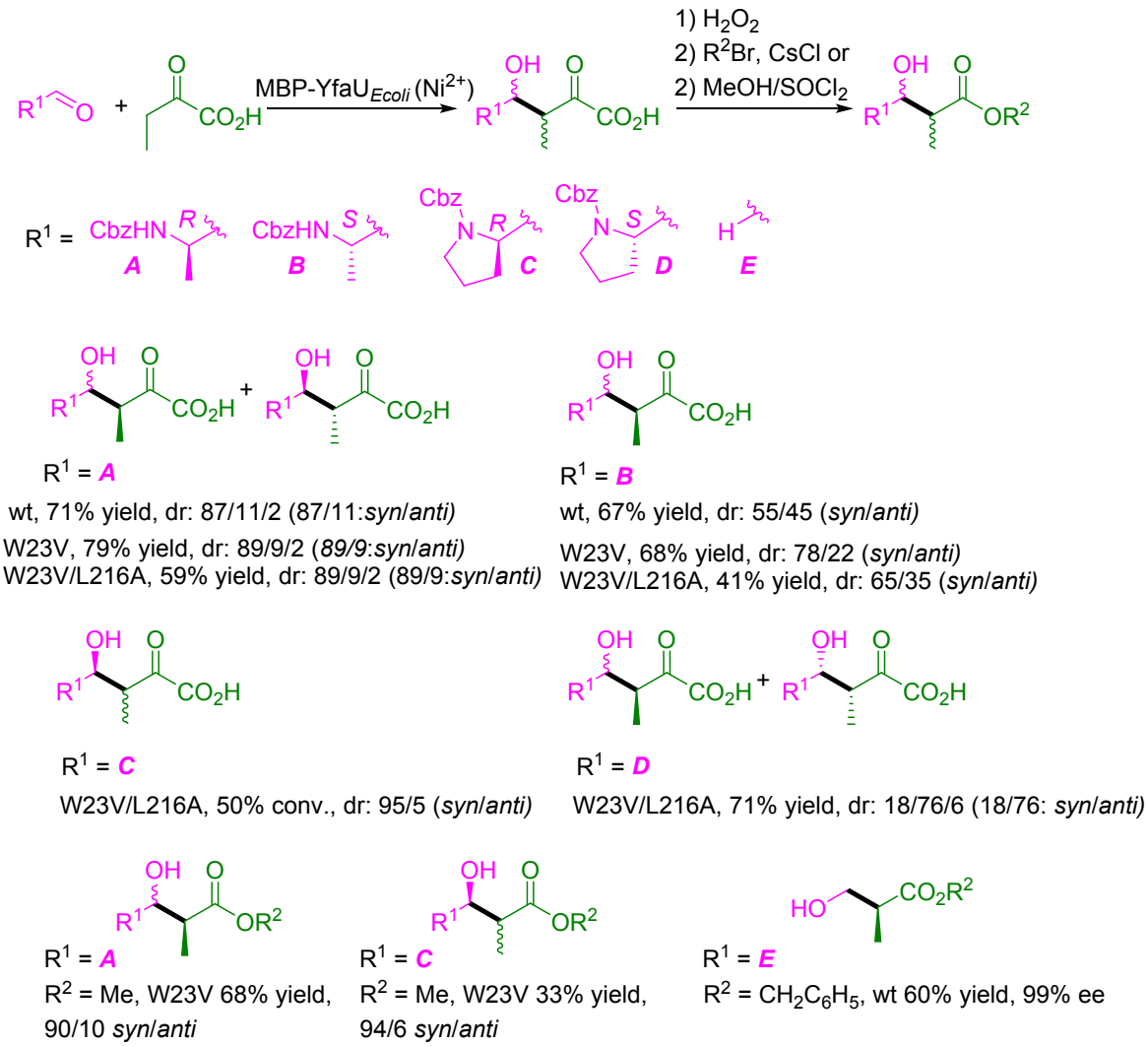


Figure 5. Models of A) wild-type $YfaU_{Ecoli}$ and B) $YfaU_{Ecoli}$ W23V/L216A complexed with 2-oxobutyrate in its enolate form (yellow C atoms) and (*R*)-*N*-Cbz-alaninal (orange C atoms) coordinated to the metal cofactor (green sphere).

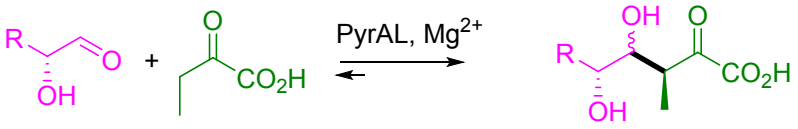
The bulkier prolinal (R^1 = **C**) derivative resulted in a lower conversion and gave rise to another minor diastereomer, while it was observed that L-aldehydes (R^1 = **B** and **D**) tended to produce a lower dr. In parallel to this work, the authors also demonstrated that formaldehyde[105] was accepted by the wild-type MBP-$YfaU_{Ecoli}$ as electrophile at 1 M concentration.[106] It was unprecedented for aldolases to maintain activity at such high concentrations of this strong electrophile. The

corresponding (3*S*)-oxoacid (R^1 = **E**) was formed in good isolated yield and excellent 99% ee (Scheme 22, bottom). The ketoacids from R^1 = **A**, **C** and **E** were then further transformed into their ester derivatives by oxidative decarboxylation.



Scheme 22. MBP- YfaU$_{Ecoli}$ (Ni^{2+}) catalyzed synthesis of α-Me substituted ketoacids and esters from 2-oxobutyrate, and *N*-Cbz protected α-amino aldehydes and formaldehyde.[101,106]

The 21 wild type aldolases described above (§ 3.5.2) that accept HPA as a nucleophile were also screened with 2-oxobutyrate.[107] A ^{1}H NMR-based assay was developed to follow their activities towards this nucleophile and to predict their stereoselectivities at C3 of the corresponding adduct. Three of them, A9E0U5, I7DKY0 and A5VH82 (Uniprot KB Id.; respectively putative hpch aldolases from *Sulfitobacter indolifex, Phaeobacter inhibens* and *Sphingomonas wittichii*; the same as in scheme 21, using the Uniprot KB id.), gave a 90/10 *syn*/*anti* aldol ratio with various hydroxylated aldehydes as electrophiles (Scheme 23). A9E0U5 and I7DKY0 have furnished the diastereomer *syn* as the major one. Interestingly, A5VH82 was *anti*-selective except when D-ribose was the electrophile.



R = -CH_2OH	A5VH82: 96% yield, 15/85 *syn*/*anti* I7DKY0: 95 % yield, 90/10 *syn*/*anti* A9E0U5: 99 % yield, 90/10 *syn*/*anti*
R = -CH_3	A5VH82: 98% yield, 25/75 *syn*/*anti* I7DKY0: 97 % yield, 90/10 *syn*/*anti* A9E0U5: 93 % yield, 80/20 *syn*/*anti*
R = (*R*)-CHOH-CH_2OH	A5VH82: 95% yield, 10/90 *syn*/*anti* I7DKY0: 91% yield, 90/10 *syn*/*anti* A9E0U5: 82% yield, 90/10 *syn*/*anti*
R = (*R,R*)-CHOH-CHOH-CH_2OH	A5VH82: 85% yield, 60/40 *syn*/*anti* A9E0U5: 95% yield, 85/15 *syn*/*anti*

Scheme 23. PyrAL catalyzed synthesis of α-Me substituted ketoacids.[107]

b) Homologous 2-oxoacids as nucleophiles

Clapés' group also studied the reaction of enzyme MBP-YfaU$_{Ecoli}$ towards a series of 2-oxoacid nucleophiles such as the keto analogue of methionine, 2-oxopentanoate, 4-(methylthio)-2-oxobutanoate, 2-oxooctanoate, and 4-methyl-2-oxopentanoate[101,106]. *N*-Cbz-alaninal, *N*-Cbz-prolinal and formaldehyde were selected as electrophiles (Schemes 24 and 25). Several variants were designed with the objective of enlarging the steric space in the active site to accommodate long and branched alkyl chains of the 2-oxoacid homologues. A crucial mutation was found to be W23V, which generated enough space for branched substituents. In addition, by combining L216A mutation to W23V, a synergistic effect was achieved, and thus bulkier nucleophile components were tolerated with *N*-Cbz-alaninal and *N*-Cbz-prolinal electrophiles (see examples of reactions in Scheme 24). It is noteworthy that branched 4-methyl-2-oxopentanoate gave the *anti* stereomer as the major product when *N*-Cbz-alaninal was *S* configured (R^1 = **B**, 5/95 dr) and a mixture of *syn*:*anti* 54:46 with the corresponding *R* enantiomer (R^1 = **A**). A linear ethyl substituent in the nucleophile component furnished a better *syn* diastereomeric ratio when using (*R*)-prolinal (R^1 = **C**, 92/8 dr) as compared with (*R*)-alaninal (R^1 = **A**, 77/23 dr). However, prolinal gave lower

conversions than alaninal with this nucleophile, the ethyl derivative being the limiting factor in the enzyme's tolerance for achieving practical preparative yields with this electrophile. These adducts were submitted to catalytic hydrogenation to provide proline and pyrrolizidine derivatives. The stereochemical and reactivity features were interpreted using computational models of wild-type YfaU$_{Ecoli}$ and the W23V/L216A variant in complexes with the pyruvate homologues and electrophiles (Figure 6).

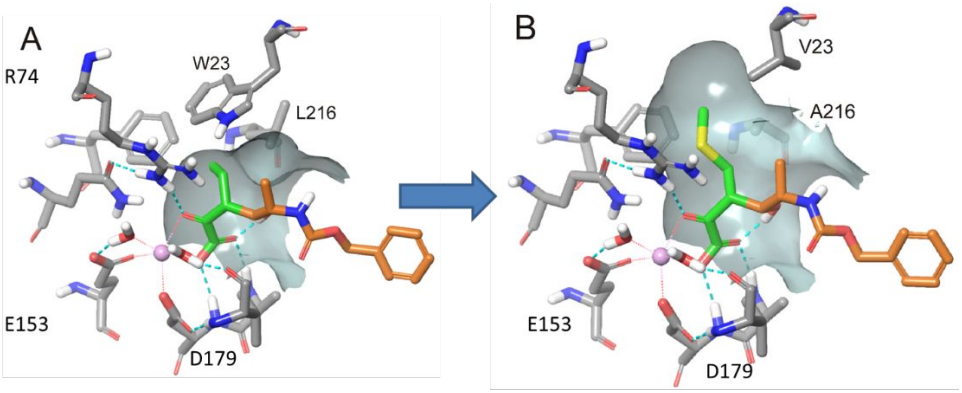
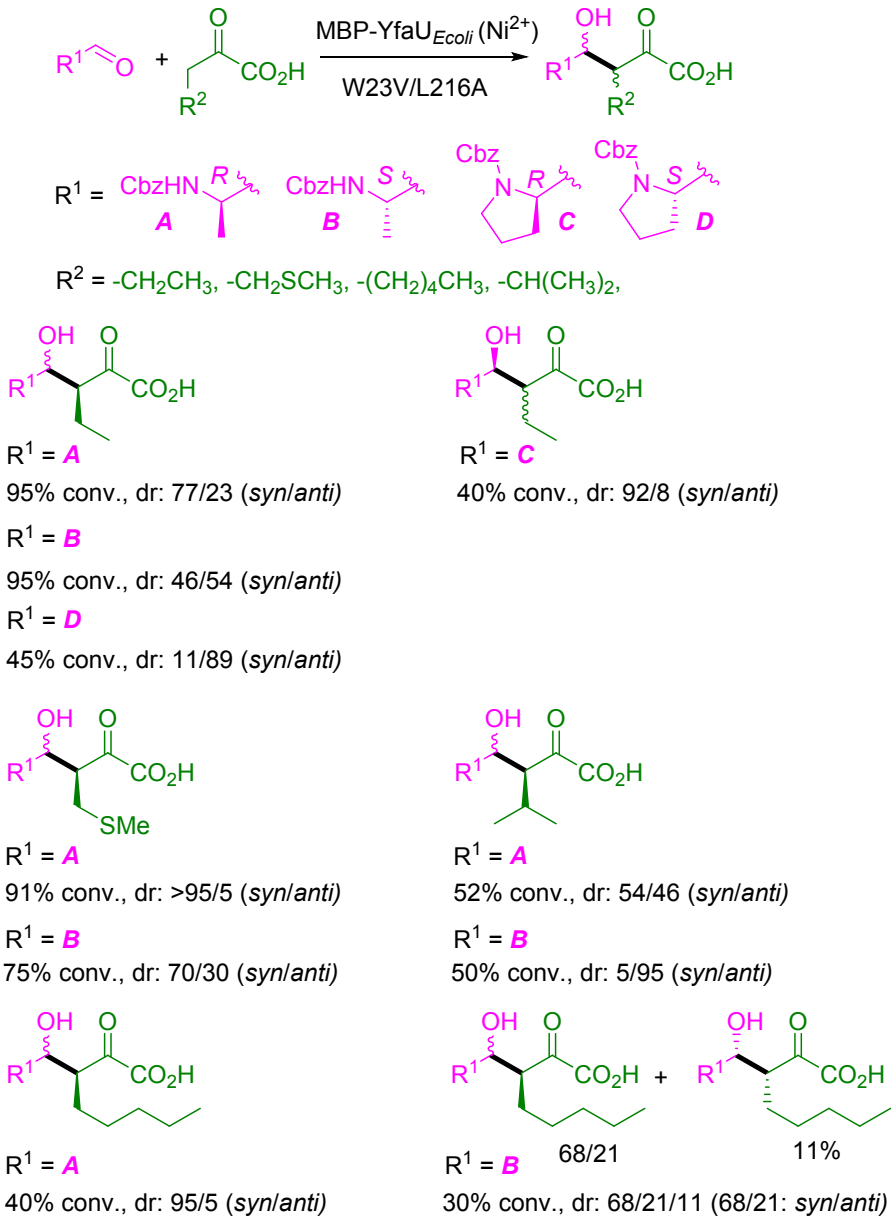
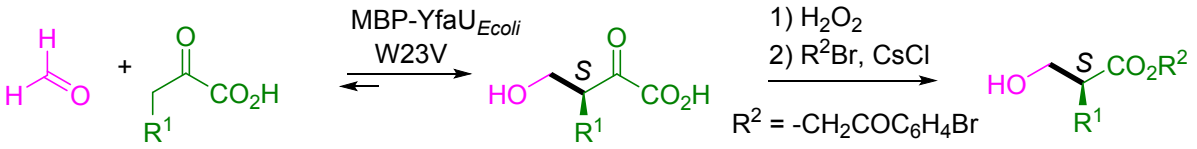


Figure 6. Example of models of A) wild-type YfaU$_{Ecoli}$ and B) YfaU$_{Ecoli}$ W23V/L216A complexed with the adduct formed between 4-(methylthio)-2-oxobutanoate (green C atoms) and (*R*)-*N*-Cbz-alaninal (orange C atoms), coordinated to the metal cofactor (purple sphere).



