CHEMICAL REVIEWS

REVIEW

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C−C Bond-Forming Lyases in Organic Synthesis

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1. INTRODUCTION

Lyases are enzymes that catalyze the bond construction and breaking by means other than hydrolysis or redox reactions. Such enzymes are particular in the sense that they only require one substrate for the reaction in one direction, but two substrates for the reverse reaction. Even though hydrolases have been the most employed biocatalysts in organic synthesis, an increasing number of recent articles deals with lyase-catalyzed synthesis of several interesting intermediates and final products.[1−3]

Lyases are classified as EC 4, and there are seven subclasses of lyases, depending on the bond type involved. EC 4.1.x.x are carbon−carbon lyases, and this is the most important and abundant subclass. This subclass has four subcategories, namely, carboxy lyases (EC 4.1.1.x), aldehyde lyases (EC 4.1.2.x), oxoacid lyases (EC 4.1.3.x), and other carbon−carbon lyases (EC 4.1.99.x).[4]

Stereoselective C−C bond construction is of utmost importance in organic synthesis.[5] In this regard, enzymes are increasingly recognized as useful catalysts for asymmetric organic preparations.[6−9] Enzymatic C−C bond formation is frequently highly chemo-, regio-, diastereo-, and enantioselective. Moreover, biocatalytic processes are performed under mild conditions and often in neutral aqueous solutions. As a result of the high selectivity and mild conditions employed, protective group chemistry can be avoided. Finally, the amount of waste generated because of avoidance of protective groups, chiral auxiliaries, and side products is minimized. Therefore, biocatalytic processes turn into an attractive, efficient, and environmentally friendly option for synthetic design.[10−12]

Despite of these advantages, the widespread use of biocatalysts in organic synthesis depends on several factors, including availability of enzymes, transferability of protocols, and reliability of obtained results.[13]

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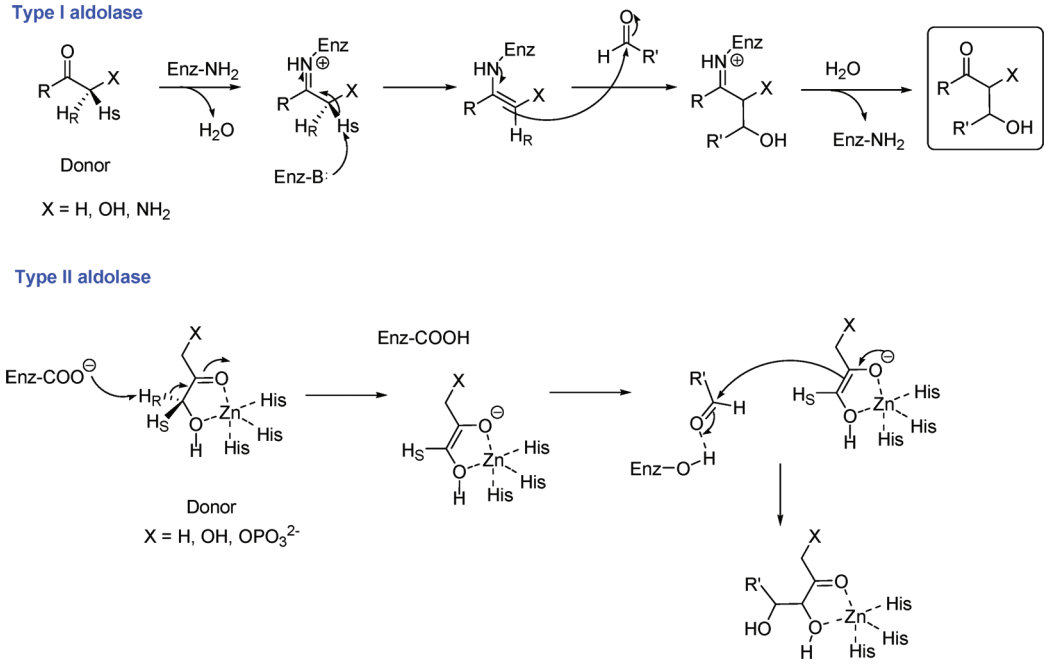

Figure 1. Mechanism of type I and II aldolases.

Several types of enzymes that catalyze C−C bond formation are known. Many of those biocatalysts are lyases.[14] Among them, aldolases and hydroxynitrile lyases (oxynitrilases) are highlighted because of their use in organic synthesis.[15] This review will focus on lyase catalyzed C−C bond formation, presenting an overview of the latest developments in the field during the past decade, with emphasis in the synthetic applications of enzymatic aldol, acyloin condensation, and cyanohydrin reactions.

2. ENZYMATIC ALDOL REACTIONS

The aldol reaction is one of the most powerful procedures for the construction of C−C bonds.[16−19] This reaction is also critical in the metabolic context, being the most important biochemical process for the production of naturally occurring carbohydrates.[20]

Improved variants of the aldol reaction, including enzymatic ones, have been developed in the last decades.[21,22] Excellent control over the stereochemistry of the reaction can be achieved by both chemical and enzymatic means.[19] The enzymatic aldol reaction is very attractive for the preparation of biologically important organic compounds, such as carbohydrates and amino acids. Those products are often structurally elaborated and water-soluble molecules, and have several functional groups. Thus, conventional synthetic approaches normally require the extensive use of protective groups and are usually more demanding in terms of stereochemical control.[20,23,24]

2.1. Enzymes Involved. Classification

Aldolases are ubiquitous enzymes belonging to the class of lyases, and found in biosynthetic pathways of carbohydrates, keto acids and amino acids, as well as in various catabolic pathways for carbohydrates.[15,25] Over forty aldolases are known,[25,26] most of them catalyzing the stereoselective aldol addition of a ketone to an aldehyde.[21,27]

As for lipases and isomerases, they do not require cofactors to display activity (except for those requiring Zn^{2+} ion and those depending on glycine that use pyridoxal phosphate). On a mechanistic basis, aldolases are divided in two types, depending on the activation mode of the donor carbonyl group (Figure 1).[25,28]

Type I aldolases activate the donor by forming a Schiff base as an intermediate. The ε-NH_2 group of a lysine residue in the active site forms the imine with the donor. This activated donor then adds stereoselectively to the acceptor aldehyde, through formation of an enamine. The enamine generation is also stereoselective because of abstraction of the *pro-S* (H_S) proton.[29]

On the other side, type II aldolases contain a Zn^{2+} cofactor in the active site. This transition metal ion is coordinated to three histidine nitrogen atoms, promoting activation of the nucleophile through formation of a chelate with both the carbonyl and the α-hydroxy group in the donor. This allows the removal of the *pro-R* (H_R) proton, generating an enediolate able to react with the acceptor.[21,30]

Type I aldolases are found mainly in animals and higher plants, whereas type II aldolases are located in microorganisms and are, in general, more stable.[21]

Functionally, aldolases can be divided in four groups, according to the structure of the donor substrate accepted by the enzyme (Table 1).

The first group uses dihydroxyacetone phosphate (DHAP) as the donor to produce a ketose 1-phosphate upon reaction with an aldehyde. Also, the dephosphorylated ketone (DHA) can be used as donor by some enzymes of this group.[14,23,31,32] The second group uses pyruvate or phosphoenolpyruvate, affording 3-deoxy 2-ketoacids. The third group employs acetaldehyde to give 2-deoxyaldehydes. Finally, the fourth group utilizes glycine as donor leading to β-hydroxy-α-amino acids.

Members of the second and the third group generate α-methylene carbonyl compounds and therefore a single stereocenter. On the other hand, aldolases belonging to the first and the fourth group form α-substituted carbonyl compounds that

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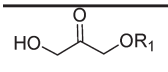
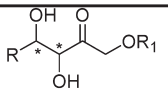
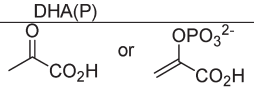
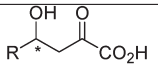
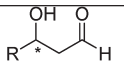
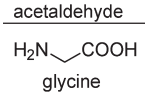
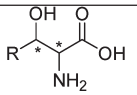
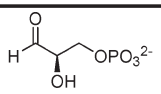
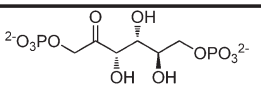
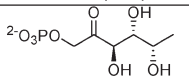
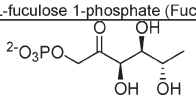
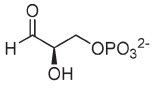
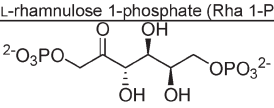
Table 1. Four Groups of Aldolases According to Their Donor Substrates

Donor	Acceptor	Product
 DHA(P)		
 Pyruvate or phosphoenolpyruvate		
 acetaldehyde		
 glycine		

R_1:$PO_3^=$ or H *: new chiral carbon atom

Table 2. Dihydroxyacetone Phosphate-Dependent Aldolases

Natural acceptor	Enzyme	Product
 D-glyceraldehyde 3-phosphate (G3P)	Fructose 1,6-biphosphate (FBP) aldolase (FruA, EC 4.1.2.13)	 D-fructose 1,6-biphosphate (FBP)
 (*S*)-lactaldehyde	L-Fuculose 1-phosphate (Fuc 1-P) aldolase (FucA, EC 4.1.2.17)	 L-fuculose 1-phosphate (Fuc 1-P)
 (*S*)-lactaldehyde	L-Rhamnulose 1-phosphate (Rha 1-P) aldolase (RhuA, EC 4.1.2.19)	 L-rhamnulose 1-phosphate (Rha 1-P)
 D-glyceraldehyde 3-phosphate (G3P)	Tagatose 1,6-diphosphate (TDP) aldolase (TagA, EC 4.1.2.40)	 D-tagatose 1,6-diphosphate (TDP)

contain two vicinal chiral centers at the newly formed C−C bond.

These enzymes generally tolerate a broad range of acceptor substrates but have rigid requirements for donor ones. Generally, stereoselectivity is controlled by the enzyme and does not depend on the structure of the substrate, producing highly predictable products. Most exceptions are found in the pyruvate-dependent aldolases.[21]

2.1.1. Dihydroxyacetone Phosphate- (DHAP) and Dihydroxyacetone- (DHA) Dependent Aldolases. This group includes several enzymes accepting a broad range of substrates. The in vivo catalyzed reactions for some known DHAP-dependent aldolases of synthetic interest are shown in Table 2.[15,21,33]

This group is the most studied so far, offering highly interesting synthetic alternatives, in particular for asymmetric synthesis of polyoxygenated compounds. When using DHAP-dependent aldolases for catalyzed C−C bond formation, two new stereocenters are formed. As a consequence, four different stereoisomers can be obtained. Different aldolases catalyzing the formation of each of those stereoisomers are commercially available. Each aldol reaction generates a single product, whose stereochemistry at C-3 and C-4 is complementary to the others (Figure 2). These enzymes have a broad substrate (acceptor aldehyde) specificity and high level of stereocontrol at C-3. However, the configuration of the stereocenter at C-4 in some cases depends on the structure of the acceptor (particularly for TagA).[34−36]

2.1.1.1. Fructose 1,6-Biphosphate (FBP) Aldolase (FruA). FBP aldolase is the most employed enzyme of this group, catalyzing in vivo the aldol addition of D-glyceraldehyde 3-phosphate (D-G3P) and DHAP to give D-fructose 1,6-biphosphate (FBP). This reaction is strongly displaced toward the synthesis ($K = 10^4$ M^{-1})[37] at standard conditions, meaning 1 M for every species, resulting in a strong dependence upon the concentration of the reactants. Although the aldolase reaction has a negative standard free energy change in the synthesis direction, at the lower concentration of reactants present in cells, the actual free energy is closer to zero and the aldolase reaction is reversible.[38]

Both type I and type II enzymes have been isolated from different prokaryotic and eukaryotic sources and thus, FBP aldolase is an ubiquitous glycolytic enzyme that plays a crucial role in glycolysis, gluconeogenesis, and fructose metabolism. The class I FBP aldolase isolated from rabbit muscle (RAMA) is the aldolase most frequently employed in organic synthesis, followed by type II aldolases from yeast and bacteria, due to their commercial availability.[39−42]

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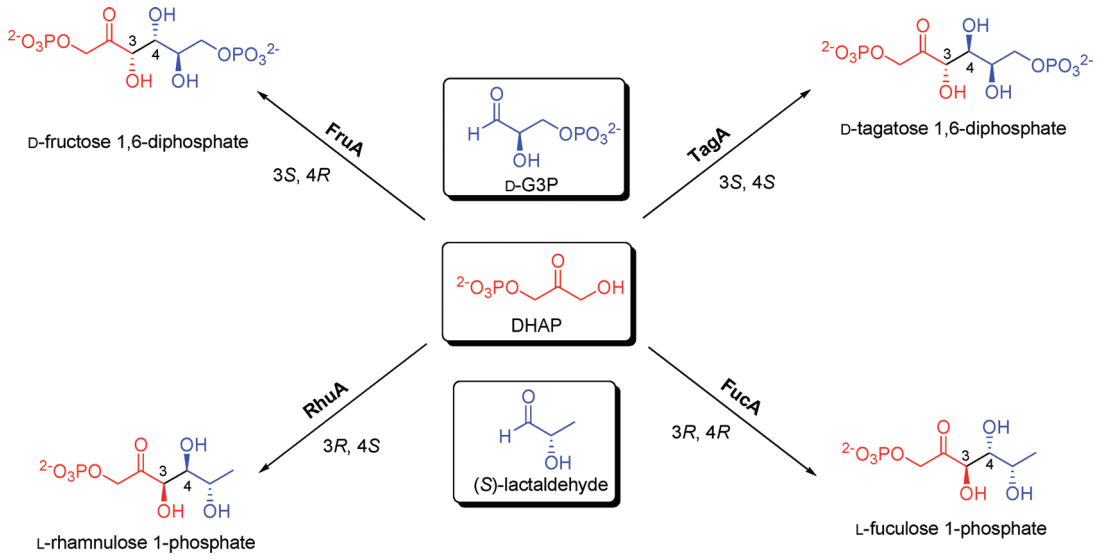

Figure 2. Stereochemical complementarity of DHAP-dependent aldolases.

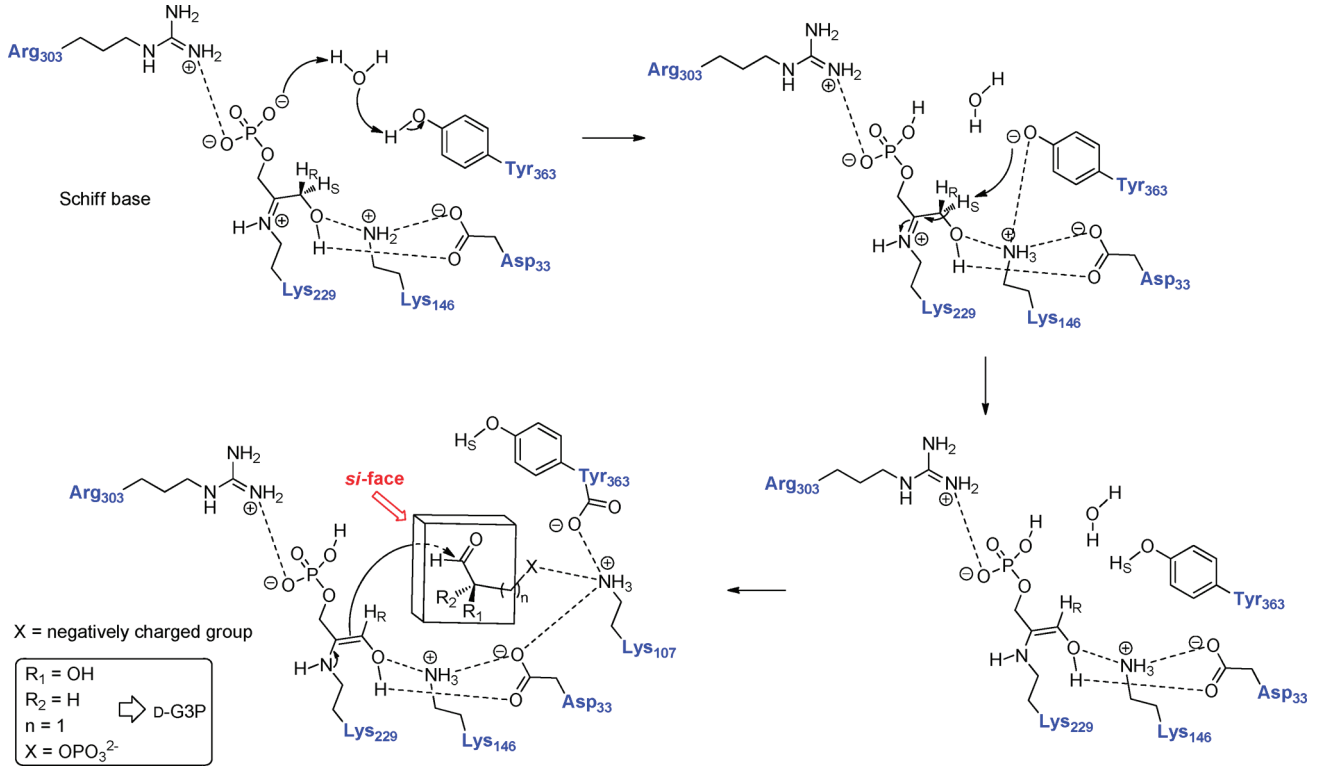

Figure 3. Proposed mechanism and key amino acids for the stereospecific abstraction of the *pro-S* (H_S) proton in the active site of RAMA and nucleophilic attack on the *si*-face of the acceptor aldehyde.