Scheme 24. MBP-YfaU$_{Ecoli}$ W23V/L216A variant catalyzed synthesis of β-alkyl substituted 2-oxoacids. Conversions are given as these compounds were then further transformed into various proline and pyrrolizidine derivatives (data not shown).[101]

In another paper, the single MBP-YfaU$_{Ecoli}$ W23V variant was able to catalyze the aldol addition of various hydrophobic nucleophiles to formaldehyde, to produce 2-substituted-3-hydroxycarboxylate esters in high isolated yields and enantiomeric excesses (Scheme 25).[106]



R^1 = -CH_2CH_3	conv 95%	81% yield, 99% ee
R^1 = -$CH(CH_3)_2$	conv 93%	75% yield, 99% ee
R^1 = -$(CH_2)_2CH_3$	conv 95%	68% yield, 99% ee
R^1 = -$(CH_2)_3CH_3$	conv 95%	65% yield, 99% ee
R^1 = -$CH_2CH{=}CH_2$	conv 95%	73% yield, 98% ee
R^1 = -$(CH_2)_2CH{=}CH_2$	conv 95%	69% yield, 95% ee
R^1 = -$(CH_2)_3CH{=}CH_2$	conv 90%	68% yield, 78% ee
R^1 = cyclopropyl	conv 97%	73% yield, 98% ee
R^1 = cyclobutyl	conv 95%	80% yield, 94% ee
R^1 = cyclohexyl	conv 85%	73% yield, 96% ee

Scheme 25. MBP-YfaU$_{Ecoli}$ W23V variant catalyzed synthesis of α-alkyl substituted esters.[106]

3.6 Conclusion

The new nucleophiles that have been found, in the last 5 years, to act as substrates for each aldolase are summarized in figure 7. As can be seen in the sections above, aldolases and engineering variants are not as specific as they are often thought to be, exhibiting, to some degree, substrate promiscuity. This promiscuity is necessary, since the existing aldolases are actually

platforms for the evolution of new activities by natural evolution.[108,109] DERA and DHA aldolases have similar catalytic mechanisms, both being class I aldolases and both having a degree of substrate promiscuity. In this sense it is not surprising to find some common structures that can act as substrates for both enzymes. But, due to their complementary stereoselectivities, DHA aldolases can produce (3*S*,4*R*) aldols while DERA produce (3*R*,4*R*) aldols. If a number of variants of DHA aldolases were built to accommodate the original nucleophiles, further work on the mutagenesis area with DERA could increase their common substrates. The case of pyruvate aldolases is somewhat different, since the presence of a carboxylic moiety with a net negative charge is incompatible with the hydrophobic environments that simple ketones and aldehydes need. However, they provide an efficient complement to the other two families by providing an access route to carboxylic acids. In addition, since their stereoselectivities are fairly diverse, they offer organic chemists a wide choice to work with.

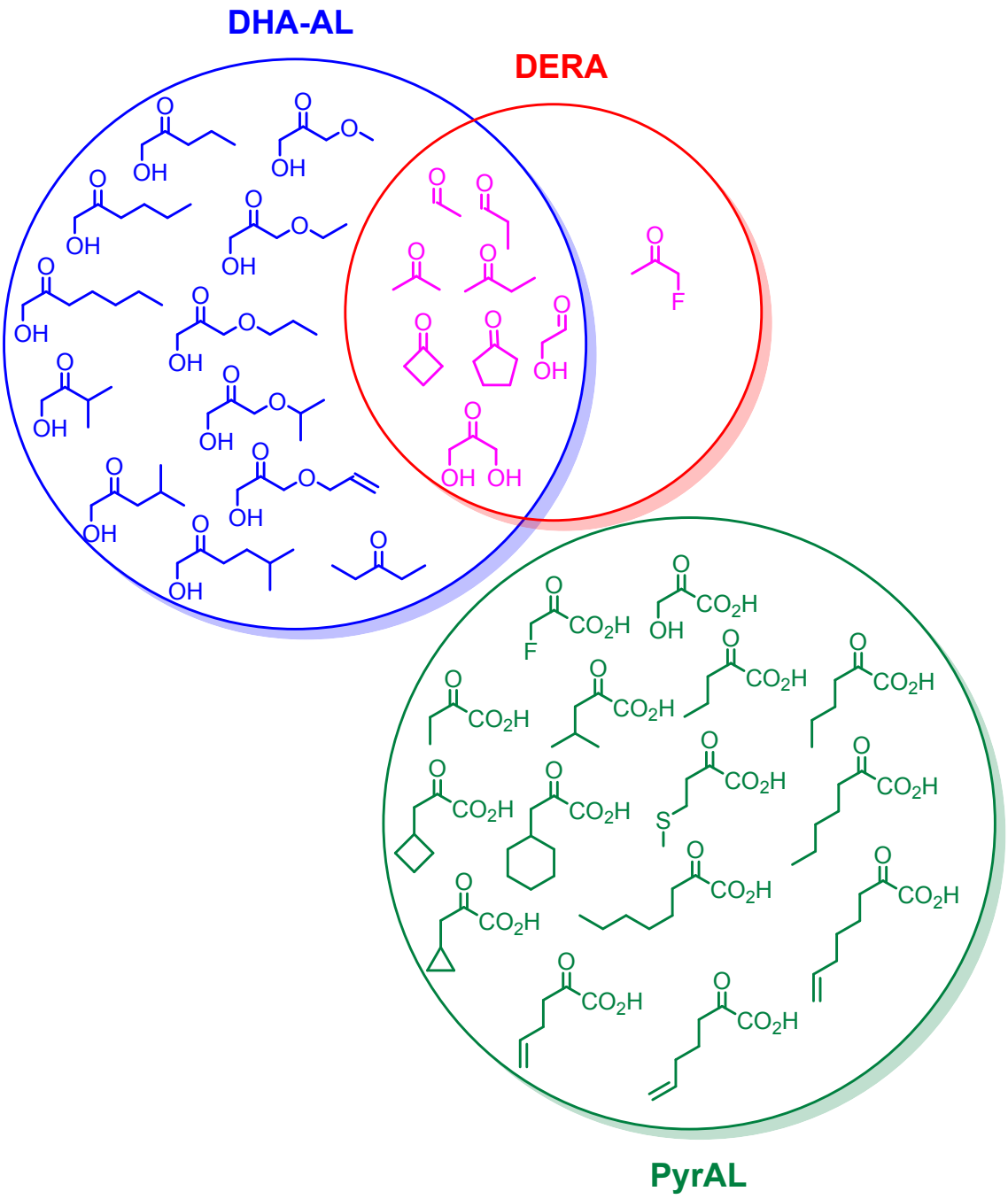


Figure 7: Nucleophiles successfully converted by aldolases in recent years.

4 Amazing electrophile substrates

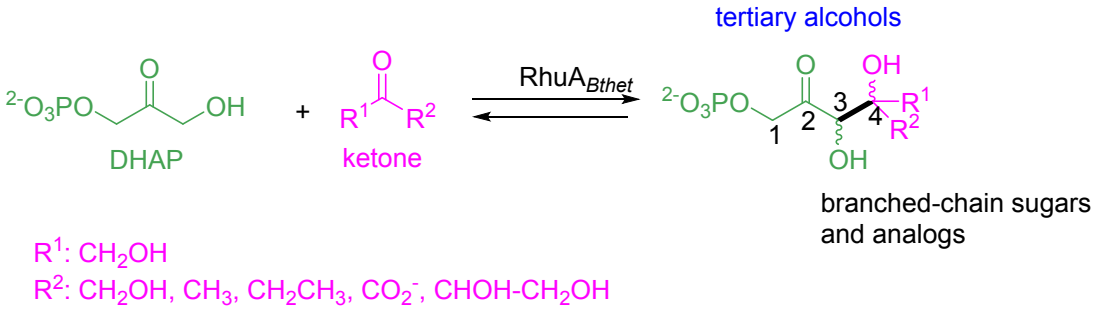
4.1 Introduction

Electrophiles for aldolases also merit discussion as, over the last five years, a significant number of unexpected molecules have been successfully converted by these enzymes. Since their inception, aldolases have been recognized as being versatile in terms of the electrophile used. This contributed to their initial success in organic synthesis. However, researchers have continued to extend their scope to other interesting electrophiles.

4.2 DHAP aldolases

Many diverse aldehyde electrophiles[1,3] have been successfully used with DHAP aldolases, reflecting the good tolerance of the electrophile binding site. Recently, a new class II DHAP aldolase of unknown function in *E. coli* metabolism was uncovered.[110] It allowed the formation of two asymmetrical carbons of (3*S*,4*R*) configurations, like FruA, but its natural electrophilic substrate remains unknown. The most promising substrate tested was L-arabinuronic acid, which demonstrated that this enzyme has a strong preference for higher order monosaccharides with a negatively charged terminus. It appears that carboxylates are better accepted than phosphates, and both are preferred over hydroxyls.

Among the DHAP aldolases, no ketones have yet been reported as electrophiles for this family of enzymes. By searching for new activity using a genome mining approach, it was discovered that some RhuA homologs were able to accept ketones, leading to the formation of tertiary alcohols (Scheme 26).[111] This was a remarkable discovery in the field of biocatalysis and in organic synthesis in general, because the preparation of enantiopure tertiary alcohols is a difficult and challenging task. In addition, tertiary alcohols are relevant compounds, being found in various molecules of interest, namely antibiotics, antitumor drugs and analgesics, and can be used as the precursors for biofuels.[112]

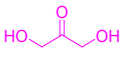
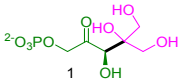
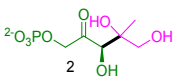
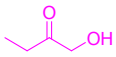
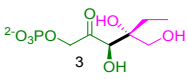
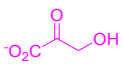
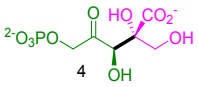
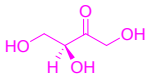
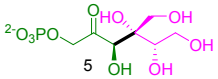


Scheme 26. RhuA$_{Bthet}$ catalyzed formation of tertiary alcohols.[111]

This new activity was particularly strong with RhuA from *Bacteroides thetaiotaomicron* (RhuA$_{Bthet}$) and took place when the reactions were carried out in an argon atmosphere. Hixon et al.[102] found that RhuA$_{Ecoli}$ was able to use O_2 as the electrophile, resulting in the oxidation of

DHAP to give hydroxypyruvaldehyde phosphate (HPP) as a byproduct. It was shown that, under anaerobic conditions, the aldolization reaction occurs only with activated ketones, i.e., those bearing an electron withdrawing group (hydroxy or carboxylate) alpha to the carbonyl. Five syntheses, at a hundred milligram scale, were conducted involving DHA, HA, HB, HPA and L-erythrulose as electrophile components, leading to the corresponding aldols in 76 to 95% yields with a uniformly 3*R* stereochemistry, along with a mainly 4*R* one (Table 2).