Normally, microbial type II aldolases are more stable in solution than their mammalian counterparts of type I. The half-life of RAMA is ~2 days in aqueous solution at pH 7.0, whereas the type II aldolase from *Escherichia coli* has a half-life of ~60 days in a 0.3 mM Zn^{2+} solution at pH 7.0.[43]

The catalytic mechanism has been extensively studied using RAMA, which differs by seven amino acids from that of humans.[44] On the basis of mutagenic and structural data, the role of the Schiff base forming lysine residue (Lys229), has been determined.[40,45]

The first part of the catalytic mechanism is outlined in Figure 3.

In RAMA, Lys229 is responsible for the activation of DHAP through Schiff base formation, while the negatively charged phosphate group of DHAP interacts with the guanidine residue of Arg303. Asp33 interacts with the C-3 hydroxyl of DHAP. The C-terminal region has an important role in catalysis as evidenced by the loss of stereospecificity in the proton exchange in the Schiff base-enamine interconversion when Tyr363 is not present. Prior to proton transfer, Tyr363 is activated as a

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phenoxide ion, which in turn abstracts the DHAP C-3 *pro-S* proton.[46−48]

Another lysine residue in the active site (Lys107), placed at 8.9 Å from the site of Schiff base formation, can stabilize the phosphate group of D-G3P (or other negatively charged group), thus playing a pivotal role in the enantioselectivity at C-2 of the aldehyde acceptor (Figure 3).[45]

In RAMA, stereoselectivity can be explained by the interaction of negatively charged groups in the acceptor aldehyde with the Lys107 residue in the active site. This in fact favors the nucleophilic attack of the enamine on the *si*-face of the aldehyde as shown in Figure 4, affording the product with (3*S*, 4*R*) stereochemistry. If the acceptor lacks negatively charged groups (such as a phosphate), or has them in a non appropriate position for interaction with Lys107, diastereoselectivity is drastically reduced. Lys107 is at the surface of the building pocket and can interact also with Asp33. Therefore, stabilization of the enamine intermediate is further assisted by Asp33.[45,48−50]

The specificity for the donor is high; only about half dozen of analogs of DHAP (carbonated homologues, thio-analogs, aza-analogs, phosphorate bioisosters and borates) have been found to be substrates for RAMA, and are poor (∼10% of the activity of DHAP) (Figure 4).[51,52]

On the other side, this aldolase accepts a wide range of aldehyde acceptor substrates, and more than a hundred different substrates have been identified. These substrates include unhindered aliphatic aldehydes, α-heteroatom-substituted aldehydes, and monosaccharides and their derivatives. However, aromatic and α,β-unsaturated aldehydes are not substrates of RAMA.[27,35,53]

The broad substrate tolerance of type I FBP aldolases, together with their highly stereoselective reactions, allowed them to be used in the synthesis of a large number of compounds. The main target has been carbohydrate chemistry. In this field, ^{13}C-labeled sugars, heterosubstituted carbohydrates, deoxysugars, fluoro sugars and higher-carbon carbohydrates (up to 9 carbon atoms), have been prepared.[40,53−57]

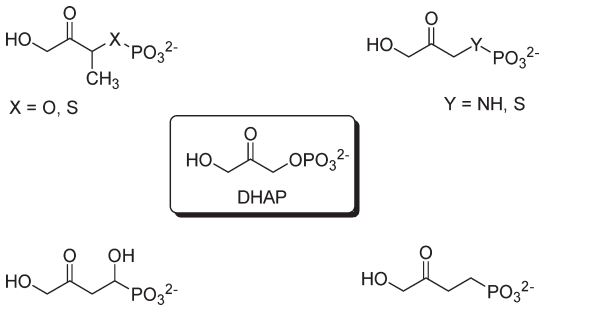

Figure 4. Analogs of DHAP accepted as donor substrates.

Even though type I FBP aldolase from rabbit muscle (RAMA) has been the most studied and used for synthetic applications, type II aldolases from prokaryotes have received attention quite recently. However, reported information is far from being abundant and complete.[58,59]

2.1.1.2. L-Fuculose 1-Phosphate (Fuc 1-P) Aldolase, L-Rhamnulose 1-phosphate (Rha 1-P) Aldolase and Tagatose 1,6-Diphosphate (TDP) Aldolase. The aldol reaction using DHAP as donor generates two chiral centers and therefore four possible stereoisomers (Figure 2). Besides FBP aldolase, there are three other enzymes able to catalyze the formation of remaining stereoisomers, namely Fuc 1-P aldolase (also referred to as FucA), Rha 1-P aldolase (RhuA, as well) and TDP aldolase (also known as TagA). The natural acceptor for both Fuc 1-P and Rha 1-P aldolases is (*S*)-lactaldehyde, whereas TDP aldolase uses D-G3P.

Fuc 1-P aldolase and Rha 1-P aldolase are found as type II enzymes in several microorganisms, and they are commercially available.[60−66] TDP aldolase is a type I aldolase that has also been isolated from several sources.[67] Also it has been found as type II enzyme from *E. coli*.[68,69]

Similar to FBP aldolase, these enzymes accept a broad range of aldehydes as the acceptor substrate, being also specific for the donor DHAP.[15,21]

The stereochemistry of the vicinal chiral centers produced normally is the same as that of the natural substrate, maintaining always the configuration at C-3 carbon. However, configuration at C-4 is less consistent and depends on the particular enzyme and the substitution pattern on the acceptor.[10]

Even though TDP aldolase accepts a variety of unphosphorylated aldehydes as substrates, products are obtained as diastereomeric mixtures. Instead of showing the expected (3*S*, 4*S*)-*erythro* configuration that is generated with the natural substrate, the major isomer of each product mixture has the (3*S*, 4*R*)-*threo* configuration.[70,71] This lack of stereoselectivity makes this enzyme less valuable in organic synthesis. However, this drawback can be overriden employing protein engineering technologies.[72]

Conversely, the configurations of the vicinal diols produced in the reaction of Fuc 1-P and Rha 1-P aldolases with (*S*)-lactaldehyde (Figure 2) do not change when using other aldehyde acceptors. Fuc 1-P aldolase generates the (3*R*, 4*R*)-diol, while Rha 1-P aldolase creates the corresponding (3*R*, 4*S*) isomer.[63,73−75] However, with some aliphatic nonhydroxylated aldehydes, selectivity in C-4 can be slightly reduced.[62,63]

Fuc 1-P and Rha 1-P aldolases exhibit a strong kinetic preference for the (*S*)-isomer of 2-hydroxyaldehydes, facilitating racemate resolutions (Figure 5).[76−78]

The group of DHAP dependent aldolases comprises other less used enzymes (Table 2), and there are no preparative uses reported.

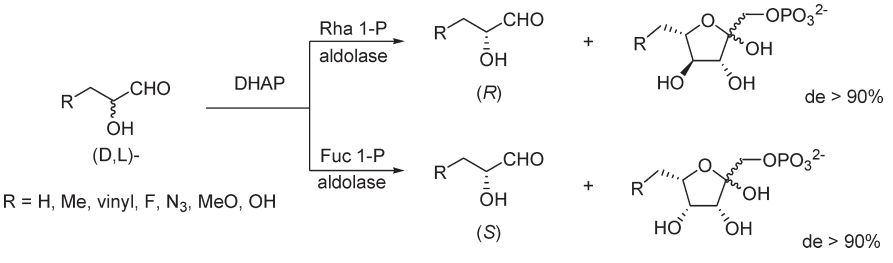

Figure 5. Kinetic enantiopreference of Rha 1-P and Fuc 1-P aldolases.

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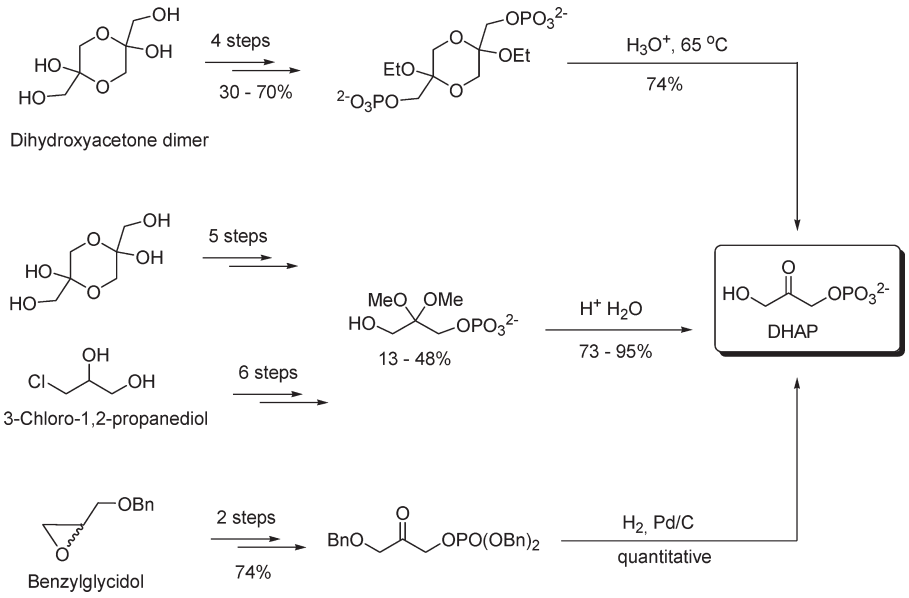

Figure 6. Chemical routes to generate DHAP.

2.1.1.3. DHAP Generation Strategies. DHAP-dependent aldolases possess the main disadvantage of strict donor substrate specificity toward DHAP. The high cost and instability of DHAP, as well as the need of removal of the phosphate group, make it more difficult to apply of the process for preparative purposes.[2] As a consequence, significant effort has been expended toward a more practical access to DHAP.

Chemical and enzymatic routes to DHAP are available and have been recently reviewed.[79]Chemical routes afford pure products and have been focused on the preparation of a stable precursor, which can be easily stored and converted into DHAP immediately before using it. Those chemical routes to DHAP are generally expensive and require complicated multistep procedures including protection/deprotection steps, as well as the use of toxic chemicals (Figure 6).[53,79−87]

DHAP can also be obtained from stable precursors through hydrolysis, by heating, acidification, or by hydrogenation. Although the final step in all cases gives excellent yields, the overall ones are only moderate.

As a consequence, more attention has been paid to enzymatic routes. However, some problems remain, mostly related to the low final concentration of the product and the inherent complications of using multienzymatic systems. There are three main general enzymatic routes to DHAP using inexpensive unphosphorylated precursors: from dihydroxyacetone,[88−90] from glycerol and glycidol via glycerol phosphate[91−93] or through multistep routes that mimic glycolysis (Figure 7).[53,91,94]

In general, these routes require the transfer of phosphate moieties from the high-energy phosphoester bond. Most of them employ ATP as phosphorylated agent, and thus regeneration of ATP becomes an important task.

As an example of the first route, dihydroxyacetone can be phosphorylated with glycerol kinase (GK) from *Saccharomyces cerevisiae* in the presence of ATP, producing DHAP with yields over 80%.[89] Also, Dihydroxyacetone kinase (DHAK) can be employed jointly with ATP.[95] Finally, an alternative approach for DHAP generation employs pyrophosphate, a cheap phosphate donor, and an Acid phosphatase (AP) from *Shigella flexneri* for transphosphorylation of dihydroxyacetone through a cascade reaction.[90] Further improvements came from directed evolution, resulting in a system six times more active when using a single mutant of the acid phosphatase.[96]

An optional route to DHAP is based in the oxidation of L-glycerol 3-phosphate catalyzed by a microbial Glycerol phosphate oxidase (GPO).[92] This reaction is coupled with H_2O_2 decomposition by catalase, thus preventing the inactivation of GPO.[93] As far as aldolases are not sensitive to oxygenated solutions, aldolic reaction can be performed in a one-pot operation. Recently, the method has been extended including an enzymatic phosphorylation of *rac*-glycerol performed with a Phytase and pyrophosphate,[91,97] or chemically through a regioselective ring-opening of the glycidol ring with an inorganic phosphate.[93]

One of the most convenient enzymatic methods is the formation of two equivalents of DHAP through retroaldolic cleavage of fructose 1,6-biphosphate, via the combined action of FBP aldolase and Triosephosphate isomerase.[53]

This step can be integrated in a one-pot eight-enzyme pathway.[94] To produce DHAP, the multienzyme system was set up starting from sucrose to generate glucose and fructose, proceeded to fructose 1,6-biphosphate and finally, to glyceraldehyde 3-phosphate and DHAP through a retro-aldolic reaction catalyzed by FBP aldolase.

An alternative to the use of DHAP is based on the reversible formation of DHA esters with inorganic anions. Such esters can replace DHAP as donor in enzymatic aldolic reactions, by adding DHA and the inorganic salt. One of the most used anion is arsenate, but the required high concentration of this toxic anion makes this approach unlikely to be used in preparative applications (Figure 8).[98,99]

Vanadates also form esters spontaneously with DHA, but they are susceptible of participating in secondary redox reactions and only have been accepted as DHAP analogs by Rha 1-P aldolase.[55]

Recently, the use of borates in the production of L-fructose and L-rhamnulose using Rha 1-P aldolase has been reported, opening interesting possibilities to this methodology.[100]

2.1.1.4. Fructose 6-Phosphate Aldolase (FSA). Even though using aldolases in organic synthesis has many advantages,

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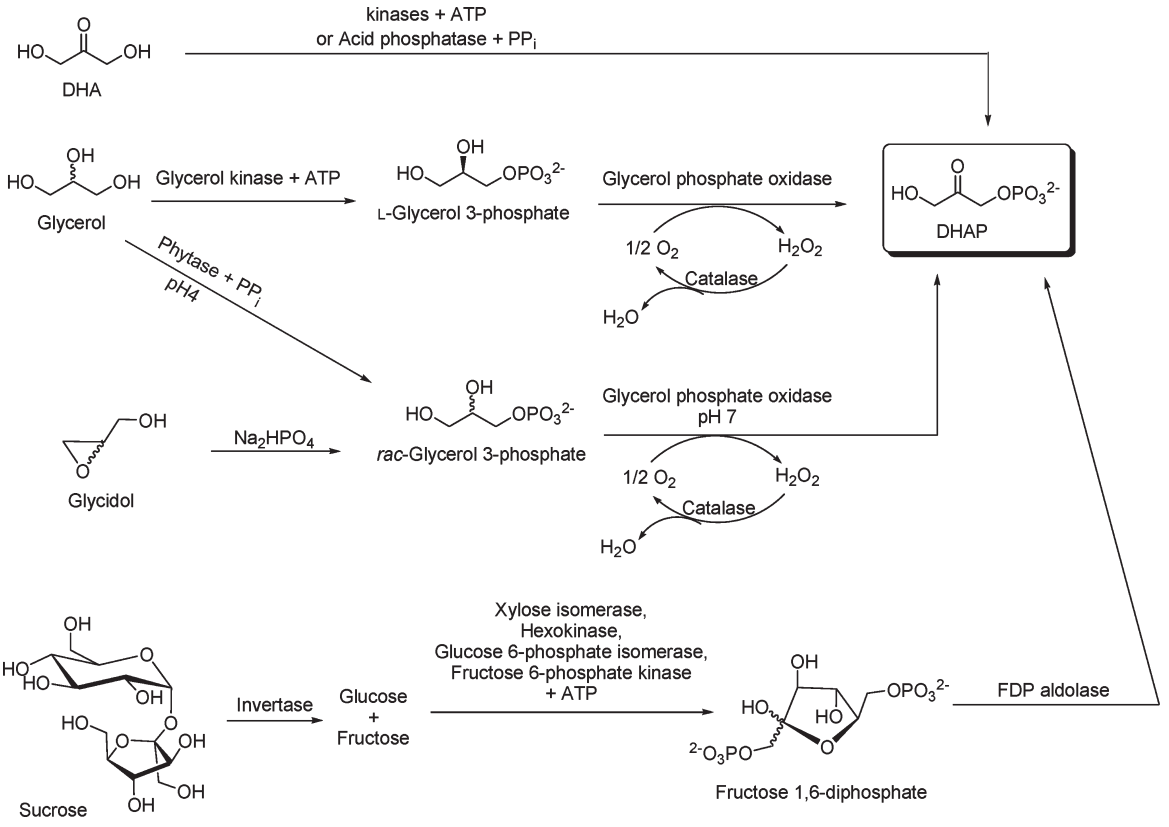

Figure 7. Enzymatic routes to DHAP. PPi = Pyrophosphate.