Table 2. RhuA$_{Bthet}$ catalyzed aldol addition of DHAP to diverse ketones as electrophiles. Reaction yield and stereoselectivity of the tertiary alcohols produced.[111]

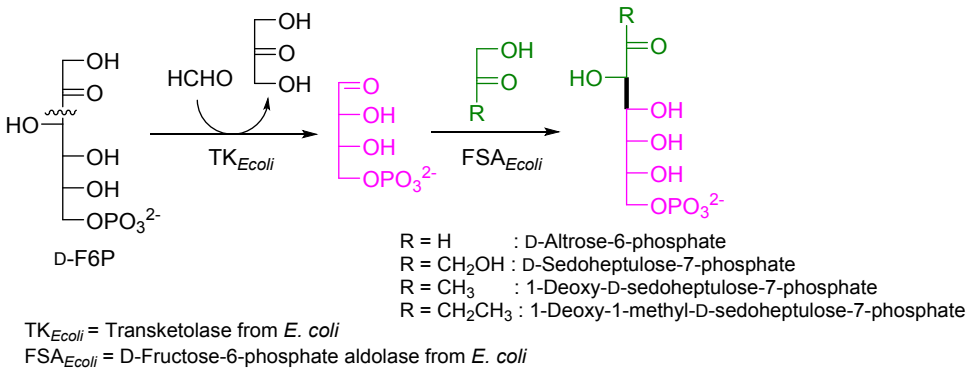
ketone	aldol[a]	yield	configuration	de (%)[b]
		90	3*R*	-
		85	3*R*,4*R* (65%) 3*R*,4*S* (35%)	30
		76	3*R*,4*R*	95
		95	3*R*,4*R*	95
		92	3*R*,4*R*	95

[a] The aldols are drawn in their linear forms for simplicity. [b] de = diastereomer excess determined by ^{1}H NMR

Later in the same year, it was shown that $FucA_{Ecoli}$ accepted DHA too, also giving compound **1** (table 2), but to a lesser degree (128 times less effective)[113] resulting in a conversion of only 17% versus 73% for $RhuA_{Ecoli}$.[111]

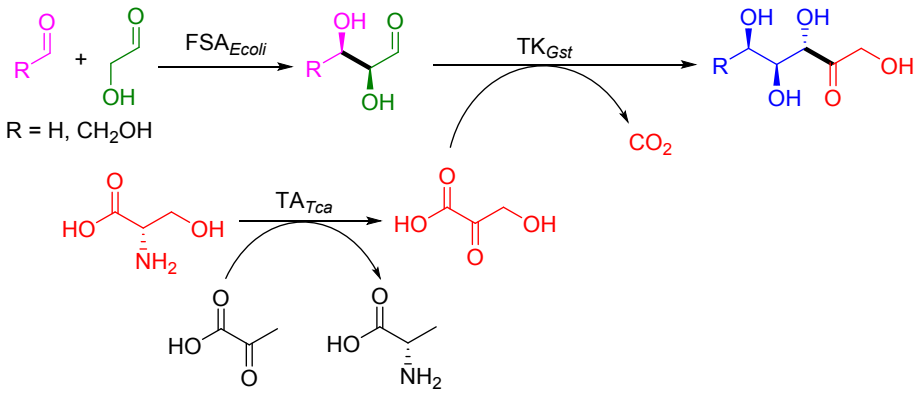
4.3 DHA aldolases

The ability of FSA_{Ecoli} to catalyze the aldol addition of aldehydes prompted research into multiple successive C-C ligation reactions. Thus, the product from one aldol addition can be utilized as the electrophile in successive C-C bond-forming reactions.[114] One interesting example is the combination of transketolase from *E. coli* with FSA_{Ecoli}. This is a similar concept to that described for DHAP-dependent aldolases (see text and Scheme 10) but using FSA_{Ecoli} instead. The catalytic activity of TK_{Ecoli} was used to generate a D-erythrose-4-phosphate (D-E4P) electrophile from D-F6P and formaldehyde. A one-pot two-step process was implemented using four FSA_{Ecoli} nucleophiles, i.e., DHA, HA, HB and HE, to produce several products of interest (Scheme 27).[70] After optimizing the amount of D-F6P, formaldehyde and FSA_{Ecoli}, the corresponding phosphorylated monosaccharides were produced in 70, 75, 65 and 61% isolated yields, respectively.



Scheme 27. Tandem TK$_{Ecoli}$-FSA$_{Ecoli}$ one-pot two-step synthesis of hexose, heptuloses and octulose phosphates.[70]

Along the same line of thought, the synthesis of ketoses has recently been carried out by a convergent generation of both nucleophile and electrophile substrates for a sequential one-pot three-step cascade.[115] This strategy comprised a combination of three thermostable enzymes including FSA$_{Ecoli}$, transketolase from *Geobacillus stearothermophilus* (TK$_{Gst}$) and L-α-transaminase from *Thermosinus carboxydivorans* (TA$_{Tca}$). FSA$_{Ecoli}$ generated the TK$_{Gst}$ electrophile by cross-aldol addition of HE to formaldehyde or homoaldol addition of HE whereas TA$_{Tca}$ provided the HPA nucleophile from the transamination reaction of L-serine to HPA using pyruvate as the amine acceptor, yielding L-Ala (Scheme 28).



Scheme 28. Synthesis of ketoses by a cascade enzymatic transformation for the generation of nucleophile and electrophile components involving FSA_{Ecoli}, TK_{Gst} and L-α-transaminase TA_{Tca}.[115]

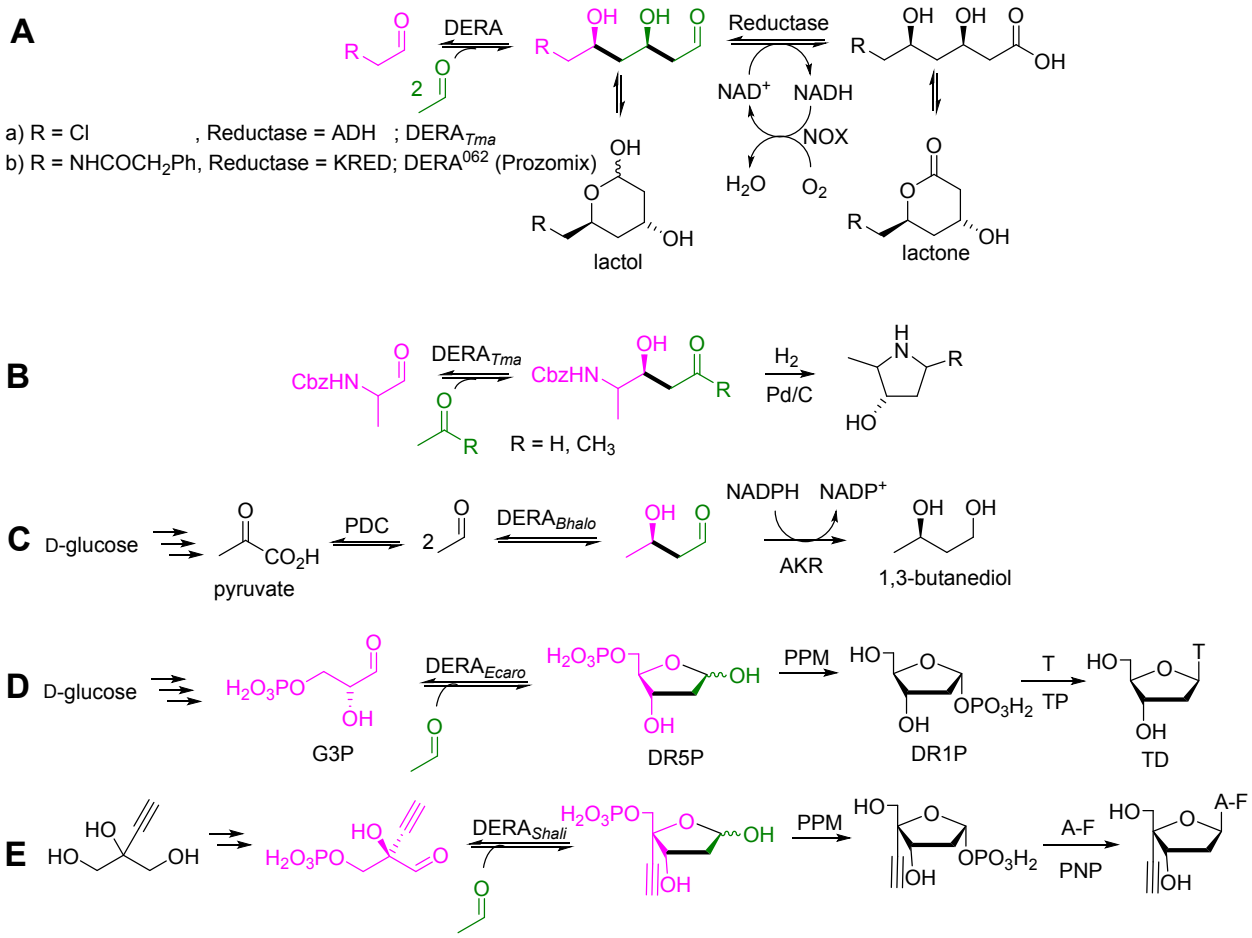
4.4 DERA

Since acetaldehyde is harmful to cells, it is thought that a natural protective mechanism might exist by which acetaldehyde inhibits DERA at high concentrations by terminating the D-R5P cleavage. This consideration has been a major hindrance to the development of eco-compatible industrial processes where acetaldehyde was required with DERA, limiting easy access to valuable building blocks. The challenge of lifting this inhibition has been revived as its cause has recently been discovered: the aldol reaction is followed by a dehydration step leading to the formation of crotonaldehyde. The latter compound is able to form a Schiff base with the catalytic lysine side chain (Lys167) within the active site, followed by a Michael addition of a thiol group of the near

cysteine residue (Cys47) to the Cβ atom of the activated crononaldehyde-Lys167 iminium complex.[116] Neither rational design nor immobilization provided satisfactory results for upscaling,[117] and only high-throughput directed evolution strategies highlighted M185V and F200I exchanges as well as a deletion of the terminal tyrosine Tyr259, leading to a 15-fold improved lactol product formation compared to wild-type DERA. DERAs from thermophilic species proved to be more stable than enzymes of mesophilic origin but were compromised by their lower intrinsic activity at the ambient reaction temperature as well as their lower level of heterologous expression.[118] Therefore, efforts are currently focused on implementation of mathematical models to optimize the process,[119] which have enabled a chlorolactol concentration of 78 g L^{-1} to be reached, with a productivity of 56 g L day^{-1} using DERA enzyme from *Thermotoga maritima* ($DERA_{Tma}$) in a fed-batch reactor (Scheme 29-Aa),[120] or a final concentration of 94 g L^{-1} along with a 229 g L day^{-1} productivity with phenylacetamide aminoprotected propanal as the electrophile (Scheme 29-Ab).[121] In these examples, cascade reactions have been implemented involving lactol production followed by its oxidation with the NAD-dependent aldehyde dehydrogenase (ADH) or ketoreductase (KRED), NADH oxidase (NOX) being employed for coenzyme regeneration, yielding a lactonized precursor of statin side chain (Scheme 29-Aa-b).[122]

N-Cbz-Aminoaldehydes of various chain lengths were also successfully converted upon $DERA_{Tma}$ catalysis, producing functionalized N-heterocycles after treatment with dihydrogen in the presence of palladium over charcoal (Scheme 29-B).[78] Machine learning methods were also implemented to aid protein engineering of $DERA_{Ecoli}$, leading to interesting variants that improved the conversion of formaldehyde as electrophile.[123] DERA has also been selected as the preferential enzyme of choice for the ecofriendly preparation of another compound of interest, (*R*)-1,3-butanediol (BDO), an essential component of pharmaceutical products and cosmetics. BDO is usually produced from petroleum-based feedstock using chemical processes, which requires harsh reaction conditions and releases toxic intermediates and by-products.[124] It can also be used as a building block for the production of synthetic polymers, pheromones, fragrances, insecticides, and antibiotics.[125–128] Structure-based site-directed mutagenesis of DERA BH1352 from *Bacillus halodurans* ($DERA_{Bhalo}$) revealed a F160Y/M173I variant able to create a six-fold increase in production of BDO compared to the wild-type, from glucose, in *E. coli* cells expressing the $DERA_{Bhalo}$ variant and the aldoketo reductase (Scheme 29-C).[129] This work follows a metabolic approach for engineering an aldolase-based pathway to improve BDO titer and yield.[130] Another biotransformation concerned the formation of D-R5P from glucose by whole *Erwinia carotovora*

cells followed by the action of phosphopentomutase and thymidine phosphorylase, producing an 85% conversion of thymidine (TD) relative to D-R5P[131] (Scheme 29-D). In a similar approach, impressive work has been devoted to the synthesis of islatravir, an HIV reverse transcriptase inhibitor, involving an in vitro three-step cascade with five engineered enzymes, including DERA from *Shewanella halifaxensis* ($DERA_{Shali}$), coupled to four auxiliary biocatalysts (Scheme 29-E). This stereoselective pathway gave the desired product in 51% overall yield, with high atom economy (no need for protecting groups) and no intermediates isolation, resulting in less than half the steps needed compared to the previous synthesis. However, the last step of the process required an additional enzymatic phosphorylation to shift the equilibrium, producing fructose-1-phosphate from fructose as a byproduct that had to be discarded from the final compound.[132]

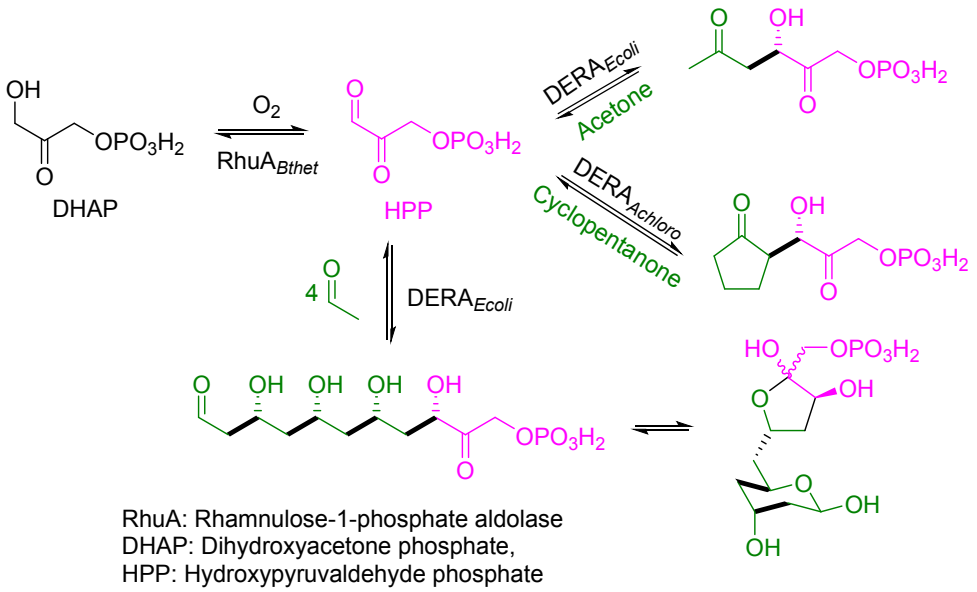


DERA: DR5P aldolase, KRED: Ketoreductase, ADH: Aldehyde dehydrogenase, NOX: NADH oxydase, PDC: Pyruvate decarboxylase, AKR: Aldoketoreductase, PPM: Phosphopentomutase, TP: Thymidine phosphorylase, PNP: Purine Nucleoside phosphorylase
G3P: D-Glyceraldehyde-3-phosphate, DR5P: 2-Deoxy-D-ribose-5-phosphate, DR1P: 2-Deoxy-D-ribose-1-phosphate, T: Thymine, TD: Thymidine, A: Adenine, A-F: Fluoroadenine