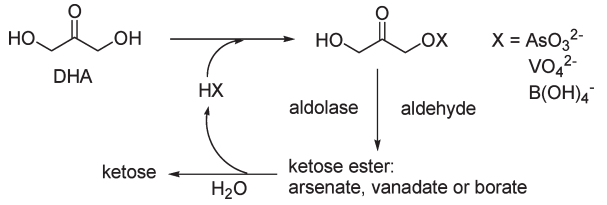

Figure 8. Alternatives to the use of DHAP.

namely, high stereospecificity, environmentally benign reaction conditions, and avoidance of protective group chemistry, some disadvantages are also found. Not all substrates are soluble in aqueous solvents, and enzymes and substrates are often expensive. In particular, for DHAP-dependent aldolases, the donor is costly and unstable (see section 2.1.1.3).[25]

As a consequence, different approaches, such as enzyme engineering, have been developed to overcome those disadvantages. Also, the discovery of new enzymes broadens the scope of enzyme-catalyzed aldol reactions.

A major discovery in this regard is fructose 6-phosphate aldolase (FSA), a novel class I aldolase isolated from *E. coli* genome.[31] This enzyme is the first example of enzymes using dihydroxyacetone (DHA) as donor substrate, instead of DHAP (Figure 9). The aldol product has the (3*S*, 4*R*) stereochemical configuration.[101]

As this enzyme uses the readily available DHA as donor substrate instead of the expensive DHAP, it has the potential to be a useful catalyst in organic synthesis. Moreover, condensation products can be readily obtained because subsequent dephosphorylation is not necessary.[23]

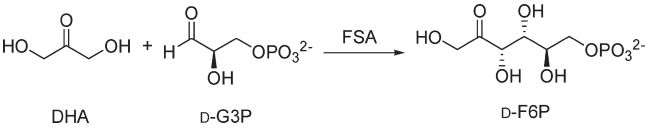

Figure 9. Reaction catalyzed by FSA.

Likewise, it has been proved that monohydroxy acetone and its homologue 1-hydroxy-2-butanone are accepted as donor substrates with equal efficiencies than the presumed endogenous DHA. This broad donor substrate specificity is unusual and can be exploited in chemoenzymatic synthesis.

In addition, this enzyme has a good tolerance to different acceptors, allowing the use of hydroxylated aldehydes of 2 or 3 carbon atoms, with or without nitrogen substituents, and also polyhydroxylated, such as D-(−)-threose.[102−104]

Recently, it has been reported the ability of FSA to catalyze the direct stereoselective self-aldol addition of glycolaldehyde to furnish D-(−)-threose (see section 2.2.1). In this reaction, glycolaldehyde acts as both the donor and the acceptor substrate for FSA.[32] In addition, FSA functions better with hydroxylacetone and glycolaldehyde than with DHA. In 2010, it was described a mutant FSA (Ala129Ser) exhibiting improved affinity toward DHA, offering complementary synthetic abilities to those of wild type FSA.[105]

The amino acidic sequence of FSA is unusual for an aldolase and, in fact, is closer to a transaldolase, although transaldolase activity was not observed for this enzyme.[31,106] An enzyme variant derived from transaldolase B of *E. coli* was described. A single mutation (Phe178Tyr) changed the enzyme from aldol transfer to an aldolase, resulting in a strongly elevated FSA activity.[107,108]

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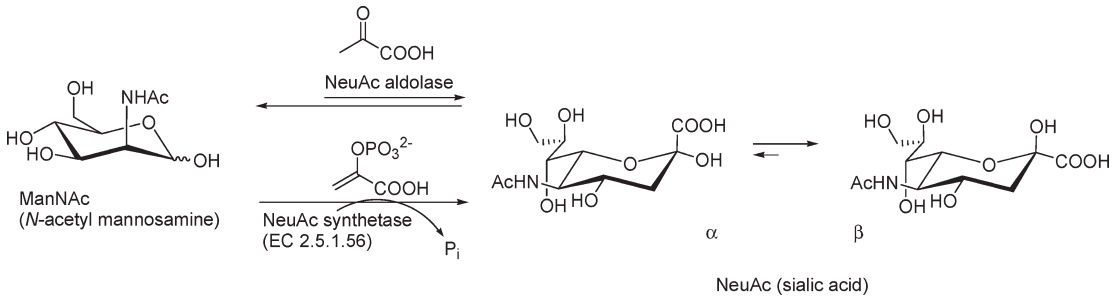
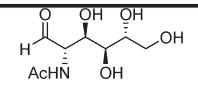
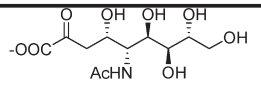
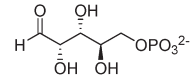
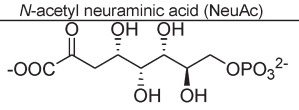
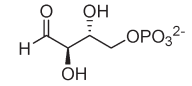
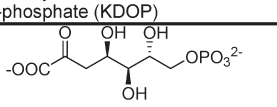


Figure 10. *N*-Acetylneuraminic acid (NeuAc) aldolase-catalyzed reaction.

Table 3. Phosphoenolpyruvate-Dependent Aldolases

Natural acceptor	Enzyme	Product
 *N*-acetyl mannosamine	*N*-Acetylneuramicic (NeuAc) synthetase (EC 2.5.1.56, former EC 4.1.3.19)	 *N*-acetyl neuraminic acid (NeuAc)
 D-arabinose 5-phosphate	3-Deoxy-D-*manno*-octulosonate 8-phosphate (KDOP) synthetase (EC 2.5.1.55, former EC 4.1.2.16)	 3-deoxy-manno-octulosonate 8-phosphate (KDOP)
 D-erythrose-4-phosphate	3-Deoxy-D-*arabino*-2-heptulosonate-7-phosphate (DAHP) synthetase (EC 2.5.1.54, former EC 4.1.2.15)	 3-deoxy-D-arabino-2-heptulosonate 7-phosphate (DAHP)

2.1.2. Pyruvate- and Phosphoenolpyruovate-Dependent Aldolases. Pyruvate-dependent aldolases have catabolic functions in vivo in the degradation of 3-deoxy-2-ulosonic acids, whereas their counterparts employing phosphoenolpyruvate as donor (carrying a high-energy phosphate bond) play roles in biosynthesis of those ketoacids. Hence, both types of enzymes can be used in organic synthesis, by shifting the equilibrium toward the condensation product. For enzymes using phosphoenolpyruvate, the shifting is achieved through irreversible releasing of inorganic phosphate, though they have not been studied with preparative purposes. Indeed, such enzymes have been relocated from the original classification as lyases (EC 4.x.x.x) to the transferases group (EC 2.x.x.x) (Figure 10 and Table 3).[35]

The group of pyruvate-dependent aldolases includes several enzymes. The in vivo catalyzed reactions for those of synthetic relevance are shown in Table 4.

In addition, an enzyme related to the pyruvate-dependent aldolases has been described. The enzyme SanM from *Streptomyces ansochromogenes*, uses 2-oxobutyrate as the donor substrate. This results in the stereoselective formation of two new stereogenic centers, thus opening new opportunities in biocatalysis.[3,109]

2.1.2.1. N-Acetylneuraminic Acid (NeuAc) Aldolase (NeuA). NeuAc aldolase, also named sialic acid aldolase, catalyzes the reversible aldol reaction of *N*-acetyl-D-mannosamine (ManNAc) and pyruvate to form *N*-acetylneuraminic acid (NeuAc or sialic acid). This aldolase has been isolated from bacteria and mammals, being always type I enzyme that forms a Schiff-base/enamine intermediate with pyruvate and thus, promoting a *si*-face attack to the aldehyde resulting in the formation of a 4*S* stereocenter for the natural substrate. NeuAc aldolase is stable in solution at ambient temperature; it is commercially available from prokaryotes (*Clostridium* spp. and *E. coli*) and has been also cloned.[110,111]

The first step of the reaction catalyzed by NeuAc aldolase is the ring-opening of the α-anomer of sialic acid affording the open form, which in turn forms a Schiff base between the C2 carbonyl group and a lysine in the active site. Further interconversion Schiff base/enamine produces the retro-aldol reaction, releasing pyruvate and *N*-acetylmannosamine (which undergo ring-closure spontaneously).[112,113]

For synthetic purposes, the equilibrium of the pyruvate dependent aldolases is shifted toward the products through the use of excess pyruvate. Purification of the products can be most easily achieved by decomposing the excess pyruvate with Pyruvate decarboxylase.[21]

NeuAc aldolase accepts only pyruvate as donor substrate, with the exception of fluoropyruvate.[114] However, the enzyme displays a broad tolerance for acceptor aldehydes, including pentoses, hexoses of D and L series, aminosugars and neutral monosaccharides.[115−118] Also, disaccharides as acceptors have been used recently, producing disaccharides containing a sialic acid residue at the reducing end (Figure 11).[119,120]

Regarding the natural substrate, ManNAc, tolerated variations including epimerization, substitution, or deletion at positions C-4, C-5, and C-6. However, a free hydroxyl group at C-3 with the mannose configuration is required for aldol reaction. Epimerization at C-2 is restricted to small polar substituents due to a remarkable decrease of reaction rates.[121−125]

Unlike most aldolases, NeuAc aldolase appears to lack stereospecificity, and therefore, the stereochemical outcome of the catalyzed reactions depends on the structure of the acceptor

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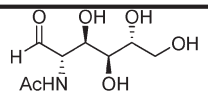
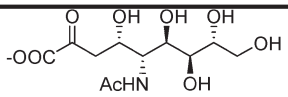
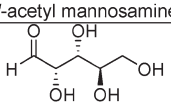
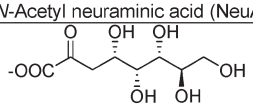
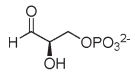
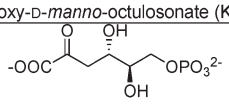
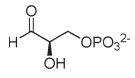
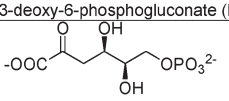
Table 4. Pyruvate-Dependent Aldolases of Synthetic Importance

Natural acceptor	Enzyme	Product
 *N*-acetyl mannosamine	*N*-Acetylneuramicic (NeuAc) aldolase (NeuA, EC 4.1.3.3)	 *N*-Acetyl neuraminic acid (NeuAc)
 D-arabinose	3-Deoxy-D-*manno*-octulosonate (KDO) aldolase (EC 4.1.2.23)	 3-Deoxy-D-*manno*-octulosonate (KDO)
 D-glyceraldehyde 3-phosphate (G3P)	2-Keto-3-deoxy-6-phosphogluconate (KDPG) aldolase (EC 4.1.2.14)	 2-Keto-3-deoxy-6-phosphogluconate (KDPG)
 D-glyceraldehyde 3-phosphate (G3P)	2-Keto-3-deoxy-6-phosphogalactonate (KDPGal) aldolase (EC 4.1.2.21)	 2-Keto-3-deoxy-6-phosphogalactonate (KDPGal)

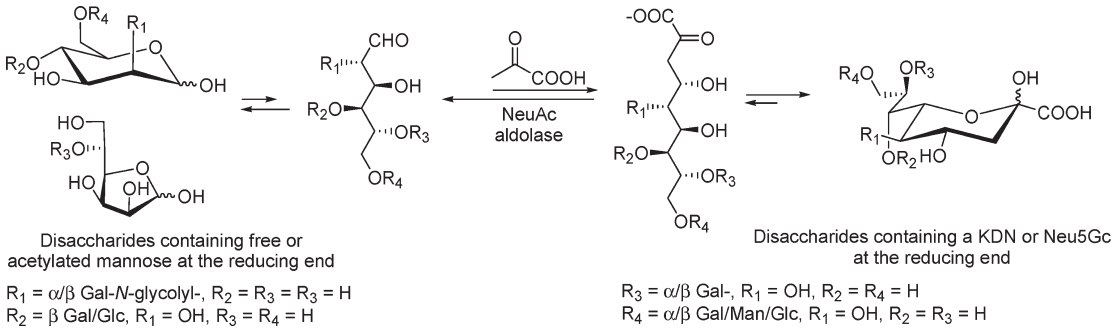


Figure 11. NeuAc aldolase catalyzed synthesis of disaccharides containing a sialic acid residue at the reducing end.

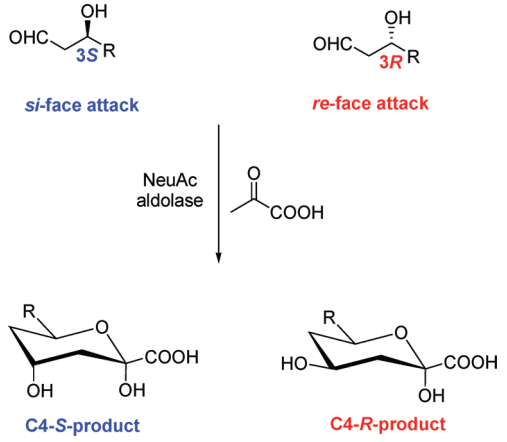


Figure 12. Stereochemical outcome of the reaction catalyzed by NeuAc aldolase.

aldehyde. With substrates with the natural *S* configuration at C-3, the carbonyl group is attacked from the *si*-face to form the new chiral center with *S* configuration. On the other side, substrates with the opposite configuration at C-3, lead to attack at the *re*-face, giving the *R* configuration at the new stereogenic center (Figure 12).[21,126]

Attack at *si*-face is proposed to be kinetically favored over attack at *re*-face. In all cases where *re*-face attack predominates, the resulting *R* product is thermodynamically favored with the newly formed stereocenter in the equatorial conformation.[127]

2.1.2.2. 3-Deoxy-D-manno-2-octulosonate (KDO) Aldolase and 3-Deoxy-D-manno-2-octulosonate-8-phosphate (KDOP) Synthetase. 3-Deoxy-D-*manno*-2-octulosonate (KDO) aldolase catalyzes the reversible condensation of pyruvate with D-arabinose to form KDO, being the enzyme responsible for the degradation of KDO in vivo.

The enzyme has been purified form bacterial sources and the *E. coli* KDO aldolase is commercially available.[128,129]

Like NeuAc aldolase, while is highly specific for pyruvate as donor substrate, KDO aldolase accepts several acceptor substrates, such as D-ribose, D-erythrose, D-lyxose, *N*-acetylmannosamine (this one at less than 5% of the natural substrate, D-arabinose), and also small substrates such as glyceraldehyde.[129]

So far, the main use of this enzyme is focused on the preparation of analogs of KDO (Figure 13).[129]

The stereochemical course of aldol reaction generally takes place at the *re*-face of the acceptor aldehyde, and therefore the newly formed stereocenter has the *S* configuration, which is complementary to that obtained with NeuAc aldolase. The enzyme is specific for substrates with the *R* configuration at C-3, and it seems to be a necessary (but not sufficient) requirement for the attack to take place. However, the stereochemical requirements at C-2 are less strict.[129]

The enzyme 3-deoxy-D-*manno*-2-octulosonate-8-phosphate (KDOP) synthetase catalyzes the irreversible condensation of

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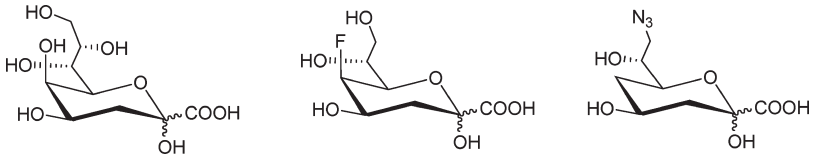

Figure 13. Analogs of KDO prepared using KDO aldolase.

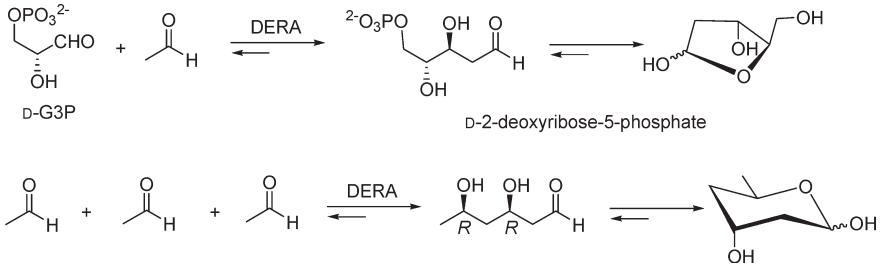

Figure 14. Aldol reactions catalyzed by DERA.

phosphoenolpyruvate and D-arabinose-5-phosphate to form KDOP. This enzyme has been isolated from bacterial sources, both as type I and II. Little is known about its substrate specificity, but preliminary results suggest that this enzyme is highly specific for its natural substrates.[130]

2.1.2.3. 2-Keto-3-deoxy-6-phosphogluconate (KDPG) aldolase and 2-Keto-3-deoxy-6-phosphogalactonate (KDPGal) Aldolase. 2-Keto-3-deoxy-6-phosphogluconate (KDPG) aldolase catalyzes the reversible condensation of pyruvate with D-G3P to produce KDPG, whereas 2-keto-3-deoxy-6-phosphogalactonate (KDPGal) aldolase catalyzes the same condensation to give KDPGal (the C-4 epimer of KDPG).

KDPG aldolases from microbial sources show high substrate specificity for the natural phosphorilated acceptor (D-G3P), but the enzyme tolerates a broad variety of substrates, though they are converted at reduced reaction rates.[131] Polar short chain aldehydes are substrates, including chloroacetaldehyde and pyridincarboxaldehydes, but simple aliphatic and aromatic aldehydes are not accepted.[132,133]

Unlike other pyruvate-dependent aldolases, KDPG aldolase operates under kinetic control, affording products possessing the *S* configuration at the C-4 with high stereoselectivity.[134,135]

KDPGal aldolase is a type I lyase with stereopreference for the 4*R* configuration, being complementary to KDPG aldolase. This enzyme accepts several non natural aldehydes, though at much lower rates (less than 5%) than the natural substrate. KDPGal aldolase, like KDPG aldolase, is highly selective for D-configuration at the C-2 of the acceptor.[136,137]

Noteworthy, the enzyme that does not use phosphorylated substrates, that is, 2-keto-3-deoxygluconate (KDG) aldolase, from the hyperthermophile *Sulfolobus solfataricus*, has no diastereoselectivity for the natural substrate. Therefore, when D-glyceraldehyde is the substrate, an epimeric mixture 1:1 on C-4, is produced.[138] However, using the acetonide derivative of glyceraldehyde, nearly complete diastereoselectivity for 4*S* configuration is obtained.[139,140]

2.1.3. Acetaldehyde-Dependent Aldolases. In the group of acetaldehyde-dependent aldolases only one enzyme is known, namely, 2-deoxyribose-5-phosphate aldolase (DERA, EC 4.1.2.4). DERA catalyzes the reversible aldol reaction of acetaldehyde and D-G3P to give 2-deoxyribose-5-phosphate, with an equilibrium constant for the synthesis of ~240 M^{-1} (Figure 14).[37] Therefore, this enzyme is special among aldolases in that it uses an aldehyde rather than a ketone as the natural donor. However, it is not unique, as far as FSA tolerates different acceptors, such as hydroxylated aldehydes of 2 or 3 carbon atoms.[102−104] Moreover, FSA uses glycolaldehyde as both the donor and the acceptor substrate.[32]

DERA is an acceptably stable type I aldolase isolated from animal tissues and microorganisms, and it is commercially available.[141−144]

The acceptor substrates are aldehydes of up to four carbon atoms, with little structural requirements. DERA tolerates unhindered aliphatic aldehydes, α-heteroatom-substituted aldehydes, and polyhydroxyaldehydes. 2-Hydroxyaldehydes are acceptably good acceptors, and the D-isomers are preferred over the L-isomers.[145] Even though the stereochemistry of the new chiral center is determined by the enzyme, giving in general the *S* configured product, reactions that lead to thermodynamically unfavorable structures may proceed with low stereoselectivity.[146]

Another distinctive feature compared to other aldolases described before is the fact that both substrates and the product are aldehydes. Due to this outcome, DERA can perform sequential aldol reactions. Starting from acetaldehyde as only substrate, a double aldolic reaction takes place to give (3*R*,5*R*)-2,4,6-trideoxyhexose. The reaction is driven by the formation of a stable hemiacetal, which precludes further condensation because it is not tolerated as acceptor substrate. This sequential aldol reaction represents a short and straightforward route to complex molecules from inexpensive and achiral starting materials.[146−148]

2.1.4. Glycine-Dependent Aldolases. The fourth group of aldolases comprises the glycine-dependent aldolases, which form β-hydroxy-α-amino-acids. These enzymes work with pyridoxal phosphate as cofactor and are classified as serine hydroxymethyltransferase (SHMT) or threonine aldolases (ThrA). Both enzymes catalyze the reversible aldol reaction yielding glycine and an aldehyde (Table 5).

There are four variants for Thr aldolase, two selective for threonine (D- and L-selective) and the other two selective for *allo*-threonine (D and L-selective). Whereas SHMT has a biosynthetical function (to transfer a formaldehyde equivalent onto glycine), Thr aldolases catalyze the degradation of threonine.[149]

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Table 5. Glycine-Dependent Aldolases

Natural acceptor	Enzyme	Product
formaldehyde	Serine hydroxymethyltransferase (SHMT) (EC 2.1.2.1)	L-Serine
acetaldehyde	L-Threonine (Thr) aldolase (ThrA, EC 4.1.2.5)	L-Threonine
acetaldehyde	D-Threonine (Thr) aldolase (ThrA, EC 4.1.2.42)	D-Threonine
acetaldehyde	L-*allo*-Threonine (Thr) aldolase (ThrA, EC 4.1.2.6)	L-*allo*-Threonine
acetaldehyde	D-*allo*-Threonine (Thr) aldolase (ThrA)	D-*allo*-Threonine

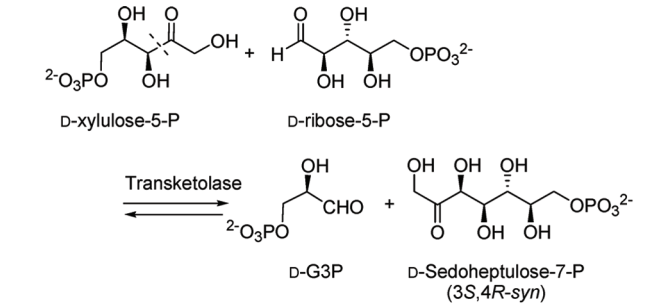
Typically, Thr aldolases display complete stereoespecificity when forming the new α-amino stereocenter (either D- or L-), but the selectivity is lower for the relative *threo*/*erythro* configuration, corresponding to threonine and *allo*-threonine.[150−154] Similarly, SHMT displays high selectivity for the L-configuration but has poor *threo*/*erythro* selectivity.[155]

For synthetic applications, it is necessary to work with an excess of glycine, to compensate the unfavorable equilibrium constant. Enzymes belonging to this group tolerate different aldehyde acceptors, such as aliphatic aldehydes (up to ten carbon atoms) and aromatic, but not α,β-unsaturated.[150,152,155−157]

2.1.5. Transketolase. Transketolase (TK, EC 2.2.1.1) catalyzes the reversible transfer of a hydroxyacetyl group among a number of phosphorylated sugars, using thiamine diphosphate (ThDP) and Mg^{2+} (or Ca^{2+}) as cofactors.[1,33,158−168] The enzyme is a transferase with homodimeric structure, molecular weight of 74 kD,[169] and two active sites located at the interface between the contacting monomers.[170] Its role in nature, as a key enzyme in the pentose-phosphate pathway, is the transfer of a 2-C hydroxyacetyl fragment from a ketose-phosphate (D-xylulose-5-phosphate, D-X5P) to an aldose phosphate (D-ribose-5-phosphate, D-R5P), thus generating D-sedoheptulose-7-phosphate and G3P (Figure 15). Although transketolase is not a lyase, the enzyme catalyzes C−C bond formation giving rise to products similar to those obtained from aldolase-mediated reactions. These reactions differ only in the nature of the donor substrate and the required cofactors.[10]

Although the activation mechanism of ThDP-dependent enzymes is well-known,[162,171] and the enamine character of the key intermediate was proven spectroscopically,[172] detailed research on the topic is in progress.[164,165,173−180]

Transketolase was identified for the first time by Racker et al.,[181,182] in *S. cerevisiae*, and subsequently found in a wide variety of sources such as other yeasts,[183,184] spinach,[185−187] wheat leaves,[186] and also mammalian cells, such as rat liver cells,[188] or human leukocytes.[189] TK from *E. coli* was also cloned and overexpressed in recombinant *E. coli* strains, and it is easily available for preparative scale reactions.[190−192] The first thiamine



ThDP-mediated activation in TK catalytic mechanism

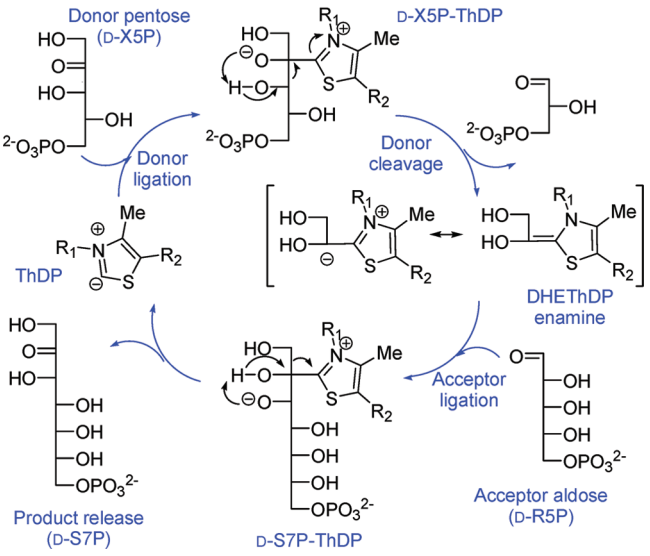


Figure 15. Ketol-transfer reaction in the oxidative pentose phosphate pathway. ThDP-mediated activation in TK catalytic mechanism.

diphosphate-dependent enzyme for which the three-dimensional structure was determined was TK from *S. cerevisiae*,[170,193,194] followed by TK from *E. coli*.[195] Later on, crystal structures of the

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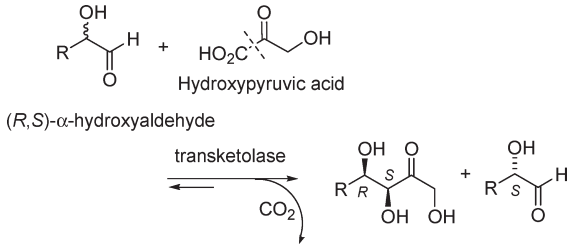

Figure 16. Kinetic resolution of racemic α-hydroxyaldehydes catalyzed by transketolase.

key intermediates in thiamine catalysis showing ThDP bound at the active site of the enzyme were determined as well.[160,163,167,196]

The enzyme has complete specificity for the newly formed stereocenter, that always has *S* configuration, resulting from the attack on the *re*-face of the aldehydes.[197−201] The enzyme has broad substrate specificity.[178] The donor function can be performed by xylulose-5-phosphate, sedoheptulose-7-phosphate, fructose-6-phosphate and hydroxypyruvate, among others. The use of hydroxypyruvate is preferred for synthetic applications, because the reaction becomes irreversible by the effective removal of the produced CO_2.[202] The best acceptor substrates are α-hydroxyaldehydes and aldoses, but the enzyme tolerates other aldehydes, including aromatics and α,β-unsaturated ones,[26,35,54,97,199,203−219] thus leading to an increased interest in industrial applications.

It is noteworthy that TK is more diastereoselective than many aldolases, accepting only α-hydroxyaldehydes with 2*R* configuration as substrates. Then, the enzyme allows to carry out kinetic resolutions of racemic mixtures of aldehydes, which can be used as (cheap) starting materials for enantiomeric synthesis.[207,208] When racemic α-hydroxyaldehydes are used, only the α-(*R*)-enantiomer is converted into the ketotriol with D-*syn* configuration (corresponding to the (3*S*,4*R*) configuration in the product), and the α-(*S*) aldehydes remain unchanged (Figure 16).

Substrate specificity of transketolase has been successfully modified from the decade of 1990 for synthetic purposes.[33,215,220−226] Recently, Hailes and co-workers have explored a series of linear and cyclic aliphatic aldehydes as substrates of genetically modified transketolase from *E. coli*, yielding high conversions and excellent stereoselectivities.[219] Although TK activity toward nonhydroxylated aliphatic aldehydes is very low,[205,209,210] the authors developed various mutants through active-site targeted saturation mutagenesis,[221] improving the desired activity. They could identify a novel TK mutant, Asp469Thr, with a nearly 5-fold increased specific activity when screened toward propionaldehyde as acceptor substrate, for the production of 1,3-dihydroxypentan-2-one.

From a synthetic viewpoint, the enzymes TK and FruA, such as RAMA, are complementary tools for the enzymatic synthesis of monosaccharides and analogs,[227] since the same type of products can be obtained from both enzymes working on different substrates. Howbeit, Kobori and co-workers stated that TK presents advantages in comparison with RAMA.[207] An aspect to take into account is the stability of both enzymes. TK from *E. coli* is more stable than the commercially available RAMA, which looses completely its activity within two days.[53] Thus, TK could become an adequate alternative to FBP aldolase as catalyst in chemoenzymatic processes.

2.2. Synthetic Applications

In Nature, aldolases are among the most important biocatalysts for C−C bond formation and are found in many biosynthetic pathways of carbohydrates, keto acids, and some amino acids.[15]

Since these enzymes have evolved to catalyze the metabolism and catabolism of highly oxygenated metabolites, they are ideally suited for the preparation of these types of compounds, which can be conducted with high stereoselectivity and, as stated before, without using protective groups. Moreover, these enzymes have also been used for the stereoselective preparation of other types of polyoxygenated compounds, such as hydroxylated carbocycles and natural products.

During the past decade, many applications of aldolases in stereoselective synthesis have been compiled in several reviews.[2,3,10,14,21,23−25,33,36,97,149,227−233] The number of known aldolases, added to their easier availability, has contributed to keep a steady flow of research in the area. This section highlights the use of aldolases (and transketolase) for preparative asymmetric synthesis. In an attempt to emphasize the preparative applications, it has been organized by type of compounds instead of describing each enzyme in particular.

2.2.1. Preparation of Monosaccharides. The most studied group of aldolases is, arguably, the group of the DHAP-dependent aldolases. Several DHAP-dependent enzymes are known, allowing for the preparation of each one of the four configurations of the two newly formed stereogenic centers (see Figure 2).

Within this group, FBP aldolase occupies a prominent position, given its broad substrate tolerance and the high stereoselectivity of its reactions. The main synthetic use is centered on the preparation of carbohydrates, where the utility of aldolases has been amply demonstrated by the extensive work of several research groups. More than 100 aldehydes have been used as acceptors for DHAP-dependent aldolases to prepare monosaccharides.[27,35,53] These aldehydes can be used in enantiopure form or as racemates, in case the enantiomer selectivity of the DHAP-dependent enzyme allows for kinetic resolution to take place. Typical applications of the DHAP aldolases include the synthesis of monosaccharides and derivatives from suitable functionalized aldehyde precursors. The ketose 1-phosphates produced are subjected to enzymatic dephosphorylation to produce the free ketosugars. Aldoses, in turn, can be obtained from them by chemical or enzymatic (using ketol isomerases) means. The synthetic applicability of this strategy using DHAP-dependent aldolases is documented in several recent reviews.[2,25,33]

Recent work in monosaccharide synthesis using these aldolases is directed mainly to the improvement of their practical preparative application, instead of preparing new products. In this regard, a chimeric enzyme containing both a DHA kinase and a DHAP-dependent FruA has been shown to be more efficient than the one-pot use of both individual enzymes.[234,235] Studies on DHAP stability showed the convenience of working at 4 °C as a good compromise between residual aldolase activity and DHAP degradation rate.[236]

The use of a multienzyme system can alter the substrate tolerance of DHAP-dependent aldolases. In particular (*E*)-α,β-unsaturated aldehydes bearing a β-electron-withdrawing group, previously reported as not accepted by RAMA, are good acceptors for RAMA in the context of a multienzyme system, giving excellent D-*threo* enantioselectivity and good conversion. The

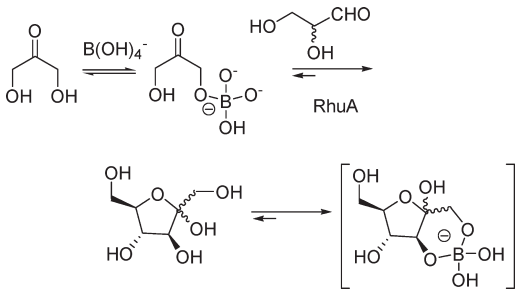

Figure 17. RhuA-catalyzed aldol addition of DHA in the presence of borate buffer.