Scheme 29. Representative examples of compounds of interest prepared by cascade reactions involving DERA and various electrophiles.[78,120,121,129,131,132]

Research is still ongoing to find new DERAs with an alternative substrate scope, as shown by the recent developments of screening assays using fluorophore-coupled substrates[133] or "real substrates" with the help of an auxiliary enzyme.[134] Interestingly, some compounds that were not thought to be enzyme substrates have turned out to be useable as such with known enzymes. For

example, hydroxypyruvaldehyde phosphate (HPP), the oxidized analogue of G3P, obtained by promiscuous oxidation of DHAP with $RhuA_{Bthet}$, was proven to be an efficient DERA electrophile. When coupled in one-pot two-step cascade reactions with $DERA_{Ecoli}$ or $DERA_{Achloro}$, employing acetone or cyclopentanone as the respective donor, unusual access to a 1,4-dicarbonyl unit was revealed through the diuloses obtained (Scheme 30).[135] Surprisingly, when performing the same reaction using acetaldehyde, an unexpected quadruple acetaldehyde addition took place rendering a 3,5,7,9-tetrahydroxy-2,11-dioxoundecyl dihydrogen phosphate (Scheme 30), which has not been described before. It was inferred that the product, observed as a thermodynamically stable doubly hemiketal form, could be the driving force for this one-pot one-step oligomerization reaction, by shifting the overall equilibrium.



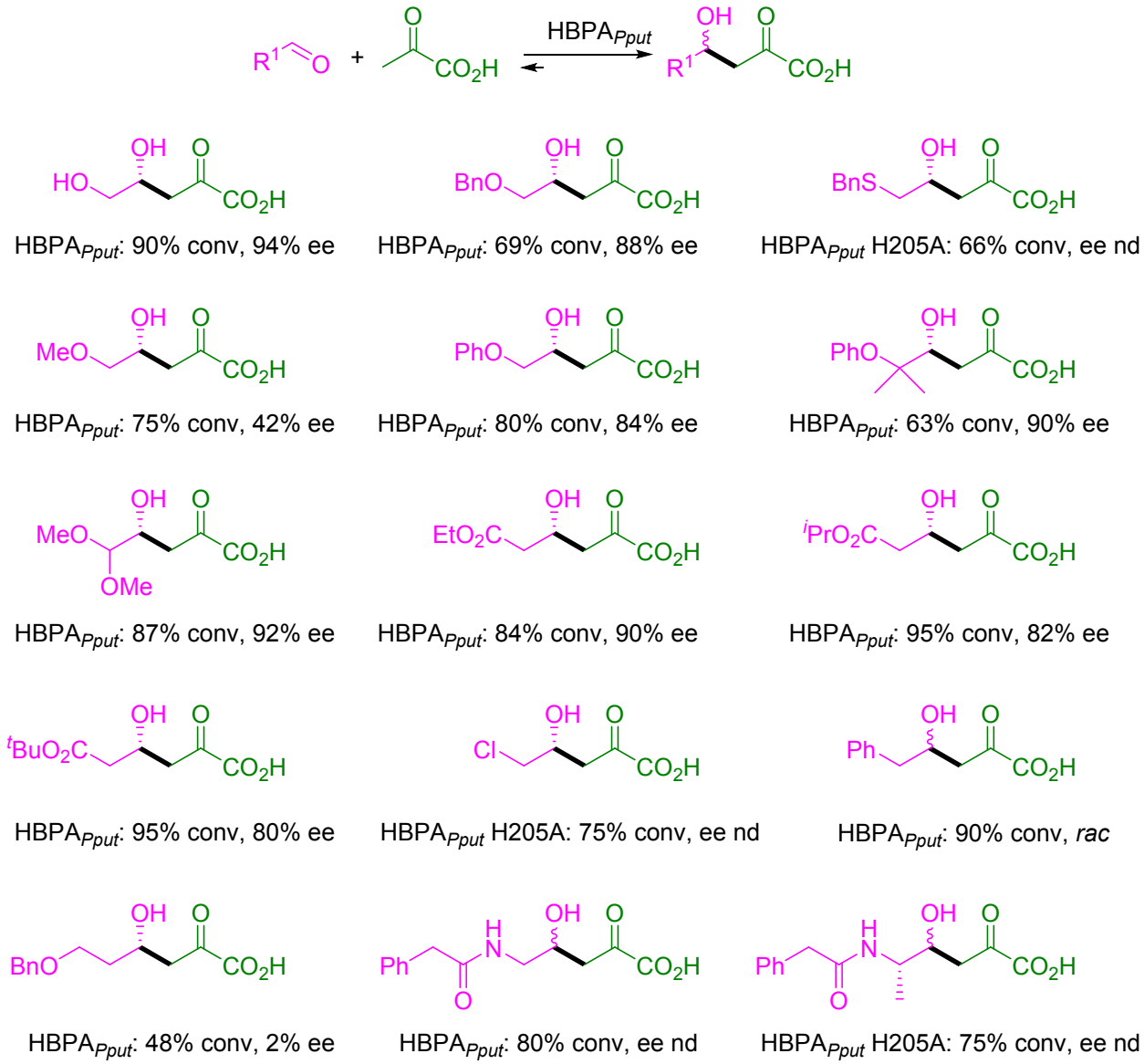
Scheme 30. Hydroxypyruvaldehyde phosphate as an acceptor platform for DERA catalyzed reactions.[135]

4.5 Pyruvate aldolases

4.5.1 Aldehydes

Recently, Clapés' group described the stereoselective aldol addition of pyruvate to aliphatic aldehydes catalyzed by class I HBPA$_{Pput}$.[136] As noted above (§ 3.5.1), despite its ability to catalyze a dehydration step (i.e., aldol condensation), the aldolase did not furnish any dehydration adducts when aliphatic, as opposed to aromatic substrates, were involved as electrophiles. The H205A variant was built to accommodate better bulkier aldehydes and to change the electrostatic environment of the active site. Good to excellent conversions were obtained using this variant, demonstrating a high substrate tolerance by the enzyme (Scheme 31). Three aldehydes (thioether,

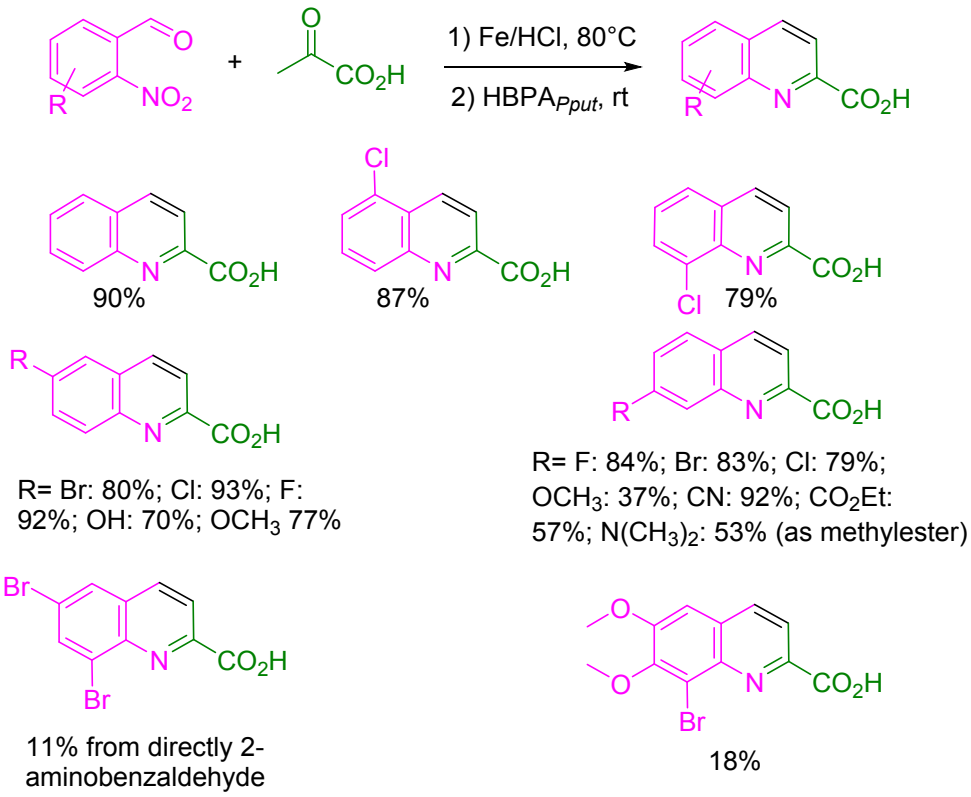
chloride and NHAcPhe substituents) also showed increased conversion. These results bring interesting new insights into the use of this aldolase in organic synthesis.



Scheme 31 Wild type HBPA$_{Pput}$ and H205A variant catalyzed aldol addition of pyruvate to various aliphatic aldehydes (*ee* measured on ester derivatives after oxidative decarboxylation, nd: not determined).[136]

In a one-pot two-step procedure, some of these ketoacids were then submitted to transamination catalyzed by transaminases to provide γ-hydroxy-α-amino acids and derivatives thereof.

On the contrary, Palmer's group has taken advantage of the dehydration properties of $HBPA_{Pput}$ to prepare quinoline derivatives.[137] When aromatic aldehydes are used as electrophiles, a dehydration step, due to the formation of conjugated compounds, leads to cinnamic acid derivatives. By introducing an amine into the *ortho* position of the benzaldehyde by chemical reduction of a nitro group, a quinoline ring can thus be prepared in a two-step process (Scheme 32). Freshly formed amine afforded better yields than starting directly from commercial amines. The authors demonstrated that the transimination (intramolecular cyclisation between the amine and the covalent imine formed via the Lys within the active site) took place prior to dehydration to provide the quinoline heterocycle. Following this method, substituted quinaldic acids were prepared from 11 to 93% yields, with the lowest yields obtained from the di-substituted benzaldehydes.

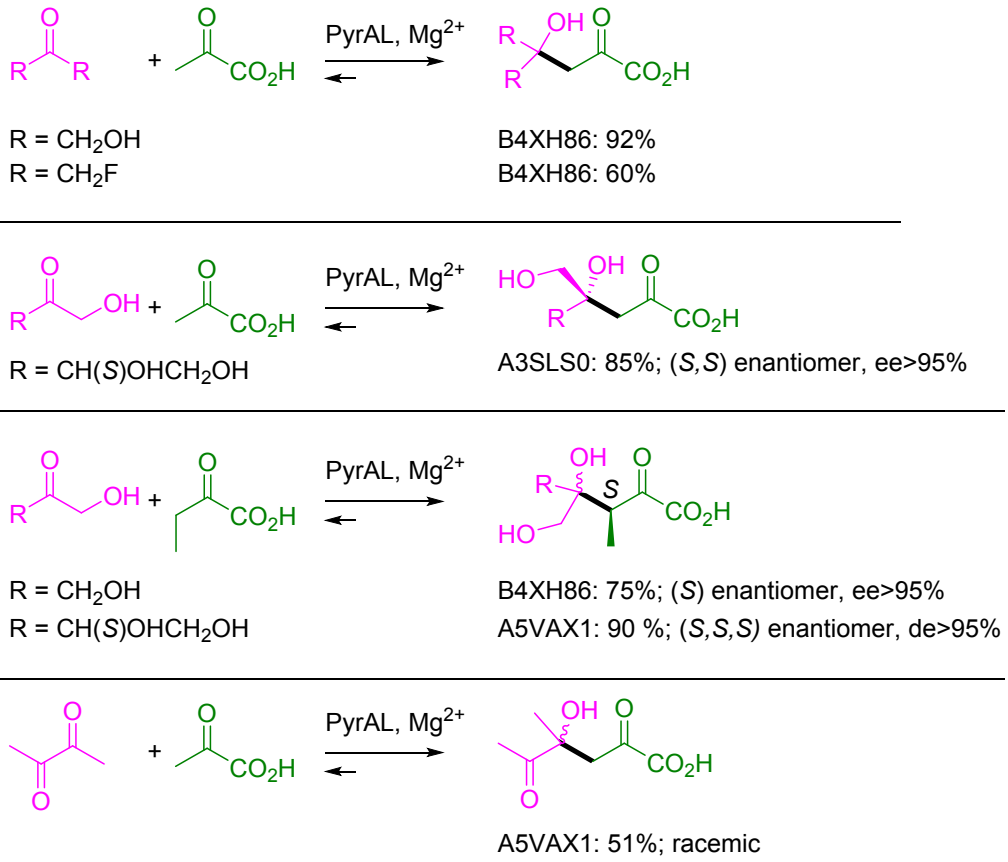


Scheme 32: HBPA$_{Pput}$ mediated chemoenzymatic synthesis of substituted quinaldic acids.[137]

4.5.2 Ketones

Since the discovery that some class II DHAP-dependent aldolases were able to accept ketones as electrophiles,[111] the question was raised as to whether other class II aldolases could catalyze aldol reactions with ketones.[138] Thus, the set of PyrALs from the PF003328 family described above (§ 3.5.2) was screened with acetone, HA and DHA. An aldol adduct was exclusively formed with DHA, the most activated ketone of the three. The best hits were kept, and preparative synthesis was run with fluorinated or hydroxylated ketones (Scheme 33, numbers cited are Uniprot KB Id.).

This study revealed that the substrate spectrum towards ketones is more restrictive for PyrAL than that for RhuA aldolases.[111] Interestingly, when 2-oxobutanoate was the nucleophile, high stereoselectivity (Scheme 33, third reaction) was shown by two PyrALs, B4XH86 and A5VAX1 (Uniprot KB Id.; respectively putative HpcH aldolases from *Actinobacillus pleuropneumoniae* and *Sphingomonas wittichii*), and the aldol adducts were isolated in good to high yields. These PyrALs have contributed to enlarging access to tertiary alcohols, whose moiety is often challenging in stereoselective synthesis.



Scheme 33. PyrAL from PF003328 catalyzed synthesis of tertiary alcohol moieties from ketones as electrophiles (A3SLS0 putative HpcH aldolase from *Roseovarius nubinhibens)*.[138]

4.6 Conclusion

Over the past five years, new outstandingly functionalized electrophiles have been identified as substrates for each aldolase family, as summarized in figure 8. Interestingly, some of them are common to several enzymes. For instance, hydroxypyruvaldehyde phosphate, the oxidized analog of G3P, can be converted by all the enzymes using G3P as natural substrate, i.e., DHAP aldolases (FruA), DERAs and DHA aldolases, giving the corresponding oxidized analogues of natural phosphorylated monosaccharides. In addition to the discovery of new aldehydic substrates, ketones have made a remarkable entry, especially with DHAP-aldolases and PyrAL, both in class II. In view of the large number of new substrates found in recent years (around thirty), PyrALs seem to be in fashion.

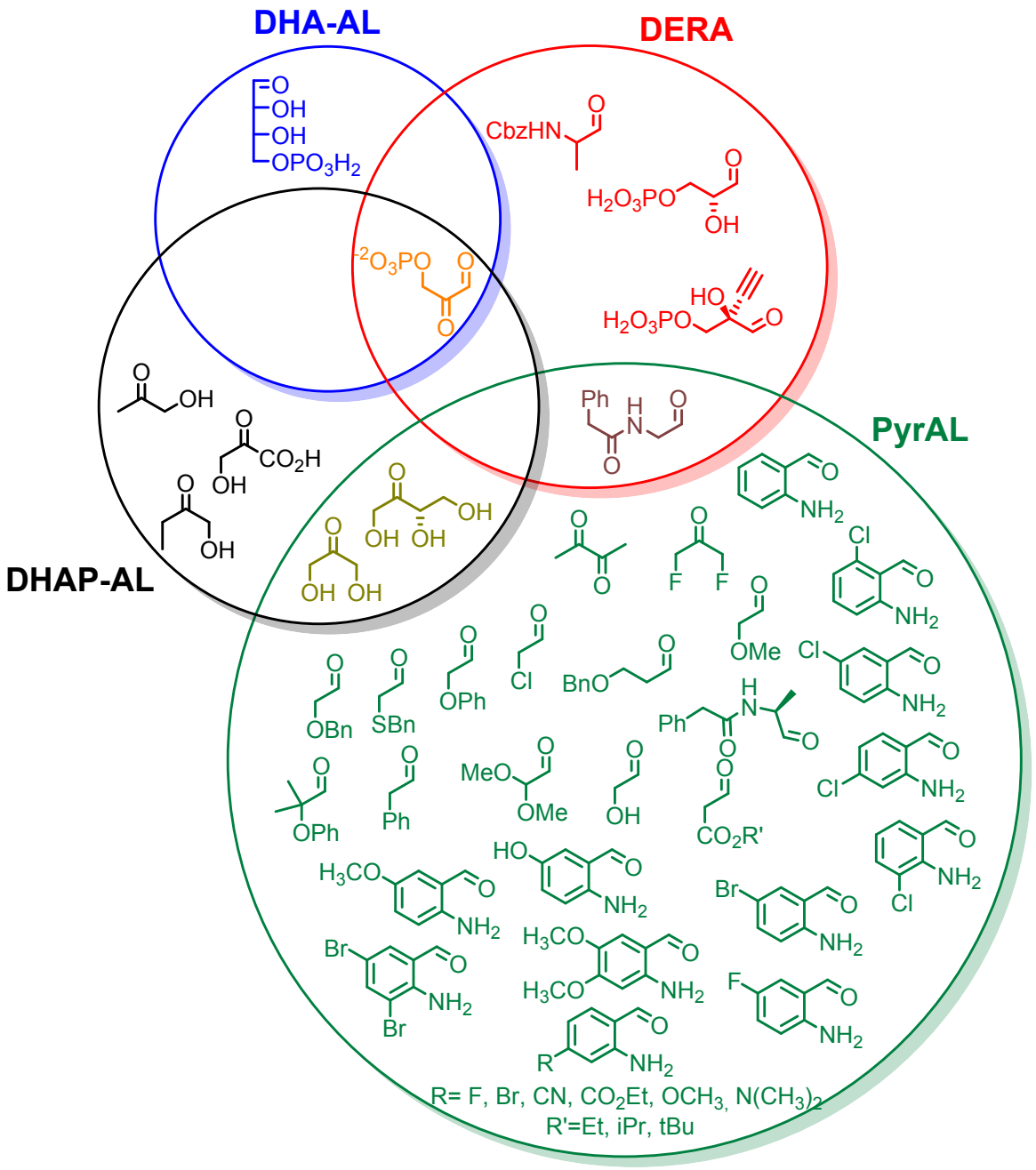


Figure 8. Electrophiles successfully converted by aldolases in recent years.

5 Conclusion and prospective

Structure-guided aldolase optimization and high-throughput screening based on mining genomes and the selection of representative enzymes among the biodiversity are successful strategies used to increase aldolase activity and discover new aldolases with novel catalytic properties. These have provided new synthetic avenues for the asymmetric crossed aldol additions of structurally varied aldehydes and ketones. Side reactions such as polymerization, homo-, and cross-aldol additions with sensitive aldehyde substrates, as well as low stereoselectivity, which often occurs in these chemical transformations[139,140], are largely overcome by the unparalleled chemo, regio and stereoselectivity, avoidance of extensive protective group chemistry, and the mild reaction conditions inherent to enzymatic reactions.

Although DHAP aldolases are the enzymes that have been the most studied over a long period of time, in recent years new advances have been made, notably with the discovery of the possible synthesis of tertiary alcohols, paving the way towards accessing new molecules of interest. The modification of these enzymes by random or directed mutagenesis techniques may allow their

potential to be expanded to less activated ketones. One family that is still under-exploited, the TagA, should reveal new insights if studied in more detail.

FSA was one of the most versatile enzymes for carbon-carbon bond formation at a preparative scale at both the nucleophile and electrophile sites. Hitherto unknown FSA activities have been revealed in recent years and, in the future, directed evolution combined with high-throughput screening strategies could also lead to the discovery of completely new activities for this highly malleable aldolase.

The strength of DERA remains in its ability to use aldehydes, in particular acetaldehyde, either as an electrophile or as a nucleophile. This has paved the way for ongoing major work into preparations to improve, for instance, statins side-chains. Obviously, acetaldehyde conversion rapidly became too restrictive to gain access to a larger panel of molecules of biological interest. Thus, it has been discovered that DERA can convert as nucleophiles other aldehydes than acetaldehyde and even ketones. On the electrophile side, other aldehydes have been discovered to be good substrates, boosting the industrial development of pathways for the synthesis of active ingredients or biofuels, where DERA operate in biocatalysed cascades. The next challenge could be to make them capable of accepting ketones as both nucleophiles and electrophiles.

PyrALs have been shown to be excellent biocatalysts for stereoselective C-C bond formation. Nucleophile promiscuity has largely been explored on natural enzymes or their variants. Improvement to their stereoselectivity could be performed by mutagenesis. Access to tertiary alcohols was made possible by the discovery that ketones were also accepted as electrophiles, although with less efficacy than DHAP-dependent RhuA. Future research could explore underused 4-hydroxy-2-oxovalerate aldolases (HOA or BphI) on both the nucleophile and electrophile sides as they have shown high stereoselectivities.[51]

The discovery of new biocatalysts, or the improvement of the catalytic properties by directed evolution and rational design will continue to supply new enzymes that might tolerate original substrates. The use of aldolases in synthetic applications is still in its infancy, but will probably take off in the near future.

REFERENCES

(1) Clapés, P.; Garrabou, X. Current Trends in Asymmetric Synthesis with Aldolases. *Adv. Synth. Catal.* **2011**, *353*, 2263–2283.

(2) Dean, S. M.; Greenberg, W. A.; Wong, C.-H. Recent Advances in Aldolase-Catalyzed Asymmetric Synthesis. *Adv. Synth. Catal.* **2007**, *349*, 1308–1320.

(3) Fesko, K.; Gruber-Khadjawi, M. Biocatalytic Methods for C-C Bond Formation. *ChemCatChem* **2013**, *5*, 1248–1272.

(4) Clapés, P. Aldol Reactions. In *Science of synthesis Biocatalysis in Organic Synthesis*; Faber, K., Fessner, W. D., Turner, N. J., Series Eds.; **2015**; Vol. 2, pp 31–92.

(5) Clapés, P. Recent Advances in Enzyme-Catalyzed Aldol Addition Reactions. In *Green Biocatalysis*; patel, R. N., Series Ed.; John Wiley & Sons, Ltd, **2016**; pp 267–306.

(6) Clapés, P. Enzymatic C-C Bond Formation. In *Organic Synthesis Using Biocatalysis - 1st Edition*; Goswami, A., Stewart, J. D., Series Eds.; Academic Press, **2016**; pp 285–337.

(7) Fessner, W.-D.; Helaine, V. Biocatalytic Synthesis of Hydroxylated Natural Products Using Aldolases and Related Enzymes. *Curr. Opin. Biotechnol.* **2001**, *12*, 574–586.

(8) Schürmann, M.; Sprenger, G. A. Fructose-6-Phosphate Aldolase Is a Novel Class I Aldolase from *Escherichia Coli* and Is Related to a Novel Group of Bacterial Transaldolases. *J. Biol. Chem.* **2001**, *276*, 11055–11061.

(9) Adams, J. P.; Brown, M. J. B.; Diaz-Rodriguez, A.; Lloyd, R. C.; Roiban, G. Biocatalysis: A Pharma Perspective. *Adv. Synth. Catal.* **2019**, *361*, 2421-2432.

(10) Li, A.; Cai, L.; Chen, Z.; Wang, M.; Wang, N.; Nakanishi, H.; Gao, X.-D.; Li, Z. Recent Advances in the Synthesis of Rare Sugars Using DHAP-Dependent Aldolases. *Carbohydr. Res.* **2017**, *452*, 108–115.

(11) Fessner, W.-D.; Eyrisch, O. One-Pot Synthesis of Tagatose 1,6-Bisphosphate by Diastereoselective Enzymatic Aldol Addition. *Angew. Chem. Int. Ed. Engl.* **1992**, *31*, 56–58.

(12) Eyrisch, O.; Sinerius, G.; Fessner, W.-D. ChemInform Abstract: Enzymes in Organic Synthesis. Part 4. Facile Enzymic de novo Synthesis and NMR Spectroscopic Characterization of D-Tagatose 1,6-Bisphosphate. ChemInform **2010**, *238*, 287-306.

(13) Fessner, W.-D.; Schneider, A.; Held, H.; Sinerius, G.; Walter, C.; Hixon, M.; Schloss, J. V. The Mechanism of Class II, Metal-Dependent Aldolases. *Angew. Chem. Int. Ed. Engl.* **1996**, *35*, 2219–2221.

(14) Choi, K. H.; Shi, J.; Hopkins, C. E.; Tolan, D. R.; Allen, K. N. Snapshots of Catalysis: The Structure of Fructose-1,6-(Bis)Phosphate Aldolase Covalently Bound to the Substrate Dihydroxyacetone Phosphate. *Biochemistry* **2001**, *40*, 13868–13875.

(15) Bilal, M.; Iqbal, H. M. N.; Hu, H.; Wang, W.; Zhang, X. Metabolic Engineering Pathways for Rare Sugars Biosynthesis, Physiological Functionalities, and Applications—a Review. *Crit. Rev. Food Sci. Nutr.* **2018**, *58*, 2768–2778.

(16) Busto, E. Recent Developments in the Preparation of Carbohydrate Derivatives from Achiral Building Blocks by Using Aldolases. *ChemCatChem* **2016**, *8*, 2589–2598.

(17) Guitart Font, E.; Sprenger, G. A. Opening a Novel Biosynthetic Pathway to Dihydroxyacetone and Glycerol in Escherichia Coli Mutants through Expression of a Gene Variant (FsaAA129S) for Fructose 6-Phosphate Aldolase. *Int. J. Mol. Sci.* **2020**, *21*, E9625.