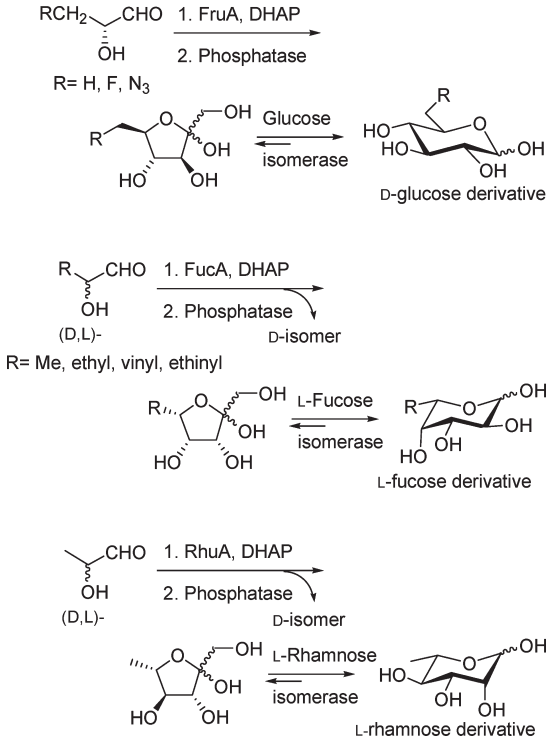

Figure 18. Synthesis of monosaccharide analogs by aldolization-ketol isomerization, including kinetic resolution of racemic aldehyde precursors.

system is composed of recombinant Dihydroxyacetone kinase (DHAK) from *Citrobacter freundii*, Fructose 1,6-biphosphate aldolase from rabbit muscle (RAMA) and Acetate kinase (AK) for adenosine triphosphate (ATP) regeneration. When using RhuA and FucA, the conversion and selectivities are sharply diminished.[237]

In an effort to circumvent the strict dependence on phosphorylated donor substrates, Wong reported that the DHAP-dependent L-Rhamnulose 1-phosphate aldolase (RhuA) can accept DHA at reasonable rates when reactions are conducted in borate buffer at 200 mM.[100] The enzyme used was a type II aldolase from *E. coli*. Presumably, DHA-borate ester is formed in situ and converted by the enzyme as a DHAP mimic. It seems that under the reaction conditions the product is further trapped because of an ensuing formation of vicinal borate diesters, thus shifting the reaction equilibrium toward the aldol product. In this way, L-fructose was prepared from racemic glyceraldehyde and DHA in the presence of RhuA and borate in 92% yield (based on L-glyceraldehyde) on a gram scale (Figure 17).[100] The procedure is a one-step operation in which the borate esters are formed in situ and hydrolyzed during the workup. Despite its simplicity, however, the use of borate buffers has not been widespread.

Work with DHAP-dependent aldolases about monosaccharides was related to the preparation of analogs, for instance the synthesis of hydrophobic analogs of ketoses modified at the terminal carbon, which was conducted using suitably substituted hydroxyaldehydes.[238] For L-fucose analogs, further biocatalytic isomerization with L-fucose isomerase afforded the corresponding aldose analogs (Figure 18).[75] This is an example of a general strategy, first described by Wong, to make aldose derivatives from the corresponding ketoses, using ketol isomerases coupled to the first enzymatic reaction.[239] Commercially available glucose isomerase catalyzes the isomerization of fructose and fructose analogs to glucose. Aldose analogs, such as 6-deoxy-, 6-fluoro-, and 6-azidoglucose have been prepared using this methodology, but the reaction is not general and no reaction is obtained with ketose analogs modified on C-5.[239] A more relaxed substrate tolerance is shown by the stereochemically complementary L-rhamnose (RhaI; EC 5.3.1.14) and L-fucose (FucI; EC 5.3.1.3) isomerases from *E. coli*.[240] Both enzymes accept substrates having a (3*R*)−OH configuration and thus, the ketose products resulting from RhuA and FucA catalyzed aldolization (both possessing (3*R*) configuration) can be converted by the isomerases to the corresponding aldose isomers.[35]

An important enzyme for the preparation of monosaccharides and analogs is Transketolase (TK),[227] which produces the same products as RAMA, but working on substrates of different length. While FruA creates two new stereogenic centers, only one is generated by the reaction of TK. However, the enantioselectivity of TK toward C-2 produces only D-*threo*-(3*S*,4*R*)-ketoses, equivalent to the product from FruA (Figure 19). This transfer of a 2-C hydroxyacetyl unit is chemically equivalent to the 3-C elongation in the FBP-catalyzed aldolization. In synthetic applications, the substitution of the phosphorylated donor by hydroxypyruvate is advisible, since the equilibrium is irreversibly shifted toward the products upon decarboxylation.

Moreover, TK is able to form directly unphosphorylated sugars; whereas a second enzymatic dephosphorylation step using phosphatases is needed when using RAMA. In addition, the catalytic potential of Transketolase is shown by the preparation of compounds that cannot be obtained with RAMA. As an example, compound (**1**) can be synthesized from (**2**) through a TK-catalyzed reaction, but not from (**3**) through a RAMA-catalyzed aldolization (Figure 19).[207]

Among the examples of synthetic applications of transketolase,[199,241] the preparation of fructose analogues, as well as other monosaccharides or their derivatives through TK-catalyzed reactions is outstanding.[200,204,211,213,216,217,242−250] In a number of these examples, the synthetic procedures were carried out using racemic 2-hydroxyaldehydes as donors, taking advantages of the discrimination ability of the enzyme, and rendering the overall condensation reaction irreversible by using hydroxypyruvate as donor substrate.

When comparing RAMA with TK, the former shows high specificity toward the donor substrates, but is more tolerant toward the acceptor aldehydes instead of its natural substrate, D-G3P, and has been used in a number of chemoenzymatic syntheses of natural compounds.

Transketolase can also be immobilized, so more efficient approaches can be achieved through high-throughput TK-catalyzed

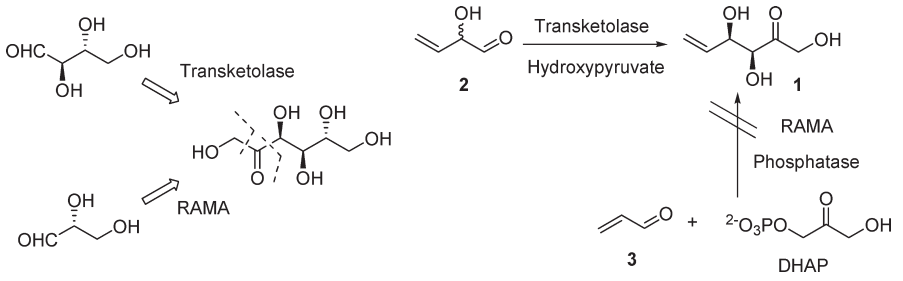

Figure 19. Different scopes in the catalytic activity of TK and RAMA.

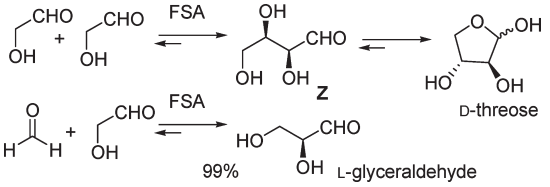

Figure 20. FSA-catalyzed aldol reactions using glycolaldehyde as the donor substrate.

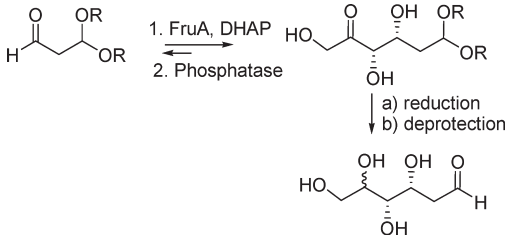

Figure 21. Inversion strategy for the synthesis of sugars.

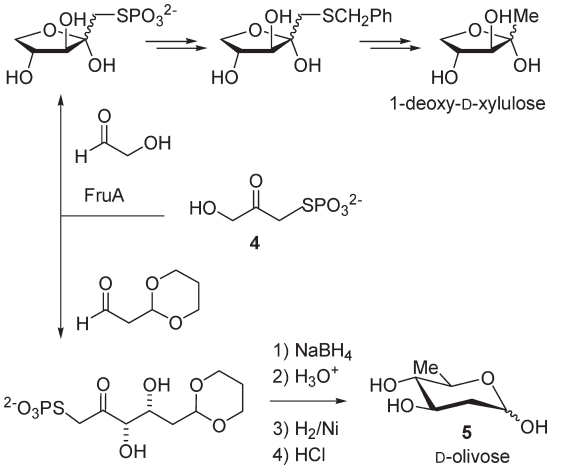

Figure 22. Synthesis of deoxysugars using a thio analog of DHAP.

bioprocesses. In this regard, Baganz and co-workers described the design of an immobilized enzyme microreactor based on a 200-μm ID fused silica capillary for quantitative kinetic analysis.[251] Continuous TK-catalyzed production of L-erythrulose from hydroxypyruvate and glycolaldehyde was achieved as well using a membrane reactor, with high conversion, stable operational points and good productivity.[252]

Another enzyme used in the preparation of monosaccharides is D-Fructose-6-phosphate aldolase (FSA). This enzyme, first reported from the *E. coli* genome,[31] is a highly promising biocatalyst which readily accepts unphosphorylated DHA and also some monohydroxylated ketones and glycolaldehyde as donors.[32] The ability of FSA to accept glycolaldehyde as alternative donor substrate is unprecedented and unexpected, and only the behavior of 2-deoxyribose-5-phosphate aldolase (DERA) could be related. This activity makes possible the enzymatic cross-aldol addition reactions of glycolaldehyde to aldehydes, thus offering new promising biocatalytic strategies for the direct and stereoselective synthesis of aldoses (instead of ketoses derived from usual ketone donors) and related complex structures (Figure 20). In particular, glycolaldehyde acts as both the donor and acceptor substrate for FSA, giving a (2*S*,3*R*)-*syn* selectivity, in agreement with previous work with other FSA-catalyzed aldol additions.[32] In addition to aldoses, the use of *N*-protected aldehydes gives access to aza sugars (see section 2.2.4).

2.2.2. Preparation of Deoxysugars. Deoxysugars have been prepared by different groups of aldolases. In particular, DHAP-dependent aldolases were used for the synthesis of ketose sugars with a deoxy carbon at C-5 or higher, using appropriate aldehydes in the aldol reaction. In turn, by using a monoprotected dialdehyde 2-deoxyaldohexoses were also prepared. In this strategy, the ω-carbon of the newly formed ketose becomes C-1 after stereoselective reduction of the ketone and further deprotection of the masked aldehyde to give the aldose (Figure 21).[253,254]

Another route to prepare deoxysugars used a phosphorothioate analog of DHAP, (**4**), which acts as substrate of FBP aldolase. Reactions with this 1-thio analog of DHAP permit added variability of functionality at a position that always bears a hydroxyl group when DHAP is used as the donor substrate. Ready desulfurization with Raney nickel provided access to deoxy sugars. In this way 1-deoxy-1-thioketoses, 1-deoxyhexoses, and 2,6-dideoxyaldohexoses like D-olivose, (**5**), were made available through enzymatic methods (Figure 22).[254−256]

Other enzymes used in the preparation of deoxysugars include D-fructose-6-phosphate aldolase (FSA), a class I aldolase from *E. coli* related to a novel group of bacterial transaldolases that catalyzes the aldol addition of DHA to glyceraldehyde-3-phosphate. FSA is ideally suited for the synthesis of deoxysugars, since it tolerates hydroxyacetone as an aldol nucleophile, giving rise to 1-deoxysugars directly.[102] In addition to simple carbonyl compounds,[32] it was also observed that small monosaccharides like D-threose are good acceptors for FSA, giving 1-deoxy-D-*ido*-hept-2-ulose (**6**) in 58% isolated yield when using hydroxyacetone as donor, thus opening new synthetic possibilities for the preparation of complex polyhydroxylated structures (Figure 23). Interestingly, no reaction was detected with DHA.[104]

Considering the type of donor, acetaldehyde-dependent aldolases are highly suited for the preparation of deoxysugars. The 2-deoxy-D-ribose-5-phosphate aldolase (DERA, EC 4.1.2.4) is

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the only representative of this group and its use in the synthesis of different types of deoxysugars, including 2-deoxy, dideoxy, trideoxy, thio, and aza sugars is well documented.[145,148,257] More recently, labeled deoxysugars ([3,4-$^{13}C_2$]-2-deoxyribose-5-phosphate) have also been prepared using labeled acceptors.[258]

Preparatively, the most attractive feature of DERA is its ability to catalyze sequential aldol additions because the aldol product is also an aldehyde, which can be accepted by the enzyme (cross-aldol). When acetaldehyde is used as the only substrate, both self-aldol and cross-aldol reaction take place to give (3*R*,5*R*)-2,4,6-trideoxyhexose (**7**) in good yield (Figure 24).[146−148] The tandem addition of two equivalents of acetaldehyde to one equivalent of chloroacetaldehyde (a particularly good acceptor) has attracted much attention since the resulting (3*R*,5*S*)-6-chloro-2,4,6-trideoxyhexose (occurring mainly as the hemiacetal (**8**) is a chiral precursor for the side chain of statins, a group of cholesterol-lowering drugs related to compactin.[259] The DERA-catalyzed reaction has recently been developed into an industrial process for the large-scale manufacture of the side chain of these drugs.[260−262]

2.2.3. Preparation of Thiosugars. In a classical procedure, thiosugars have been prepared by reaction of 2- and 3-thiolated aldehydes with different DHAP-dependent aldolases (Figure 25).[263−265] In addition, reduction with triethylsilane in the presence of boron trifluoride etherate gives the corresponding 1-deoxythioaldose.

However, because of the low tolerance of these enzymes to thiolated aldehydes, it is usually preferred to conduct the reaction with a halogenated aldehyde and then convert the product to a thiol by substitution.[227] Also, the resulting thio ketose can be enzymatically isomerized to a ω-thioaldose (Figure 26).[246] The thio aldose (**10**) is an inhibitor of β-D-xylosidase.

2.2.4. Preparation of Iminosugars (Azasugars, Including Pyrrolizidine and Indolizidine Alkaloids). Iminocyclitols (or iminosugars) enjoy considerable attention since many of them can act as inhibitors of glycosidases, glycosyltransferases, or metalloproteinases, and thus possess therapeutic potential for a number of diseases related to glycosylation disorders.[266] In order to prepare them, a suitable synthetic equivalent of amino aldehydes is needed. From an enzymatic standpoint, azido aldehydes are well accepted by many aldolases giving synthetically useful yields.[267] Thus, they have been regularly used for the preparation of an ample array of aza sugars, namely, 1-deoxy azasugars of the nojirimycin type, aza-analogs of acetylglucosamine, polyhydroxylated pyrrolidine, piperidine, and azepanes, and recent accounts are available.[144,232] The developed strategy includes a stereoselective aldol addition mediated by DHAP aldolases followed by azide hydrogenation with concomitant intramolecular reductive amination.[144] The reductive amination with Pd/C is highly diastereoselective, hydrogen being added to the face opposite to the newly formed hydroxyl group originated from the aldehyde (C-4), regardless of the relative stereochemistry of the other substituents. Hence, the stereochemistry observed at C-2 is controlled exclusively by the configuration at C-4.[268] The cyclization produces a hydroxymethyl branched iminocyclitol. Alternatively, the use of ketol isomerases allows for the preparation of nonbranched compounds by subsequent ring expansion by one carbon atom. Using different DHAP-dependent aldolases, diastereomeric piperidine- and pyrrolidine derived iminocyclitol were prepared in good yields.[144,269,270] Using 3-azido aldehydes and ketol isomerases, seven-membered ring are obtained, as exemplified in the preparation of 3,4,5,6-tetrahydroxyazepane (**15**).[271] Representative examples are shown (Figure 26).

Taking advantage of the ability of DHAP-dependent RhuA to accept DHA at reasonable rates when reactions are conducted in borate buffer at 200 mM, several L-iminocyclitols with (3*S*,4*S*)-*syn* selectivity were prepared in a concise procedure, avoiding the enzymatic dephosphorylation step. In the case of α-azidoaldehydes, RhuA preferentially accepts the D-isomer of the aldehyde over the L-isomer (in a ratio dependent on the substitution on C-β) (Figure 27).[100]

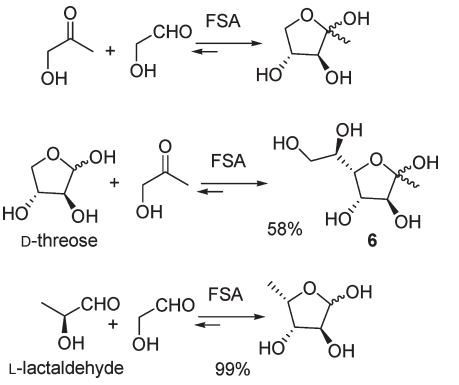

Figure 23. FSA-catalyzed preparation of deoxysugars.

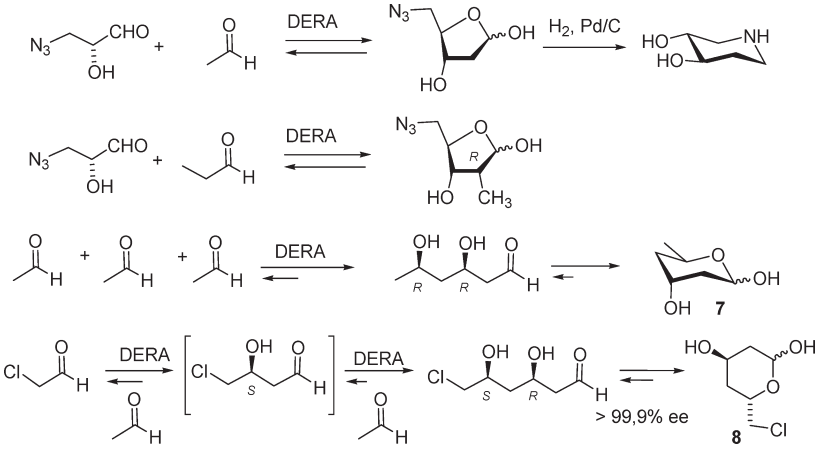

Figure 24. DERA-catalyzed preparation of deoxysugars.