(18) Thorell, S.; Schürmann, M.; Sprenger, G. A.; Schneider, G. Crystal Structure of Decameric Fructose-6-Phosphate Aldolase from Escherichia Coli Reveals Inter-Subunit Helix Swapping as a Structural Basis for Assembly Differences in the Transaldolase Family. *J. Mol. Biol.* **2002**, *319*, 161–171.

(19) Concia, A. L.; Lozano, C.; Castillo, J. A.; Parella, T.; Joglar, J.; Clapés, P. D-Fructose-6-Phosphate Aldolase in Organic Synthesis: Cascade Chemical-Enzymatic Preparation of Sugar-Related Polyhydroxylated Compounds. *Chem. Weinh. Bergstr. Ger.* **2009**, *15*, 3808–3816.

(20) Garrabou, X.; Castillo, J. A.; Guérard-Hélaine, C.; Parella, T.; Joglar, J.; Lemaire, M.; Clapés, P. Asymmetric Self- and Cross-Aldol Reactions of Glycolaldehyde Catalyzed by D-Fructose-6-Phosphate Aldolase. *Angew. Chem. Int. Ed.* **2009**, *48*, 5521–5525.

(21) Castillo, J. A.; Guérard-Hélaine, C.; Gutiérrez, M.; Garrabou, X.; Sancelme, M.; Schürmann, M.; Inoue, T.; Hélaine, V.; Charmantray, F.; Gefflaut, T.; Hecquet, L.; Joglar, J.; Clapés, P.; Sprenger, G. A.; Lemaire, M. A Mutant D-Fructose-6-Phosphate Aldolase (Ala129Ser) with Improved Affinity towards Dihydroxyacetone for the Synthesis of Polyhydroxylated Compounds. *Adv. Synth. Catal.* **2010**, *352*, 1039–1046.

(22) Guérard-Hélaine, C.; de Berardinis, V.; Besnard-Gonnet, M.; Darii, E.; Debacker, M.; Debard, A.; Fernandes, C.; Hélaine, V.; Mariage, A.; Pellouin, V.; Perret, A.; Petit, J.-L.; Sancelme, M.; Lemaire, M.; Salanoubat, M. Genome Mining for Innovative Biocatalysts: New Dihydroxyacetone Aldolases for the Chemist's Toolbox. *ChemCatChem* **2015**, *7*, 1871–1879.

(23) Stellmacher, L.; Sandalova, T.; Leptihn, S.; Schneider, G.; Sprenger, G. A.; Samland, A. K. Acid-Base Catalyst Discriminates between a Fructose 6-Phosphate Aldolase and a Transaldolase. *ChemCatChem* **2015**, *7*, 3140–3151.

(24) Tittmann, K. Sweet Siblings with Different Faces: The Mechanisms of FBP and F6P Aldolase, Transaldolase, Transketolase and Phosphoketolase Revisited in Light of Recent Structural Data. *Bioorganic Chem.* **2014**, *57*, 263–280.

(25) Yang, X.; Ye, L.; Li, A.; Yang, C.; Yu, H.; Gu, J.; Guo, F.; Jiang, L.; Wang, F.; Yu, H. Engineering of D-Fructose-6-Phosphate Aldolase A for Improved Activity towards Cinnamaldehyde. *Catal. Sci. Technol.* **2017**, *7*, 382–386.

(26) Pricer, W. E.; Horecker, B. L. Deoxyribose Aldolase from Lactobacillus Plantarum. *J. Biol. Chem.* **1960**, *235*, 1292–1298.

(27) Tozzi, M. G.; Camici, M.; Mascia, L.; Sgarrella, F.; Ipata, P. L. Pentose Phosphates in Nucleoside Interconversion and Catabolism. *FEBS J.* **2006**, *273*, 1089–1101.

(28) Racker, E. Enzymatic Synthesis and Breakdown of Desoxyribose Phosphate. *J. Biol. Chem.* **1952**, *196*, 347–365.

(29) Hoffee, P.; Snyder, P.; Sushak, C.; Jargiello, P. Deoxyribose-5-P Aldolase: Subunit Structure and Composition of Active Site Lysine Region. *Arch. Biochem. Biophys.* **1974**, *164*, 736–742.

(30) Schulte, M.; Petrović, D.; Neudecker, P.; Hartmann, R.; Pietruszka, J.; Willbold, S.; Willbold, D.; Panwalkar, V. Conformational Sampling of the Intrinsically Disordered C-Terminal Tail of DERA Is Important for Enzyme Catalysis. *ACS Catal.* **2018**, *8*, 3971–3984.

(31) Ma, H.; Szeler, K.; Kamerlin, S. C. L.; Widersten, M. Linking Coupled Motions and Entropic Effects to the Catalytic Activity of 2-Deoxyribose-5-Phosphate Aldolase (DERA). *Chem. Sci.* **2016**, *7*, 1415–1421.

(32) Bisterfeld, C.; Classen, T.; Küberl, I.; Henßen, B.; Metz, A.; Gohlke, H.; Pietruszka, J. Redesigning Aldolase Stereoselectivity by Homologous Grafting. *PLOS ONE* **2016**, *11*, e0156525.

(33) Rosen, O. M.; Hoffee, P.; Horecker, B. L. The Mechanism of Action of Aldolases: VII. Formation of a 2-Methyl-2-Deoxypentose catalyzed by deoxyribose 5-phosphate Aldolase. *J. Biol. Chem.* **1965**, *240*, 1517–1524.

(34) Wong, C.-H.; Garcia-Junceda, E.; Chen, L.; Blanco, O.; Gijsen, H. J. M.; Steensma, D. H. Recombinant 2-Deoxyribose-5-Phosphate Aldolase in Organic Synthesis: Use of Sequential Two-Substrate and Three-Substrate Aldol Reactions. *J. Am. Chem. Soc.* **1995**, *117*, 3333–3339.

(35) Barbas, C. F.; Wang, Y. F.; Wong, C. H. Deoxyribose-5-Phosphate Aldolase as a Synthetic Catalyst. *J. Am. Chem. Soc.* **1990**, *112*, 2013–2014.

(36) Valino, A. L.; Iribarren, A. M.; Lewkowicz, E. New Biocatalysts for One Pot Multistep Enzymatic Synthesis of Pyrimidine Nucleoside Diphosphates from Readily Available Reagents. *J. Mol. Catal. B Enzym.* **2015**, *114*, 58–64.

(37) Valino, A. L.; Palazzolo, M. A.; Iribarren, A. M.; Lewkowicz, E. Selection of a New Whole Cell Biocatalyst for the Synthesis of 2-Deoxyribose 5-Phosphate. *Appl. Biochem. Biotechnol.* **2012**, *166*, 300–308.

(38) Gijsen, H. J. M.; Wong, C.-H. Unprecedented Asymmetric Aldol Reactions with Three Aldehyde Substrates Catalyzed by 2-Deoxyribose-5-Phosphate Aldolase. *J. Am. Chem. Soc.* **1994**, *116*, 8422–8423.

(39) Gijsen, H. J. M.; Wong, C.-H. Sequential One-Pot Aldol Reactions Catalyzed by 2-Deoxyribose-5-Phosphate Aldolase and Fructose-1,6-Diphosphate Aldolase. *J. Am. Chem. Soc.* **1995**, *117*, 2947–2948.

(40) Liu, J.; Wong, C.-H. Aldolase-Catalyzed Asymmetric Synthesis of Novel Pyranose Synthons as a New Entry to Heterocycles and Epothilones. *Angew. Chem. Int. Ed.* **2002**, *41*, 1404–1407.

(41) Brovetto, M.; Gamenara, D.; Saenz Méndez, P.; Seoane, G. A. C−C Bond-Forming Lyases in Organic Synthesis. *Chem. Rev.* **2011**, *111*, 4346–4403.

(42) Machajewski, T. D.; Wong, C.-H. The Catalytic Asymmetric Aldol Reaction. *Angew. Chem. Int. Ed Engl.* **2000**, *39*, 1352–1375.

(43) Wu, X.; Jiang, J.; Chen, Y. Correlation between Intracellular Cofactor Concentrations and Biocatalytic Efficiency: Coexpression of Diketoreductase and Glucose Dehydrogenase for the Preparation of Chiral Diol for Statin Drugs. *ACS Catal.* **2011**, *1*, 1661–1664.

(44) Haridas, M.; Abdelraheem, E. M. M.; Hanefeld, U. 2-Deoxy-d-Ribose-5-Phosphate Aldolase (DERA): Applications and Modifications. *Appl. Microbiol. Biotechnol.* **2018**, *102*, 9959–9971.

(45) Gijsen, H. J. M.; Qiao, L.; Fitz, W.; Wong, C.-H. Recent Advances in the Chemoenzymatic Synthesis of Carbohydrates and Carbohydrate Mimetics. *Chem. Rev.* **1996**, *96*, 443–474.

(46) Tobert, J. A. Lovastatin and beyond: The History of the HMG-CoA Reductase Inhibitors. *Nat. Rev. Drug Discov.* **2003**, *2*, 517–526.

(47) Zdenko, C. Historic Overview and Recent Advances in the Synthesis of Super-Statins. *Curr. Org. Chem.* **2010**, *14*, 816–845.

(48) Windle, C. L.; Müller, M.; Nelson, A.; Berry, A. Engineering Aldolases as Biocatalysts. *Curr. Opin. Chem. Biol.* **2014**, *19*, 25–33.

(49) Samland, A. K.; Sprenger, G. A. Microbial Aldolases as C–C Bonding Enzymes—Unknown Treasures and New Developments. *Appl. Microbiol. Biotechnol.* **2006**, *71*, 253–264.

(50) Mistry, J.; Chuguransky, S.; Williams, L.; Qureshi, M.; Salazar, G. A.; Sonnhammer, E. L. L.; Tosatto, S. C. E.; Paladin, L.; Raj, S.; Richardson, L. J.; Finn, R. D.; Bateman, A. Pfam: The Protein Families Database in 2021. *Nucleic Acids Res.* **2021**, *49*, D412–D419.

(51) Baker, P.; Seah, S. Y. K. Rational Design of Stereoselectivity in the Class II Pyruvate Aldolase BphI. *J. Am. Chem. Soc.* **2012**, *134*, 507–513.

(52) Mazurkewich, S.; Wang, W.; Seah, S. Y. K. Biochemical and Structural Analysis of RraA Proteins To Decipher Their Relationships with 4-Hydroxy-4-Methyl-2-Oxoglutarate/4-Carboxy-4-Hydroxy-2-Oxoadipate Aldolases. *Biochemistry* **2014**, *53*, 542–553.

(53) Pickl, M. Recent Trends in the Stereoselective Synthesis of (Poly)-Substituted 2-Oxo Acids by Biocatalyzed Aldol Reaction. *Curr. Opin. Green Sustain. Chem.* **2021**, *30*, 100476.

(54) Garrabou, X.; Calveras, J.; Joglar, J.; Parella, T.; Bujons, J.; Clapés, P. Highly Efficient Aldol Additions of DHA and DHAP to N-Cbz-Amino Aldehydes Catalyzed by L-Rhamnulose-1-Phosphate and L-Fuculose-1-Phosphate Aldolases in Aqueous Borate Buffer. *Org. Biomol. Chem.* **2011**, *9*, 8430–8436.

(55) Sugiyama, M.; Hong, Z.; Whalen, L. J.; Greenberg, W. A.; Wong, C.-H. Borate as a Phosphate Ester Mimic in Aldolase-Catalyzed Reactions: Practical Synthesis of L-Fructose and L-Iminocyclitols. *Adv. Synth. Catal.* **2006**, *348*, 2555–2559.

(56) Garrabou, X.; Joglar, J.; Parella, T.; Crehuet, R.; Bujons, J.; Clapés, P. Redesign of the Phosphate Binding Site of L-Rhamnulose- 1-Phosphate Aldolase towards a Dihydroxyacetone Dependent Aldolase. *Adv. Synth. Catal.* **2011**, *353*, 89–99.

(57) Schümperli, M.; Pellaux, R.; Panke, S. Chemical and Enzymatic Routes to Dihydroxyacetone Phosphate. *Appl. Microbiol. Biotechnol.* **2007**, *75*, 33–45.

(58) Gao, C.; Li, Z.; Zhang, L.; Wang, C.; Li, K.; Ma, C.; Xu, P. An Artificial Enzymatic Reaction Cascade for a Cell-Free Bio-System Based on Glycerol. *Green Chem.* **2015**, *17*, 804–807.

(59) Hettwer, J.; Oldenburg, H.; Flaschel, E. Enzymic Routes to Dihydroxyacetone Phosphate or Immediate Precursors. *J. Mol. Catal. B Enzym.* **2002**, *19–20*, 215–222.

(60) Hartley, C. J.; French, N. G.; Scoble, J. A.; Williams, C. C.; Churches, Q. I.; Frazer, A. R.; Taylor, M. C.; Coia, G.; Simpson, G.; Turner, N. J.; Scott, C. Sugar Analog Synthesis by in Vitro Biocatalytic Cascade: A Comparison of Alternative Enzyme Complements for Dihydroxyacetone Phosphate Production as a Precursor to Rare Chiral Sugar Synthesis. *PLOS ONE* **2017**, *12*, e0184183.

(61) Fessner, W.-D.; Sinerius, G. Synthesis of Dihydroxyacetone Phosphate (and Isosteric Analogues) by Enzymatic Oxidation; Sugars from Glycerol. *Angew. Chem. Int. Ed. Engl.* **1994**, *33*, 209–212.