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Even though azido aldehydes are good acceptors for aldolases, their limited commercial availability, toxicity, and low stability, have enabled other synthetic equivalents to be tested to this end. In particular several *N*-protected amino aldehydes have been used in the aldolization, and best results were obtained for the benzyloxycarbonyl (Cbz) group. Potential problems derived from the poor water solubility of these derivatives were solved by using a water-in-oil emulsion, where the rate and solubility of the reactants are good, without loss of enzymatic activity.[74,232,268] The strategy using *N*-Cbz amino aldehydes is the same as for azido aldehydes; thus the aldol adducts from the enzymatic addition are first dephosphorylated and then hydrogenated over Pd/C. Under these conditions the Cbz group removal and the reductive amination take place in one pot, the iminosugars being obtained upon lyophilization. As stated previously, the delivery of hydrogen occurs from the face opposite to the C-4 hydroxyl group. With the protected aldehydes derived from 3-aminopropanal, glycine and both enantiomers of alanine, the DHAP-dependent aldolases RAMA, RhuA and FucA (both derived from *E. coli*) gave the corresponding iminocyclitols in good yields (Figure 28).[73,74] RAMA was the most stereoselective, giving single diastereomers of the polyhydroxylated pyrrolidines ((3*R*,4*R*)-*syn*). Interestingly, RhuA gave a single diastereomer from (*S*)-Cbz-alaninal, whereas only 34% de was obtained from the enantiomer. FucA was much less sensitive to the configuration of the protected alaninal, giving the configuration (3*S*,4*R*)-*anti* in both instances.

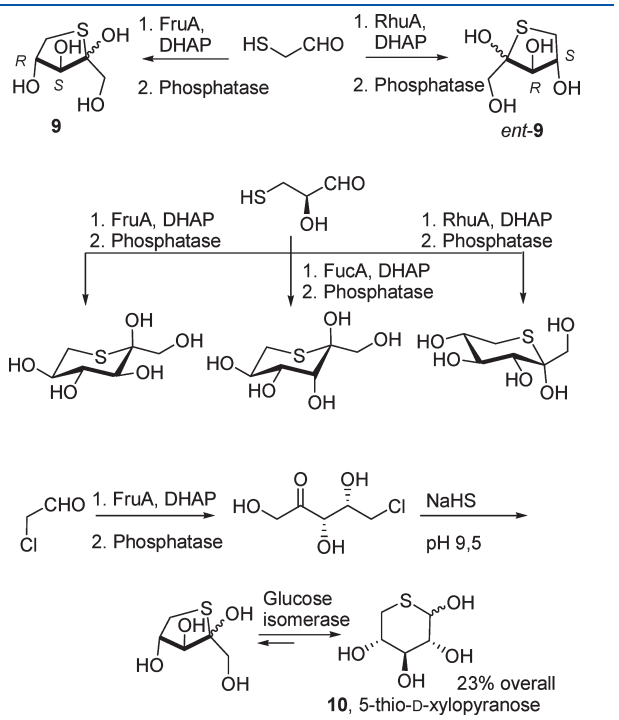

Figure 25. Synthesis of thio sugars.

The substrate tolerance of these enzymes was further investigated, using linear, and branched substituents on the α-carbon (Figure 28).[272] Type II aldolases were more versatile than RAMA, but whereas RhuA accepted both linear and branched substituents on C-2, FucA tolerated only linear substrates. Conversely, FucA was the most stereoselective biocatalyst (90−100% (3*S*, 4*R*)-*anti*-adducts). Some of these derivatives proved good inhibitors for α-L-fucosidase and α-L-rhamnosidase. A thorough kinetic modeling of the FucA-mediated aldol addition of (*S*)-Cbz-alaninal to DHAP revealed that DHAP is also degraded by type II aldolases and this reaction can be minimized by operating a 4 °C, leading to higher conversions.[236,273]

A particular class of iminocyclitols is constituted by polyhydroxylated pyrrolizidine alkaloids. Some of them have been prepared using DHAP-dependent aldolases. For instance, (−)-hyacinthacine A_2 has been prepared by a concise two-step aldol

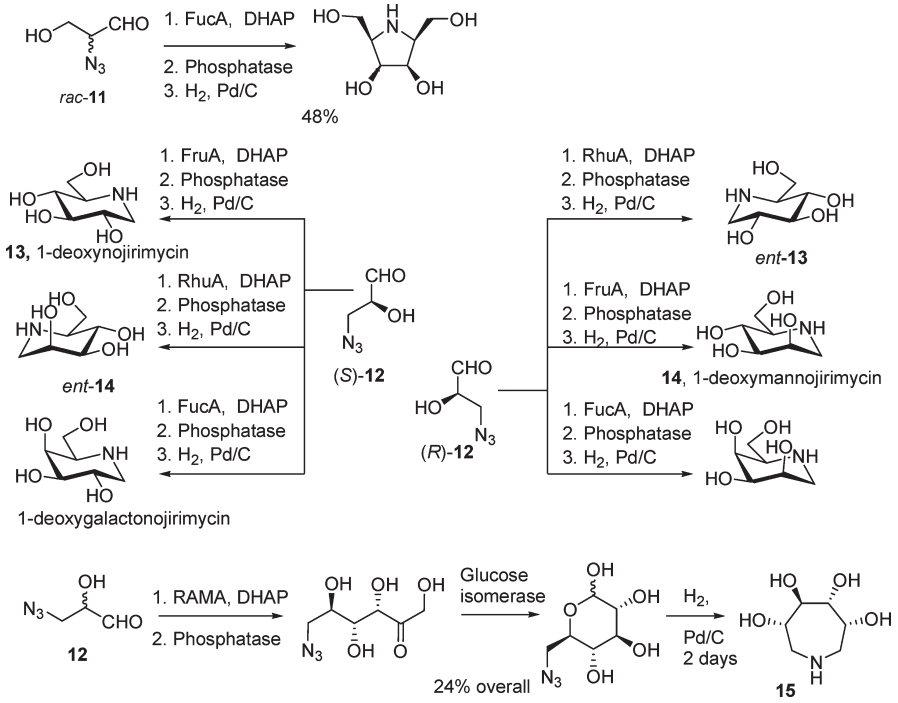

Figure 26. Synthesis of five-, six-, and seven-membered iminocyclitols from azido aldehydes.

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addition of DHAP to *N*-Cbz-protected prolinal, catalyzed by RhuA from *E. coli*, followed by a reductive amination (Figure 29).[274] As noted previously with this enzyme, the stereoselectivity on C-4 is dependent on the configuration of the starting *N*-protected aldehyde,[275] giving better selectivity for the (*S*)-prolinal (**16**). These aldehydes were not accepted by RAMA or FucA. Regarding the product, it is interesting to note that (−)-hyacinthacine A_2 is a good α-D-glucosidase inhibitor whereas the natural (+)-isomer is not. However, linear substrates were accepted by FBP-aldolase to furnish australine, (**17**), and 3-epiaustraline. The bicyclic core resulted from a double reductive amination on the former DHAP ketone and on an aldehyde masked as a vinyl group in the linear acceptor (Figure 29).[276]

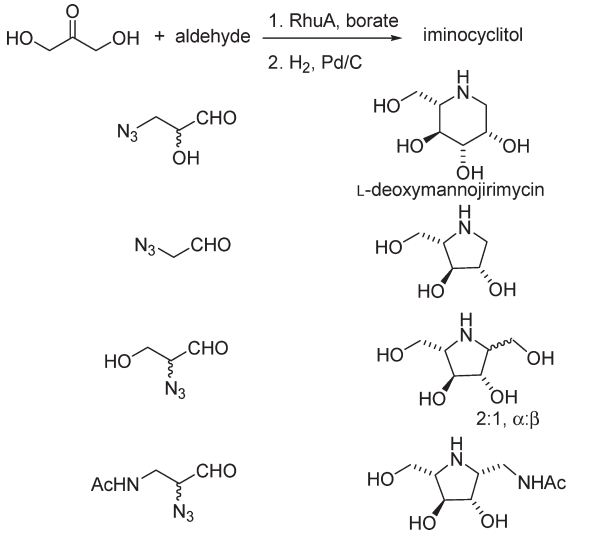

Figure 27. Synthesis of L-iminocyclitols using borate as a phosphate ester mimic.

Other enzymes have been very successful in the preparation of iminosugars, namely, D-Fructose-6-phosphate aldolase (FSA) and DERA, and even TK, pyruvate, and glycine aldolases. Compared to the DHAP-dependent aldolases, the use of FSA offers the advantage of working with nonphosphorylated substrates on a similar set of acceptors. The first example of the use of FSA in iminosugar synthesis was a straightforward procedure for the stereoselective synthesis of D-fagomine (**18**), a piperidine-type iminosugar first isolated from buckwheat seeds of *Fagopyrum esculentum* Moench. The strategy included the stereoselective aldol addition of DHA to *N*-Cbz-3-amino propanal followed by selective catalytic reductive amination to afford the iminosugar in an 89% isolated yield (Figure 30).[103] Further examples using azido and *N*-Cbz aldehydes as acceptors and DHA, hydroxyacetone and 1-hydroxybutanone as donors have been recently reported. The efficiency of the procedure is reflected in the high overall yields obtained for the preparation of the inhibitors Miglustat and Miglitol, 30% and 33%, respectively from the *N*-Cbz-protected aldehyde (Figure 31).[104,106] Unbranched piperidine derivatives were obtained when using glycolaldehyde as the donor substrate.[32]

Transketolase was used in the early 2000 for the preparation of effective glycosidase-inhibitors with iminosugar structure. The TK-catalyzed reaction of *rac*-3-azido- and 3-benzyloxy glyceraldehyde with hydroxypyruvate gave the corresponding ketose derivatives which, upon hydrogenation, afforded the hydroxylated pyrrolidines (Figure 31).[214]

The use of DERA in the preparation of iminocyclitols was reported some years ago, using (*S*)-3-azido-2-hydroxypropanal as acceptor and either acetaldehyde or acetone as donors, affording the iminocyclitols in 74% and 61% yield, respectively. Other compounds gave lower yields.[145]

By that time, a derivative of the indolizidine alkaloid castanospermine was prepared using NeuA-catalyzed aldol reaction of pyruvate with *N*-Cbz-D-mannosamine (**19**) as a key step. Intramolecular reductive amination of the deprotected aldolase product gave a pyrrolidine, which was then converted to the

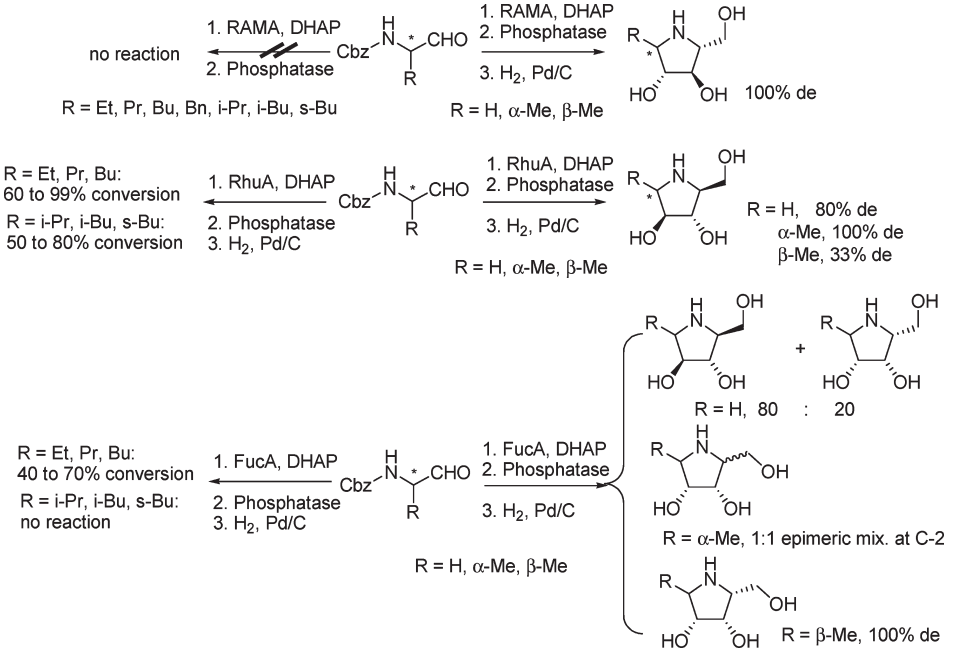

Figure 28. Synthesis of iminocyclitols from Cbz-*N*-protected aldehydes.

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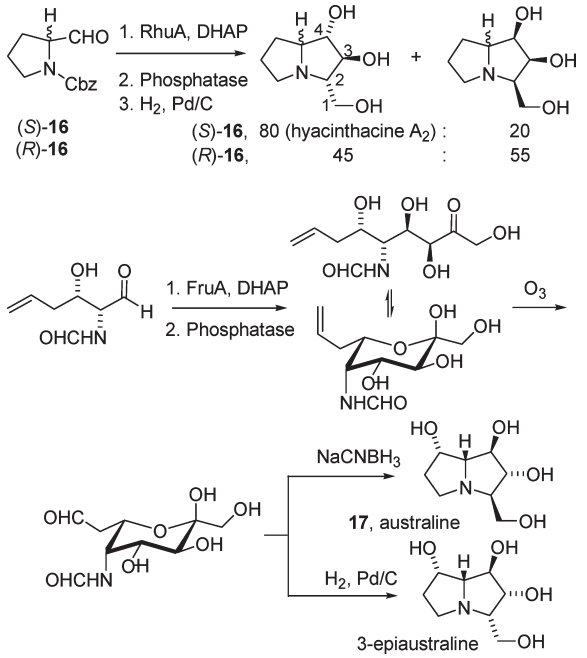

Figure 29. DHAP-dependent aldolase mediated synthesis of pyrrolizidine alkaloids.

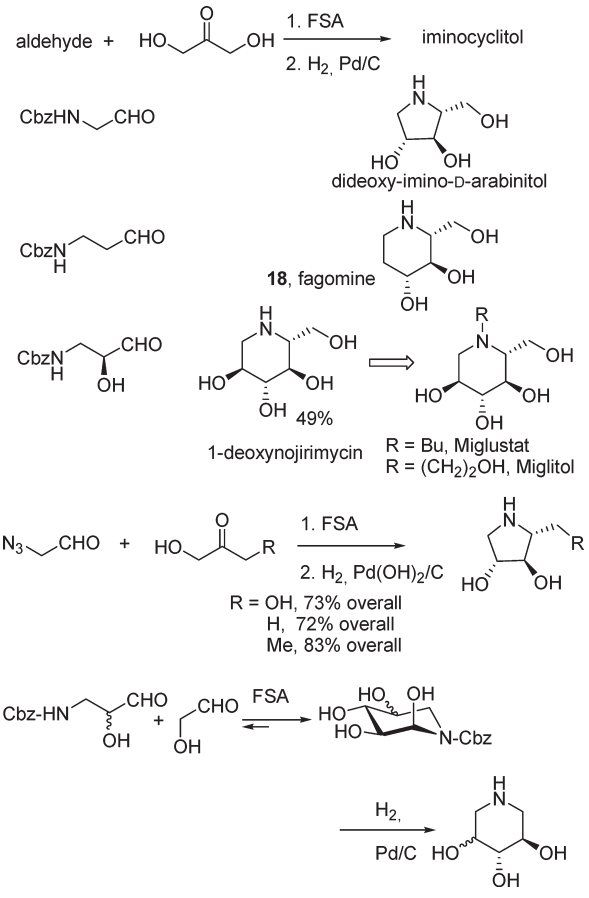

Figure 30. FSA-mediated synthesis of iminocyclitols.

iminocyclitol derivative 3-(hydroxymethyl)-6-epicastanospermine (Figure 32).[277]

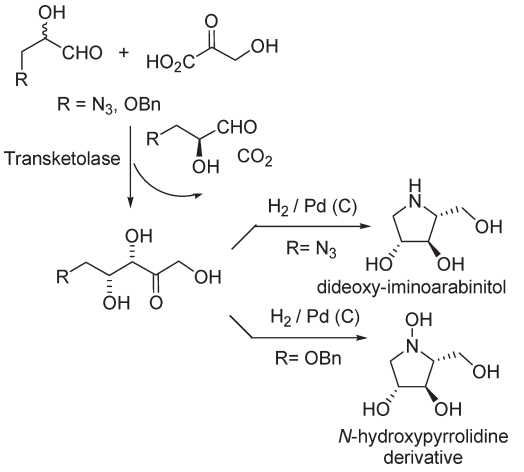

Figure 31. TK-mediated synthesis of iminocyclitols.