(62) Koga, Y.; Morikawa, M.; Haruki, M.; Nakamura, H.; Imanaka, T.; Kanaya, S. Thermostable Glycerol Kinase from a Hyperthermophilic Archaeon: Gene Cloning and Characterization of the Recombinant Enzyme. *Protein Eng. Des. Sel.* **1998**, *11*, 1219–1227.

(63) Brockamp, H. P.; Kula, M. R. Purification and Characterization of a Class I Fructose 1,6-Bisphosphate Aldolase from Staphylococcus Carnosus. *Appl. Microbiol. Biotechnol.* **1990**, *34*, 287–291.

(64) Li, Z.; Li, F.; Cai, L.; Chen, Z.; Qin, L.; Gao, X.-D. One-Pot Multienzyme Synthesis of Rare Ketoses from Glycerol. *J. Agric. Food Chem.* **2020**, *68*, 1347–1353.

(65) van Herk, T.; Hartog, A. F.; Schoemaker, H. E.; Wever, R. Simple Enzymatic in Situ Generation of Dihydroxyacetone Phosphate and Its Use in a Cascade Reaction for the Production of Carbohydrates: Increased Efficiency by Phosphate Cycling. *J. Org. Chem.* **2006**, *71*, 6244–6247.

(66) van Herk, T.; Hartog, A. F.; van der Burg, A. M.; Wever, R. Regioselective Phosphorylation of Carbohydrates and Various Alcohols by Bacterial Acid Phosphatases; Probing the Substrate Specificity of the Enzyme from Shigella Flexneri. *Adv. Synth. Catal.* **2005**, *347*, 1155–1162.

(67) Schoevaart, R.; van Rantwijk, F.; Sheldon, R. A. A Four-Step Enzymatic Cascade for the One-Pot Synthesis of Non-Natural Carbohydrates from Glycerol. *J. Org. Chem.* **2000**, *65*, 6940–6943.

(68) Wang, W.; Yang, J.; Sun, Y.; Li, Z.; You, C. Artificial ATP-Free in Vitro Synthetic Enzymatic Biosystems Facilitate Aldolase-Mediated C–C Bond Formation for Biomanufacturing. *ACS Catal.* **2020**, *10*, 1264–1271.

(69) Fessner, W.-D.; Walter, C. "Artificial Metabolisms" for the Asymmetric One-Pot Synthesis of Branched-Chain Saccharides. *Angew. Chem. Int. Ed. Engl.* **1992**, *31*, 614–616.

(70) Guérard-Hélaine, C.; De Sousa Lopes Moreira, M.; Touisni, N.; Hecquet, L.; Lemaire, M.; Hélaine, V. Transketolase-Aldolase Symbiosis for the Stereoselective Preparation of Aldoses and Ketoses of Biological Interest. *Adv. Synth. Catal.* **2017**, *359*, 2061–2065.

(71) Yang, J.; Zhu, Y.; Men, Y.; Sun, S.; Zeng, Y.; Zhang, Y.; Sun, Y.; Ma, Y. Pathway Construction in Corynebacterium Glutamicum and Strain Engineering To Produce Rare Sugars from Glycerol. *J. Agric. Food Chem.* **2016**, *64*, 9497–9505.

(72) Chen, Z.; Li, Z.; Li, F.; Wang, M.; Wang, N.; Gao, X.-D. Cascade Synthesis of Rare Ketoses by Whole Cells Based on L-Rhamnulose-1-Phosphate Aldolase. *Enzyme Microb. Technol.* **2020**, *133*, 109456.

(73) Güclü, D.; Szekrenyi, A.; Garrabou, X.; Kickstein, M.; Junker, S.; Clapés, P.; Fessner, W.-D. Minimalist Protein Engineering of an Aldolase Provokes Unprecedented Substrate Promiscuity. *ACS Catal.* **2016**, *6*, 1848–1852.

(74) Gutierrez, M.; Parella, T.; Joglar, J.; Bujons, J.; Clapés, P. Structure-Guided Redesign of d-Fructose-6-Phosphate Aldolase from E. Coli: Remarkable Activity and Selectivity towards Acceptor Substrates by Two-Point Mutation. *Chem. Commun.* **2011**, *47*, 5762–5764.

(75) Roldán, R.; Sanchez-Moreno, I.; Scheidt, T.; Hélaine, V.; Lemaire, M.; Parella, T.; Clapés, P.; Fessner, W.-D.; Guérard-Hélaine, C. Breaking the Dogma of Aldolase Specificity: Simple Aliphatic Ketones and Aldehydes Are Nucleophiles for Fructose-6-Phosphate Aldolase. *Chem. - Eur. J.* **2017**, *23*, 5005–5009.

(76) Roldán, R.; Hernandez, K.; Joglar, J.; Bujons, J.; Parella, T.; Sánchez-Moreno, I.; Hélaine, V.; Lemaire, M.; Guérard-Hélaine, C.; Fessner, W.-D.; Clapés, P. Biocatalytic Aldol

Addition of Simple Aliphatic Nucleophiles to Hydroxyaldehydes. *ACS Catal.* **2018**, *8*, 8804–8809.

(77) Lehwess-Litzmann, A.; Neumann, P.; Parthier, C.; Lüdtke, S.; Golbik, R.; Ficner, R.; Tittmann, K. Twisted Schiff Base Intermediates and Substrate Locale Revise Transaldolase Mechanism. *Nat. Chem. Biol.* **2011**, *7*, 678–684.

(78) Roldán, R.; Hernández, K.; Joglar, J.; Bujons, J.; Parella, T.; Fessner, W.-D.; Clapés, P. Aldolase-Catalyzed Asymmetric Synthesis of N-Heterocycles by Addition of Simple Aliphatic Nucleophiles to Aminoaldehydes. *Adv. Synth. Catal.* **2019**, *361*, 2673–2687.

(79) Mukherjee, S.; Yang, J. W.; Hoffmann, S.; List, B. Asymmetric Enamine Catalysis. *Chem. Rev.* **2007**, *107*, 5471–5569.

(80) Ajikumar, P. K.; Tyo, K.; Carlsen, S.; Mucha, O.; Phon, T. H.; Stephanopoulos, G. Terpenoids: Opportunities for Biosynthesis of Natural Product Drugs Using Engineered Microorganisms. *Mol. Pharm.* **2008**, *5* (2), 167–190. https://doi.org/10.1021/mp700151b.

(81) J, P. Biosynthesis of Polyketides by Trans-AT Polyketide Synthases. *Nat. Prod. Rep.* **2010**, *27*, 996–1047.

(82) Falbe, J.; Bahrmann, H.; Lipps, W.; Mayer, D.; Frey, G. D. Alcohols, Aliphatic. In *Ullmann's Encyclopedia of Industrial Chemistry*; Wiley-VCH Verlag GmbH & Co. KGaA, Ed.; Wiley-VCH Verlag GmbH & Co. KGaA: Weinheim, Germany, **2013**, 1–26.

(83) Junker, S.; Roldan, R.; Joosten, H.-J.; Clapés, P.; Fessner, W.-D. Complete Switch of Reaction Specificity of an Aldolase by Directed Evolution In Vitro: Synthesis of Generic Aliphatic Aldol Products. *Angew. Chem. Int. Ed.* **2018**, *57*, 10153–10157.

(84) Chambre, D.; Guérard-Hélaine, C.; Darii, E.; Mariage, A.; Petit, J.-L.; Salanoubat, M.; Berardinis, V. de; Lemaire, M.; Hélaine, V. 2-Deoxyribose-5-Phosphate Aldolase, a Remarkably Tolerant Aldolase towards Nucleophile Substrates. *Chem. Commun.* **2019**, *55*, 7498–7501.

(85) Chen, L.; Dumas, D. P.; Wong, C. H. Deoxyribose 5-Phosphate Aldolase as a Catalyst in Asymmetric Aldol Condensation. *J. Am. Chem. Soc.* **1992**, *114*, 741–748.

(86) Chokhawala, H. A.; Cao, H.; Yu, H.; Chen, X. Enzymatic Synthesis of Fluorinated Mechanistic Probes for Sialidases and Sialyltransferases. *J. Am. Chem. Soc.* **2007**, *129*, 10630–10631.

(87) Beliczey, J.; Kragl, U.; Liese, A.; Wandrey, C.; Hamacher, K.; Coenen, H. H.; Tierling, T. Method for Making Fluorinated Sugars Having a Side Chain and Use Thereof. US6355453B1, March 12, 2002.

(88) Watts, A.; Withers, S. The Synthesis of Some Mechanistic Probes for Sialic Acid Processing Enzymes and the Labeling of a Sialidase from Trypanosoma Rangeli. *Can. J. Chem.* 2011, *82*, 1581–1588.

(89) Windle, C. L.; Berry, A.; Nelson, A. Aldolase-Catalysed Stereoselective Synthesis of Fluorinated Small Molecules. *Curr. Opin. Chem. Biol.* 2017, *37*, 33–38.

(90) Stockwell, J.; Daniels, A. D.; Windle, C. L.; Harman, T. A.; Woodhall, T.; Lebl, T.; Trinh, C. H.; Mulholland, K.; Pearson, A. R.; Berry, A.; Nelson, A. Evaluation of Fluoropyruvate as Nucleophile in Reactions Catalysed by N-Acetyl Neuraminic Acid Lyase Variants: Scope, Limitations and Stereoselectivity. *Org. Biomol. Chem.* 2016, *14*, 105–112.

(91) Howard, J. K.; Müller, M.; Berry, A.; Nelson, A. An Enantio- and Diastereoselective Chemoenzymatic Synthesis of α-Fluoro β-Hydroxy Carboxylic Esters. *Angew. Chem. Int. Ed.* 2016, *55*, 6767–6770.

(92) Stolz, A. Degradation of Substituted Naphthalenesulfonic Acids by Sphingomonas Xenophaga BN6. *J. Ind. Microbiol. Biotechnol.* 1999, *23*, 391–399.

(93) Eaton, R. W. *Trans-o* -Hydroxybenzylidenepyruvate Hydratase-Aldolase as a Biocatalyst. *Appl. Environ. Microbiol.* 2000, *66*, 2668–2672.

(94) Fang, J.; Hait, D.; Head-Gordon, M.; Chang, M. C. Y. Chemoenzymatic Platform for Synthesis of Chiral Organofluorines Based on Type II Aldolases. *Angew. Chem.* 2019, *131*, 11967–11971.

(95) Shelton, M. C.; Cotterill, I. C.; Novak, S. T. A.; Poonawala, R. M.; Sudarshan, S.; Toone, E. J. 2-Keto-3-Deoxy-6-Phosphogluconate Aldolases as Catalysts for Stereocontrolled Carbon−Carbon Bond Formation. *J. Am. Chem. Soc.* 1996, *118*, 2117–2125.

(96) Henderson, D. P.; Shelton, M. C.; Cotterill, I. C.; Toone, E. J. Stereospecific Preparation of the N-Terminal Amino Acid Moiety of Nikkomycins KX and KZ via a Multiple Enzyme Synthesis. *J. Org. Chem.* 1997, *62*, 7910–7911.

(97) Henderson, D. P.; Cotterill, I. C.; Shelton, M. C.; Toone, E. J. 2-Keto-3-Deoxy-6-Phosphogalactonate Aldolase as a Catalyst for Stereocontrolled Carbon−Carbon Bond Formation. *J. Org. Chem.* 1998, *63*, 906–907.

(98) Griffiths, J. S.; Wymer, N. J.; Njolito, E.; Niranjanakumari, S.; Fierke, C. A.; Toone, E. J. Cloning, Isolation and Characterization of the Thermotoga Maritima KDPG Aldolase. *Bioorg. Med. Chem.* **2002**, *10*, 545–550.

(99) Berardinis, V. de; Guérard-Hélaine, C.; Darii, E.; Bastard, K.; Hélaine, V.; Mariage, A.; Petit, J.-L.; Poupard, N.; Sánchez-Moreno, I.; Stam, M.; Gefflaut, T.; Salanoubat, M.; Lemaire, M. Expanding the Reaction Space of Aldolases Using Hydroxypyruvate as a Nucleophilic Substrate. *Green Chem.* **2017**, *19*, 519–526.

(100) Marsden, S. R.; Mestrom, L.; Bento, I.; Hagedoorn, P.; McMillan, D. G. G.; Hanefeld, U. CH-π Interactions Promote the Conversion of Hydroxypyruvate in a Class II Pyruvate Aldolase. *Adv. Synth. Catal.* **2019**, *361*, 2649–2658.

(101) Hernández, K.; Joglar, J.; Bujons, J.; Parella, T.; Clapés, P. Nucleophile Promiscuity of Engineered Class II Pyruvate Aldolase YfaU from E. Coli. *Angew. Chem. Int. Ed Engl.* **2018**, *57*, 3583–3587.

(102) Hixon, M.; Sinerius, G.; Schneider, A.; Walter, C.; Fessner, W. D.; Schloss, J. V. Quo Vadis Photorespiration: A Tale of Two Aldolases. *FEBS Lett.* **1996**, *392*, 281–284.

(103) Rea, D.; Hovington, R.; Rakus, J. F.; Gerlt, J. A.; Fülöp, V.; Bugg, T. D. H.; Roper, D. I. Crystal Structure and Functional Assignment of YfaU, a Metal Ion Dependent Class II Aldolase from *Escherichia Coli* K12. *Biochemistry* **2008**, *47*, 9955–9965.

(104) Hernandez, K.; Bujons, J.; Joglar, J.; Charnock, S. J.; Domínguez de María, P.; Fessner, W. D.; Clapés, P. Combining Aldolases and Transaminases for the Synthesis of 2-Amino-4-Hydroxybutanoic Acid. *ACS Catal.* **2017**, *7*, 1707–1711.