Some deoxyiminosugars have been prepared by chemical elaboration of β-hydroxy-α-aminoacids obtained through enzymatic means using glycine-dependent enzymes. Recent examples include iminodigitoxose and deoxyimino-digitoxose (for details, see the corresponding section).[278]

2.2.5. Preparation of Phosphonated Sugars. Phosphonate analogs, resulting from the formal replacement of the P−O bond of a phosphate by a P−C bond, have attracted attention because of their stability toward phosphatase degradation, which makes them good candidates as potential inhibitors or regulators of metabolic processes. The phosphonate group can be supplied by the aldehyde acceptor, giving ω-phosphonates or, alternatively, is introduced by the DHAP bioisoster (**20**), giving 1-phosphonates.[279] Such isosteric replacement of the phosphate ester oxygen by a methylene group is usually well tolerated by type I and type II aldolases, and only some specific enzymes failed to accept the less polar phosphonate (**20**) (Figure 33).[280,281]

Also, iminocyclitols possessing a phosphonic acid moiety were prepared using DHAP-dependent aldolases and linear phosphonate-containing azido aldehydes. Following the aldol reaction performed with FBP aldolase and FucA, the resulting azido ketone was hydrogenated over Pd/C to produce the iminocyclitols (Figure 34).[282]

Other aza sugar phosphonic acids of the pyrrolidine type resulted from the reaction of 2-azidopropanal with the bioisoster of DHAP (**20**) under FruA catalysis, followed by hydrogenation.[283]

2.2.6. Preparation of Higher Carbon Sugars, Analogs, and Sugar Mimics. The ability of FBP aldolase to accept aldehydes with different size and polarity is exemplified by the preparation of a number of sugar derivatives and analogs. In particular fructose containing a perfluorinated alkyl chain (**21**),[42] oligosaccharide mimics (**22**),[284] spiro and branched compounds (**23**−**26**),[94,285] homo-*C*-nucleoside analogs (**27**),[286] have been prepared some years ago using FBP aldolases (Figure 35)

Higher carbon sugars of the bicyclic type (**28**) have been obtained from methyl galactoside through a multienzymatic system, achieving the C-6 terminal oxidation to aldehyde (using galactose oxidase) and further RhuA-mediated stereoselective aldolization with DHAP, which was also produced enzymatically. The whole scheme can be performed in a one-pot process (Figure 36).[287] Similar structures can be produced

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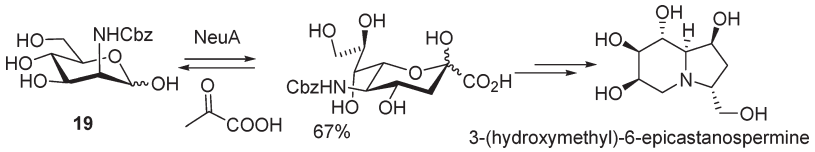

Figure 32. Synthesis of iminocylitol derivatives using pyruvate-dependent enzymes.

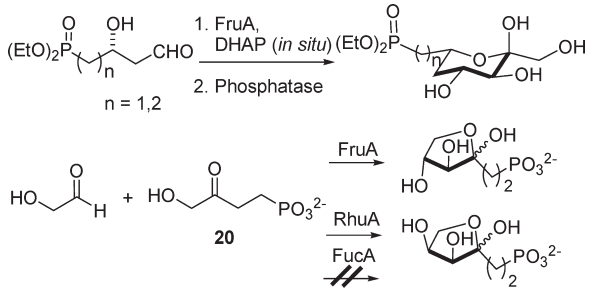

Figure 33. Strategies for the preparation of phosphonated sugars.

using dialdehydes as substrates, which undergo double (tandem) aldolization incorporating two DHAP residues. In this way several *C*-linked disaccharide mimics and higher carbon sugar analogs became available in a concise fashion. The starting dialdehyde is usually prepared by oxidative cleavage of a hydroxylated cycloalkene. Despite their complexity, the cyclic nature of the products makes possible to obtain a single diastereomer through thermodynamic control, even when starting from racemic precursors.[288] When working with reactive dialdehyde acceptors, using recombinant FruA from *Staphylococcus carnosus* may be more convenient than RAMA, due to the extraordinary process stability of the former.[289] With non-hydroxylated dialdehydes, the RAMA catalyzed aldolization stops after the first reaction, giving the mono adduct. Remote stereocenters have no influence on the outcome, and thus a 1:1 mixture of diastereomeric aldehydes (**30**) is produced from *meso*-**29** (Figure 36).[290]

2.2.6.1. Preparation of Sialic Acid and Related Compounds. Sialic acid, or *N*-acetylneuraminic acid, is the most important of the 3-deoxy-2-ulosonic acids of natural origin, more than forty of which are known.[291] They are involved in a wide range of biochemical events, mainly related to cellular recognition, communication and adhesion,[292] and thus are subject of intense research, due to the possibility of ample therapeutic applications. In this context the use of pyruvate-dependent aldolases becomes a valuable tool to access to this class of oxo-acids. *N*-Acetylneuraminic acid aldolase (NeuA) and its mutants are among the most studied and have been extensively used for the synthesis of sialic acid, (**31**), and its analogs.[33,293,294]

The industrial production of sialic acid is based on the NeuA-catalyzed aldol addition of pyruvate to *N*-acetylmannosamine, which is produced in situ from the inexpensive glucose derivative by *N*-acylglucosamine 2-epimerase (EC 5.1.3.8). The operation is amenable of optimization, according to the enzymatic source and method of isolation.[295,296] In addition, a coupled whole-cell system can be used instead of the free enzyme process.[297] A variation was reported utilizing a coupled whole cell biocatalytic system where stable and cheaper lactate was oxidized by means of a lactate oxidase into pyruvate (to reduce inhibition effects), which reacted in situ with *N*-acetyl-D-mannosamine (ManNAc) upon NeuA catalysis. Two type of cells were used, *Pseudomonas stutzeri* SDM and *E. coli* BL21 (DE3) (pET15b-nanA), and the mannosamine derivative was obtained by previous base-catalyzed epimerization of *N*-acetylglucosamine (GlcNAc), which afforded 5.7:1 ratio of ManNAc/GlcNAc (Figure 37).[298]

The ability of NeuA to accept 3-fluoropyruvate as donor substrate was used in the preparation of 3-fluorinated sialic acid derivatives, to be used as mechanistic probes for structural and kinetic studies (Figure 38).[114] Also, for similar purposes, cytidine monophosphate (CMP)-activated 7-fluorosialic acid was prepared from fluorinated *N*-acetylmannose and pyruvate, followed by CMP activation using CMP-sialic acid synthetase. Both enzymatic steps were conducted in a one-pot reaction.[299]

NeuA displays a fairly broad tolerance for sugars and derivatives as aldehyde acceptors, provided that are larger o equal to pentoses.[117,118,127] A number of modifications are tolerated in the positions C-2, C-4, or C-6, giving rise to sialic acids with different substitutions at C-5, C-7, or C-9 (Figure 38).[35,300−304] Also, the *N*-acetyl group can be altered, maintaining the activity.[121,123] Stable isotope labeling (^{13}C or ^{2}H) of sialic acid and KDN via NeuA-catalyzed aldol reaction using labeled ManNAc and/or pyruvate has been reported.[305,306]

In 2006, it was reported that a disaccharide can be used as an acceptor for NeuA to create glycosylated sialic acids. The synthesis of an unusual disaccharide component of the cell wall of *Streptomyces* sp. MB-8 was accomplished in high yield from β-D-Gal*p*-(1−6)-D-Man*p* and pyruvate using a sialic acid aldolase (Figure 39).[120] Other disaccharides, containing a reducing D-Man or D-ManNAc moiety, were also accepted by the enzyme.[119] The strategy could be applied to the synthesis of sialooligosaccharides (tri- and tetrasaccharides) using a one-pot two-enzyme system containing a CMP-sialic acid synthase (from *Neissera meningitidis*) and sialyltransferases (from two microbial sources).[122] This methodology was further exploited in the chemoenzymatic production of more complex oligosaccharides,[307] sialoside libraries,[308] and to prepare protein-sialoglycoside conjugates as potential vaccines.[309]

The broad substrate tolerance of NeuA to sugar acceptors was used in the generation of sialic acids and analogs for screening of a dynamic combinatorial library.[310]

An application of the fluorous tagging method to the chemoenzymatic synthesis of biologically active carbohydrates was realized in the synthesis of 9-substituted sialic acid as a perfluorinated acetal. The fluorous tag on C-6 of the acceptor is tolerated by the enzyme and allows the rapid and efficient purification of the product by fluorous reverse-phase silica gel, after which is easily deprotected.[311]

Pyruvate aldolases have been subject of improvement by directed evolution (Figure 40).[293] Starting out from the structure of a potent inhibitor of influenza sialidase (**32**), the enzyme NeuA has been evolved, using structure-guided saturation mutagenesis, to increase its activity toward aldehyde substrates of general structure (**33**). One variant, Glu192Asn, showed a 49-fold improvement in catalytic efficiency toward the target analogue and also had broad scope, which was exploited in the parallel synthesis of a small library of sialic acid analogues of

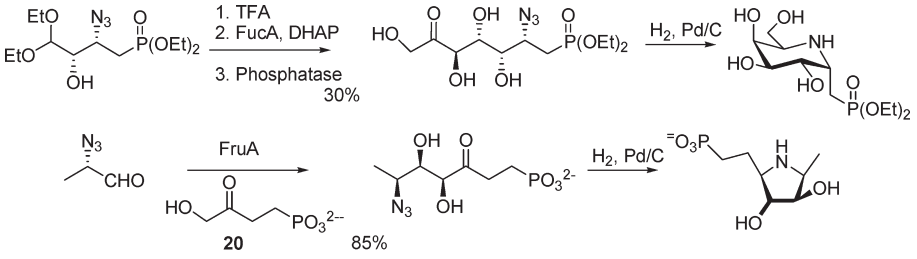

Figure 34. Phosphonated sugar derivatives prepared by using DHAP aldolases.

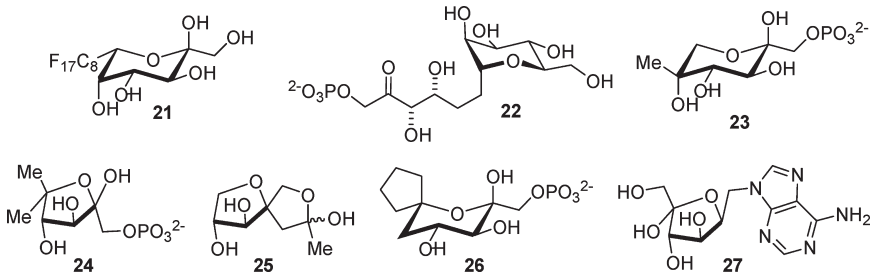

Figure 35. Sugar analogs and derivatives prepared by using DHAP aldolases.

general structure (**34**).[312] Related compounds, such as **35**, are potent and selective inhibitors of influenza A sialidase.[294]

Also, a pair of stereochemical complementary NeuA variants was created by directed evolution. These enzymes allow the selective preparation of either 4*S* or 4*R* isomers of sialic acid and derivatives.[313] In another example of directed evolution, NeuA was transformed in L-KDO aldolase after eight amino acid changes.[314]

Recently, a recombinant NeuA from *Pasteurella multocida* was characterized to show a higher expression level and a broader substrate tolerance as compared to NeuA from *E. coli*, and was used for the synthesis of the naturally occurring 8-*O*-methylsialic acid.[315]

In addition to sialic acid, other higher carbon 3-deoxysugars are important constituents of complex carbohydrates. For example, 3-deoxy-D-*manno*-octulosonic acid (KDO) is an essential component of lipopolysaccharides in Gram-negative bacteria, 3-deoxy-D-*glycero*-D-*galacto*-nonulosonic acid (KDN) is found in the carbohydrates of the bacterial cell wall. Recently an efficient preparation of this type of compounds was reported, exploiting the promiscuous aldolase activity of the enzyme macrophomate synthase (MPS, from *Macrophoma commelinae*) to add pyruvate enolate diastereoselectively to a wide range of *O*-protected aldoses (Figure 41). The enzyme catalyzes the reaction between oxaloacetate and differentially protected pentoses and hexoses in an efficient and stereoselective fashion, giving products with preferred 4*S* configuration, regardless of substrate bias (the diastereomeric ratio at C-4 ranges from 8:1 to >19:1).[316] Interestingly, protected glyceraldehyde and simpler substrates are converted with only modest selectivity (see below).

2.2.6.2. Preparation of Simpler (Shorter Chain) Ulosonic Acids. Short chain ulosonic acids are produced by other pyruvate-dependent aldolases, namely, 2-keto-3-deoxy-6-phospho-D-gluconate (KDPG) aldolase, 2-keto-3-deoxy-6-phospho-D-galactonate (KDPGal) aldolase, and 2-keto-3-deoxy-D-gluconate (KDG) aldolase. The rather high specificity of KDPG- and KDPGal aldolases for their natural phosphorylated acceptor makes them good candidates for directed evolution studies, which resulted in mutants with improved selectivity for nonphosphorylated aldehydes.[317,318]

Despite the specificity of KDPG aldolase for its natural acceptor, D-glyceraldehyde-3-phosphate, a series of polar, short chain aldehydes are converted at slower but useful rates.[131−135] The reaction is highly stereoselective, giving short chain ulosonic acids with the 4*S* configuration (Figure 42). The complementary enzyme is KDPGal aldolase, which uses the same phosphorylated acceptor and shows stereopreference for products with the (4*R*) configuration.[136,137] KDPG aldolase from *Thermotoga maritima* and KDPGal aldolase from *E. coli* yielded the unbranched Nikkomycin K side chain precursor (**37**) and its mirror image *ent*-(**37**), respectively, from 2-pyridincarboxaldehyde (**36**) with high enantioselectivity (Figure 42).[133,319,320]

Using site-directed mutagenesis to perturb the phosphate-binding pocket of KDPG aldolase, the catalytic efficiency of the Ser184Leu variant for the synthesis of (**37**) was improved >40-fold because of an increased selectivity for nonphosphorylated acceptors.[318]

The KDG aldolase, from the hyperthermophile *S. solfataricus*, is a promising biocatalyst since it does not use a phosphorylated acceptor; however synthetic applications have been hampered by the very low stereoselectivity displayed on C-4.[138] Interestingly, the use of a bulky protecting group allows the preparation of ulosonic acids with high selectivity for the 4*S* configuration, similar to that of KDPG aldolase (Figure 42).[139]

Macrophomate synthase (MPS) from *M. commelinae* catalyzes the synthesis of macrophomate (**39**) via formation of two C−C bonds in a multistep reaction cascade from oxaloacetate (**40**) and 2-pyrone (**41**). Although long considered a rare case of enzymatic diels-alderase reactivity; recently, it has been discovered that this enzyme is able to form pyruvate enolate from oxaloacetate, followed by stereoselective aldol addition to a variety of aromatic and aliphatic aldehydes (Figure 43).[321] These findings strongly corroborate an alternative two-step Michael-aldol sequence as the most plausible mechanism of macrophomate synthesis and are of relevance for understanding the divergent evolution of type II pyruvate aldolases.

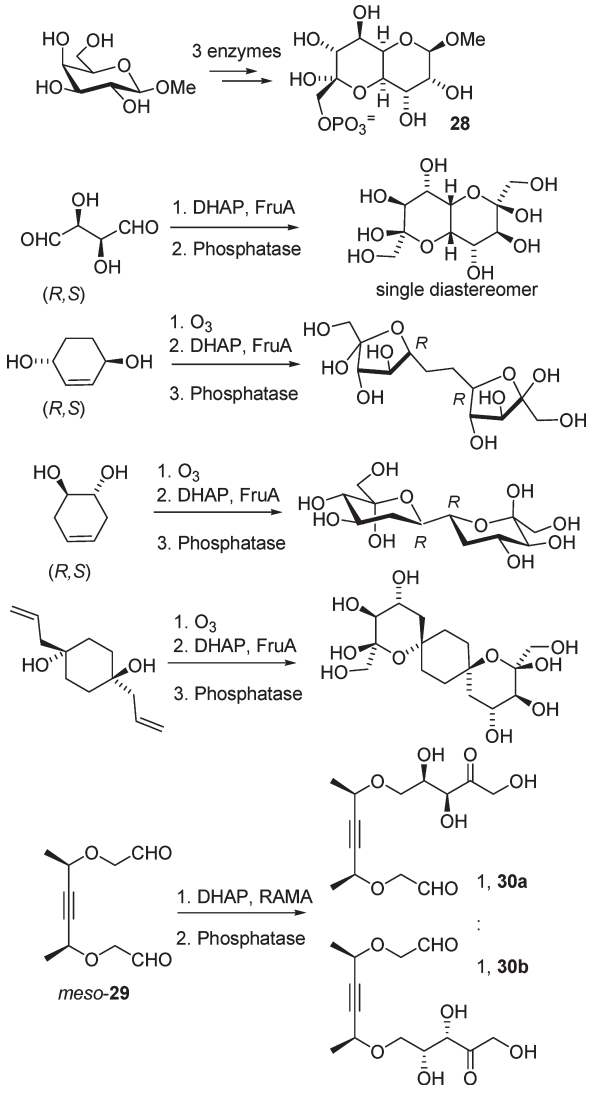

Figure 36. Synthesis of *C*-linked disaccharide mimics and higher carbon sugar analogs using DHAP aldolases.