(105) Desmons, S.; Fauré, R.; Bontemps, S. Formaldehyde as a Promising C_1 Source: The Instrumental Role of Biocatalysis for Stereocontrolled Reactions. *ACS Catal.* **2019**, *9*, 9575–9588.

(106) Marín-Valls, R.; Hernández, K.; Bolte, M.; Joglar, J.; Bujons, J.; Clapés, P. Chemoenzymatic Hydroxymethylation of Carboxylic Acids by Tandem Stereodivergent Biocatalytic Aldol Reaction and Chemical Decarboxylation. *ACS Catal.* **2019**, *9*, 7568–7577.

(107) Laurent, V.; Uzel, A.; Hélaine, V.; Nauton, L.; Traïkia, M.; Gefflaut, T.; Salanoubat, M.; de Berardinis, V.; Lemaire, M.; Guérard-Hélaine, C. Exploration of Aldol Reactions Catalyzed by Stereoselective Pyruvate Aldolases with 2-Oxobutyric Acid as Nucleophile. *Adv. Synth. Catal.* **2019**, *361*, 2713–2717.

(108) Arnold, F. H. Directed Evolution: Bringing New Chemistry to Life. *Angew. Chem. Int. Ed.* **2018**, *57*, 4143–4148.

(109) Renata, H.; Wang, Z. J.; Arnold, F. H. Expanding the Enzyme Universe: Accessing Non-Natural Reactions by Mechanism-Guided Directed Evolution. *Angew. Chem. Int. Ed.* **2015**, *54*, 3351–3367.

(110) Huddleston, J. P.; Thoden, J. B.; Dopkins, B. J.; Narindoshvili, T.; Fose, B. J.; Holden, H. M.; Raushel, F. M. Structural and Functional Characterization of YdjI, an Aldolase of Unknown Specificity in Escherichia Coli K12. *Biochemistry* **2019**, *58*, 3340–3353.

(111) Laurent, V.; Darii, E.; Aujon, A.; Debacker, M.; Petit, J.-L.; Hélaine, V.; Liptaj, T.; Breza, M.; Mariage, A.; Nauton, L.; Traïkia, M.; Salanoubat, M.; Lemaire, M.; Guérard-Hélaine, C.; de Berardinis, V. Synthesis of Branched-Chain Sugars with a DHAP-Dependent Aldolase: Ketones Are Electrophile Substrates of Rhamnulose-1-Phosphate Aldolases. *Angew. Chem. Int. Ed.* **2018**, *57*, 5467–5471.

(112) Müller, M. Enzymatic Synthesis of Tertiary Alcohols. *ChemBioEng Rev.* **2014**, *1*, 14–26.

(113) Yang, J.; Zhu, Y.; Qu, G.; Zeng, Y.; Tian, C.; Dong, C.; Men, Y.; Dai, L.; Sun, Z.; Sun, Y.; Ma, Y. Biosynthesis of Dendroketose from Different Carbon Sources Using in Vitro and in Vivo Metabolic Engineering Strategies. *Biotechnol. Biofuels* **2018**, *11*, 290.

(114) Schmidt, N. G.; Eger, E.; Kroutil, W. Building Bridges: Biocatalytic C–C-Bond Formation toward Multifunctional Products. *ACS Catal.* **2016**, *6*, 4286–4311.

(115) Lorillière, M.; Guérard-Hélaine, C.; Gefflaut, T.; Fessner, W.-D.; Clapés, P.; Charmantray, F.; Hecquet, L. Convergent in Situ Generation of Both Transketolase Substrates via Transaminase and Aldolase Reactions for Sequential One-Pot, Three-Step Cascade Synthesis of Ketoses. *ChemCatChem* **2020**, *12*, 812–817.

(116) Dick, M.; Hartmann, R.; Weiergräber, O. H.; Bisterfeld, C.; Classen, T.; Schwarten, M.; Neudecker, P.; Willbold, D.; Pietruszka, J. Mechanism-Based Inhibition of an Aldolase at High Concentrations of Its Natural Substrate Acetaldehyde: Structural Insights and Protective Strategies. *Chem. Sci.* **2016**, *7*, 4492–4502.

(117) Grabner, B.; Pokhilchuk, Y.; Gruber-Woelfler, H. DERA in Flow: Synthesis of a Statin Side Chain Precursor in Continuous Flow Employing Deoxyribose-5-Phosphate Aldolase Immobilized in Alginate-Luffa Matrix. *Catalysts* **2020**, *10*, 137.

(118) Schürmann, M. An Aldolase for the Synthesis of the Statin Side Chain. In *Industrial Enzyme Applications*; Vogel, A., May, O., Eds.; John Wiley & Sons, Ltd: Chichester, UK, 2019; pp 385–403.

(119) Ručigaj, A.; Krajnc, M. Kinetic Modeling of a Crude DERA Lysate-Catalyzed Process in Synthesis of Statin Intermediates. *Chem. Eng. J.* 2015, *259*, 11–24.

(120) Švarc, A.; Blažević, Z. F.; Vasić-Rački, Đ.; Szekrenyi, A.; Fessner, W.-D.; Charnock, S. J.; Presečki, A. V. 2-Deoxyribose-5-Phosphate Aldolase from Thermotoga Maritima in the Synthesis of a Statin Side-Chain Precursor: Characterization, Modeling and Optimization. *J. Chem. Technol. Biotechnol.* 2019, *94*, 1832–1842.

(121) Švarc, A.; Fekete, M.; Hernandez, K.; Clapés, P.; Findrik Blažević, Z.; Szekrenyi, A.; Skendrović, D.; Vasić-Rački, Đ.; Charnock, S. J.; Presečki, A. V. An Innovative Route for the Production of Atorvastatin Side-Chain Precursor by DERA-Catalysed Double Aldol Addition. *Chem. Eng. Sci.* 2021, *231*, 116312.

(122) Švarc, A.; Findrik Blažević, Z.; Vasić-Rački, Đ.; Charnock, S. J.; Vrsalović Presečki, A. A Multi-Enzyme Strategy for the Production of a Highly Valuable Lactonized Statin Side-Chain Precursor. *Chem. Eng. Res. Des.* 2020, *164*, 35–45.

(123) Voutilainen, S.; Heinonen, M.; Andberg, M.; Jokinen, E.; Maaheimo, H.; Pääkkönen, J.; Hakulinen, N.; Rouvinen, J.; Lähdesmäki, H.; Kaski, S.; Rousu, J.; Penttilä, M.; Koivula, A. Substrate Specificity of 2-Deoxy-D-Ribose 5-Phosphate Aldolase (DERA) Assessed by Different Protein Engineering and Machine Learning Methods. *Appl. Microbiol. Biotechnol.* 2020, *104*, 10515–10529.

(124) V. Makshina, E.; Dusselier, M.; Janssens, W.; Degrève, J.; A. Jacobs, P.; F. Sels, B. Review of Old Chemistry and New Catalytic Advances in the On-Purpose Synthesis of Butadiene. *Chem. Soc. Rev.* 2014, *43*, 7917–7953.

(125) Matsuyama, A.; Yamamoto, H.; Kawada, N.; Kobayashi, Y. Industrial Production of (R)-1,3-Butanediol by New Biocatalysts. *J. Mol. Catal. B Enzym.* 2001, *11*, 513–521.

(126) Yamamoto, H.; Matsuyama, A.; Kobayashi, Y. Synthesis of (R)-1,3-Butanediol by Enantioselective Oxidation Using Whole Recombinant Escherichia Coli Cells Expressing (S)-Specific Secondary Alcohol Dehydrogenase. *Biosci. Biotechnol. Biochem.* 2002, *66*, 925–927.

(127) Ichikawa, N.; Sato, S.; Takahashi, R.; Sodesawa, T. Catalytic Reaction of 1,3-Butanediol over Solid Acids. *J. Mol. Catal. Chem.* 2006, *256*, 106–112.

(128) Sabra, W.; Groeger, C.; Zeng, A.-P. Microbial Cell Factories for Diol Production. *Adv. Biochem. Eng. Biotechnol.* **2016**, *155*, 165–197.

(129) Kim, T.; Stogios, P. J.; Khusnutdinova, A. N.; Nemr, K.; Skarina, T.; Flick, R.; Joo, J. C.; Mahadevan, R.; Savchenko, A.; Yakunin, A. F. Rational Engineering of 2-Deoxyribose-5-Phosphate Aldolases for the Biosynthesis of (R)-1,3-Butanediol. *J. Biol. Chem.* **2020**, *295*, 597–609.

(130) Nemr, K.; Müller, J. E. N.; Joo, J. C.; Gawand, P.; Choudhary, R.; Mendonca, B.; Lu, S.; Yu, X.; Yakunin, A. F.; Mahadevan, R. Engineering a Short, Aldolase-Based Pathway for (R)-1,3-Butanediol Production in Escherichia Coli. *Metab. Eng.* **2018**, *48*, 13–24.

(131) Valino, A. L.; Iribarren, A. M.; Lewkowicz, E. New Biocatalysts for One Pot Multistep Enzymatic Synthesis of Pyrimidine Nucleoside Diphosphates from Readily Available Reagents. *J. Mol. Catal. B Enzym.* **2015**, *114*, 58–64.

(132) Huffman, M. A.; Fryszkowska, A.; Alvizo, O.; Borra-Garske, M.; Campos, K. R.; Canada, K. A.; Devine, P. N.; Duan, D.; Forstater, J. H.; Grosser, S. T.; Halsey, H. M.; Hughes, G. J.; Jo, J.; Joyce, L. A.; Kolev, J. N.; Liang, J.; Maloney, K. M.; Mann, B. F.; Marshall, N. M.; McLaughlin, M.; Moore, J. C.; Murphy, G. S.; Nawrat, C. C.; Nazor, J.; Novick, S.; Patel, N. R.; Rodriguez-Granillo, A.; Robaire, S. A.; Sherer, E. C.; Truppo, M. D.; Whittaker, A. M.; Verma, D.; Xiao, L.; Xu, Y.; Yang, H. Design of an in Vitro Biocatalytic Cascade for the Manufacture of Islatravir. *Science* **2019**, *366*, 1255–1259.

(133) Bisterfeld, C.; Kullartz, I. K. née; Dick, M.; Pietruszka, J. A Fluorogenic Screening for Enantio- and Diastereoselectivity of 2-Deoxy-d-Ribose-5-Phosphate Aldolases. *Synlett* **2016**, *27*, 11–16.

(134) Jiao, X.-C.; Pan, J.; Kong, X.-D.; Xu, J.-H. Protein Engineering of Aldolase LbDERA for Enhanced Activity toward Real Substrates with a High-Throughput Screening Method Coupled with an Aldehyde Dehydrogenase. *Biochem. Biophys. Res. Commun.* **2017**, *482*, 159–163.

(135) Laurent, V.; Hélaine, V.; Vergne-Vaxelaire, C.; Nauton, L.; Traikia, M.; Petit, J.-L.; Salanoubat, M.; de Berardinis, V.; Lemaire, M.; Guérard-Hélaine, C. Achiral Hydroxypyruvaldehyde Phosphate as a Platform for Multi-Aldolases Cascade Synthesis of Diuloses and for a Quadruple Acetaldehyde Addition Catalyzed by 2-Deoxyribose-5-Phosphate Aldolases. *ACS Catal.* **2019**, *9*, 9508–9512.

(136) Moreno, C. J.; Hernández, K.; Charnok, S. J.; Gittings, S.; Bolte, M.; Joglar, J.; Bujons, J.; Parella, T.; Clapés, P. Synthesis of γ-Hydroxy-α-Amino Acid Derivatives by Enzymatic Tandem Aldol Addition–Transamination Reactions. *ACS Catal.* **2021**, *11*, 4660–4669.

(137) Fansher, D. J.; Granger, R.; Kaur, S.; Palmer, D. R. J. Repurposing an Aldolase for the Chemoenzymatic Synthesis of Substituted Quinolines. *ACS Catal.* **2021**, *11*, 6939–6943.

(138) Laurent, V.; Gourbeyre, L.; Uzel, A.; Hélaine, V.; Nauton, L.; Traïkia, M.; de Berardinis, V.; Salanoubat, M.; Gefflaut, T.; Lemaire, M.; Guérard-Hélaine, C. Pyruvate Aldolases Catalyze Cross-Aldol Reactions Between Ketones: Highly Selective Access to Multi-Functionalized Tertiary Alcohols. *ACS Catal.* **2020**, *10*, 2538–2543.

(139) Martínez, A.; Zumbansen, K.; Döhring, A.; Gemmeren, M. van; List, B. Improved Conditions for the Proline-Catalyzed Aldol Reaction of Acetone with Aliphatic Aldehydes. *Synlett* **2014**, *25*, 932–934.

(140) Scheffler, U.; Mahrwald, R. Asymmetric Organocatalyzed Direct Aldol Additions of Enolizable Aldehydes. *Synlett* **2011**, *2011*, 1660–1667.

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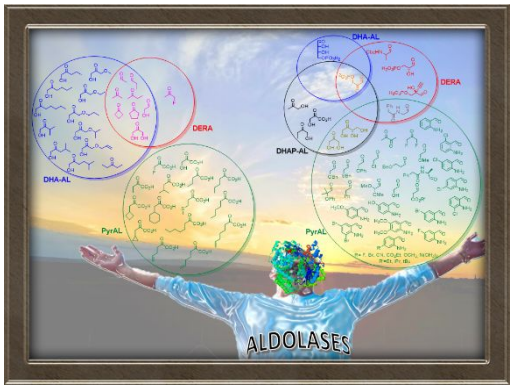
TOC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