Some ulosonic acids were prepared using a TK-catalyzed reaction as the key step. This interesting approach, developed by by Bolte et al., uses enzymes not involved in their biosynthetic pathways. The synthesis of (**42**), precursor of the corresponding 3-deoxy-2-ulosonic acid, was conducted by Bolte using xylulose-5-phosphate and the adequate α-hydroxyaldehyde (**43**) (Figure 44).[216] Other ulosonic acid analogs were prepared later using this methodology.[217]

2.2.7. Preparation of Carbocycles. RAMA has also been used for the preparation of five- and six-membered carbocycles. Using the formyl phosphonate (**44**) as acceptor, the resulting oxophosphonate (**45**) is cyclized in situ, through a Horner−Wadsworth−Emmons olefination, to the polyhydroxylated cyclopentene (**46**). The product was isolated in 71% yield in a one-pot synthetic sequence that included a olefination in a slightly acidic aqueous medium (Figure 45).[322] Unfortunately, this methodology failed for the preparation of cyclohexenes.

An interesting chemoenzymatic approach to carbocycles was reported by Lemaire, in the preparation of aminocyclitol analogues of valiolamine in just two synthetic steps.[323,324] RAMA-catalyzed aldolization of 3-hydroxy-4-nitrobutanal is followed by

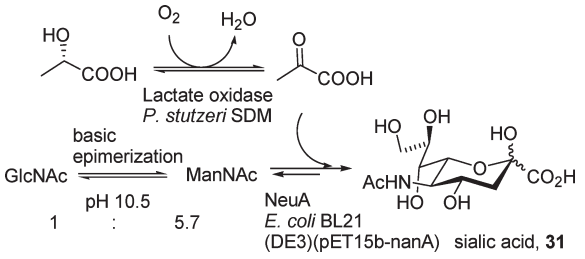

Figure 37. Coupled whole-cell biocatalytic synthesis of sialic acid.

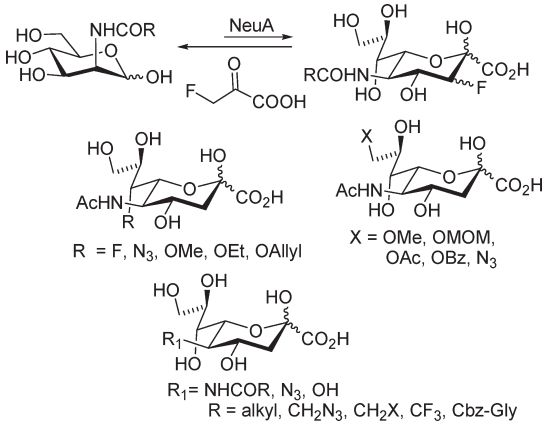

Figure 38. Sialic acid derivatives prepared by using NeuA.

a rapid intramolecular Henry reaction to give analogs of the glycosidase inhibitor valiolamine (**47**) (Figure 45).[325] In the procedure, two carbon−carbon bonds are formed in one-pot with high stereoselectivity.

2.2.8. Preparation of β-Hydroxy-α-amino Acids and Related Amino Alcohols. β-Hydroxy-α-amino acids constitute an important class of natural compounds with biological activity on their own and also as constituents of more complex natural products, such as antibiotics and immunosuppressants. Their metabolism involves pyridoxal phosphate-dependent enzymes, classified as serine hydroxymethyltransferase (SHMT) or the lyases threonine aldolases (ThrA). Since two new stereogenic centers are formed when using the lyases, four different products with complementary stereochemistry can be formally obtained from a single aldehyde using either L-threonine and D-threonine or the corresponding *allo*-threonine selective aldolases. Recent accounts on the four types of ThrA are available.[152−154]

Both L- and D-ThrA have been exploited for preparative purposes in the last years. Using a recombinant D-ThrA from *Alcaligenes xylosoxidans* a derivative of phenylserine, (**48**), which is an intermediate to the enantiomer of the antibiotic thiamphenicol, was obtained with excellent selectivity (>99% ee, 92% de) (Figure 46). On the other hand, its enantiomer was obtained by L-ThrA catalysis with low diastereoselectivity (<20% de).[152]

An *anti*-selective L-ThrA from *Candida humicola* has been used in the preparation of β-hydroxy-α-amino acids from D-glyceraldehyde acetonide, which were further elaborated into dihydroxyprolines (**49**) and (**50**) (Figure 46). Other α-oxygenated aldehydes were substrate of the enzyme in the preparation of thymine polyoxin C,[326] and in the first enantioselective synthesis of iminodigitoxose and imino-deoxydigitoxose.[278] Also, a synthon for the preparation of the immunosuppressant lipid mycestericin D was obtained by enzyme-catalyzed aldol

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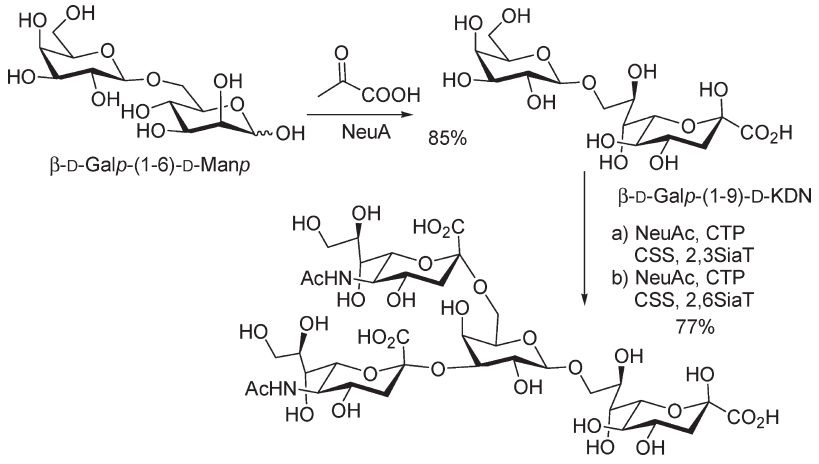

Figure 39. Disaccharides as acceptors of NeuA to form glycosylated sialic acids. CTP: cytidine triphosphate, CSS: CMP-sialic acid synthetase 2,3SiaT: multifunctional 2,3-sialyltransferase; 2,6SiaT: α-2,6-sialyltransferase.

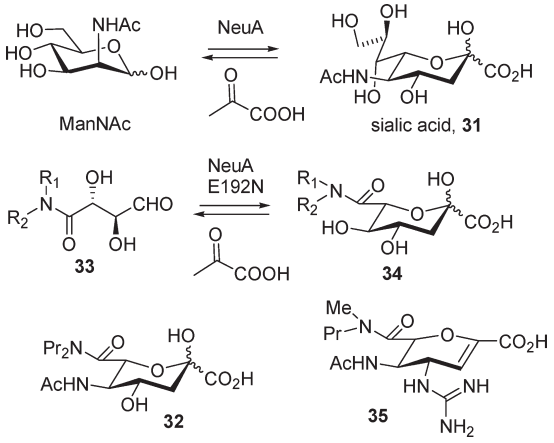

Figure 40. Directed evolution of NeuA aiming at the preparation of sialidase inhibitors.

reaction of glycine with 4-benzyloxybutanal under kinetic control (Figure 46).[255,327]

Both L- and D-selective ThrA were used in several works, concerning the preparation of γ-halogenated and long-chain L- and D-3-alkylserine derivatives as potential intermediates for sphingolipid synthesis.[157]

The densely functionalized β-hydroxy-α,ω-diamino acids (**52**), valuable intermediates for the synthesis of many bioactive compounds, can be approached by a combination of two diastereocomplementary enzymes (Figure 47). By varying the reaction temperature, a novel serine hydroxymethyltransferase from *Streptococcus thermophilus* (SHMT) and L-ThrA from *E. coli* turned out to be stereocomplementary biocatalysts, for the concise synthesis of L-*anti* and L-*syn* β-hydroxy-α,ω-diamino acid derivatives (**52**).[328] From these compounds, high yielding regiospecific formation of 2-oxazolidinone derivatives allows for protection and further elaboration of the compounds.

A recombinant L-ThrA from *E. coli* also accepted aldehydes bearing a carboxylic acid of type (**53**) to give ω-carboxy-β-hydroxy-L-α-amino acids (**54**) with acceptable yields but low *syn*/*anti* selectivity (Figure 47).[329]

Because of the reversible nature of the aldol reaction, enzymes with low stereoselectivity will typically lead to thermodynamic

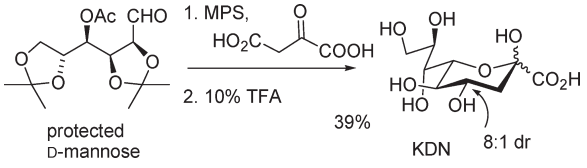

Figure 41. Synthesis of protected C8 and C9 3-deoxysugars using macrophomate synthase (MPS).

mixtures of *syn*/*anti* adducts.[154] This can be turned to an advantage by the design of a diastereoselective dynamic kinetic discrimination process. In particular, by coupling L-ThrA and a diastereoselective L-tyrosine decarboxylase (L-TyrDC), the *syn*/*anti* isomeric mixture resulting from the aldol reaction is enriched in the *anti* isomer by preferential decomposition of the L-*syn* isomer through an irreversible decarboxylation, leading to the aromatic (*R*)-2-amino-1-phenylethanol (**57**) in 78% ee and high yield (Figure 48).[330]

In a closely related strategy, the same group used a three-enzyme combination of L-ThrA, D-ThrA, and L-TyrDC to effect a dynamic kinetic de-epimerization of diastereomers (DYKAT) to produce (*R*)-(**57**) in 58% isolated yield and >99% ee when starting from racemic *syn*-phenylserine, *rac*-(**55**) (Figure 48).[330−332]

The specificity of ThrA or SHMT can also be exploited for kinetic resolutions of stereoisomer mixtures. Best results are obtained for aromatic β-hydroxy-α-amino acids, which are important synthons for pharmaceuticals (Figure 49). Hence, a D-ThrA from *Arthrobacter* sp. DK-38 has been shown to be particularly useful for the resolution of racemic *syn*-aryl serines (**58**) by retroaldolization under kinetic control to furnish enantiomerically pure L-*syn*-amino acids in 50% yield and almost 100% ee.[333] Also, a D-ThrA from *A. xylosoxidans* has been used for the resolution of racemic *syn*-β-(3,4-methylenedioxyphenyl)serine (**59**) by retro-aldol cleavage to furnish the desired L-*syn* isomer (**60**).[334] This compound is an intermediate in route to L-*syn*-3,4-dihydroxyphenylserine (DOPS), used for the treatment of Parkinson's disease (Figure 49).

An improved application of aldolase catalysis at industrial scale has been recently reported for the synthesis of L-*syn*-3,4-dihydroxyphenylserine (DOPS), by employing a recombinant *E. coli* expressing L-ThrA genes cloned from *Streptomyces avelmitilis*

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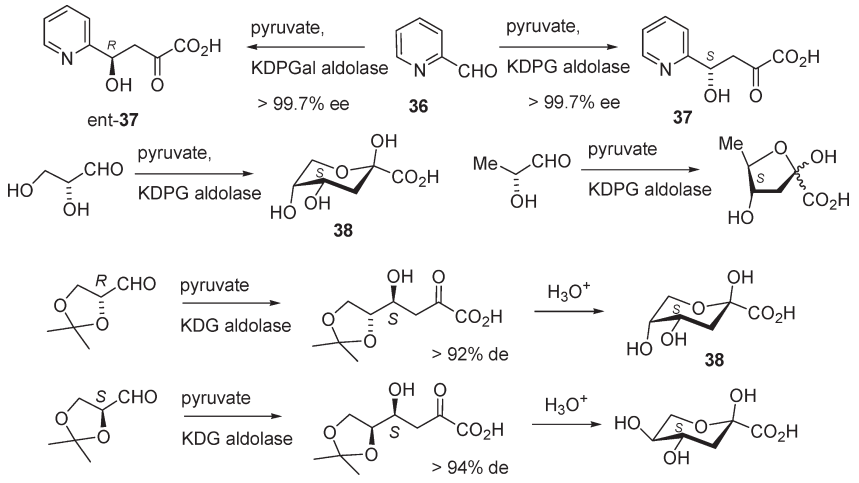

Figure 42. Preparation of ulosonic acids using KDPG and KDPGal aldolases.

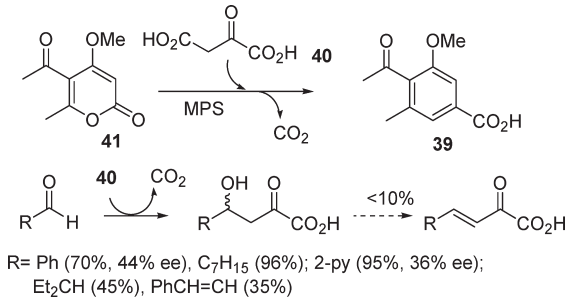

Figure 43. Aldolase activity of macrophomate synthase (MPS).

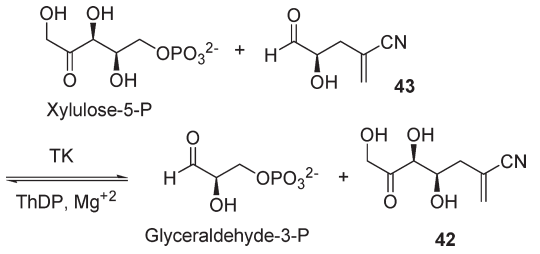

Figure 44. Synthesis of a precursor of 3-deoxy-D-arabino-2-heptulosonic acid using a TK-catalyzed reaction.

MA-4680 in a whole-cell, high-density bioreactor, to achieve productivities around 8 g L^{-1}.[335] Another application was the production of L-*allo*-threonine mediated by SHMT from *E. coli* in a enzyme membrane reactor, reaching a space-time yield above 220 g L^{-1} d^{-1}.[336]

A reengineered alanine racemase from *Geobacillus stearothermophilus*, which had been converted into a ThrA by single point mutation (Tyr265Ala)[337] has been shown to accept α-substituted β-phenylserines, which are not substrates for natural ThrAs, as substrates for aldol cleavage.[338] Similar to natural ThrA, the engineered ThrA exhibits a high stereoselectivity at Cα (for D-amino acids) but a low preference at Cβ. Various aromatic aldehydes were accepted, affording D-amino acids exclusively (>99% ee), in moderate to high Cβ selectivity in favor of the *syn* diastereomer (40−97% de).[339] Together with an improved Tyr265Lys mutant, both enzymes show a >7.9:1 preference for the D-*syn* isomer of β-phenylserine and are therefore more diastereoselective than many natural pyridoxal-5′-phosphate (PLP)-dependent aldolases. Remarkably, D-alanine was an alternative donor, thereby yielding α-branched amino acids.[340]

A recently discovered PLP-dependent α-methylserine aldolase from different microorganisms[341,342] catalyzes the addition of L-alanine and L-2-aminobutyric acid to formaldehyde as an acceptor, resulting in an aldolase-mediated production of α-branched amino acids (Figure 50). The enzyme from *Ralstonia* sp. was applied in a whole-cell catalyzed stereospecific synthesis of α-methyl-L-serine on a 30 mmol scale.

The expansion of this concept to a general synthesis of α-substituted-β-hydroxy-α-amino acids has been reported by the group of Griengl.[343] Two aldolases (L-*allo* threonine aldolase from *Aeromonas jandaei* and D-threonine aldolase from *Pseudomonas* sp.) are able to catalyze the reaction of D-amino acids with both aromatic and aliphatic aldehydes to give α-branched hydroxyamino acids (Figure 50). Remarkably, only the D-enantiomers of alanine, serine, and cysteine were suitable amino acid donors. Highest conversions were obtained when using D-alanine as donor. Both enzymes exhibited a high stereoselectivity at Cα (>99% ee for L- and D-products, respectively) but a low preference at Cβ (between 6 and 65% de for L-*anti* and up to 95% de for D-*syn*, respectively)

For the aldolase-catalyzed preparation of amino acids, Thr-dependent aldolases are largely preferred, since they furnish the amino functionality in addition to the C−C bond. However, other types of aldolases have been used, provided that another system supplies the amino group, like a transaminase. A 4-hydroxy-3-methyl-2-keto-pentanoate aldolase from *Arthrobacter simplex* AKU 626, which catalyzes the addition of 2-ketobutyrate (**61**) to acetaldehyde, has been applied to the coupled enzymatic synthesis of 4-hydroxyisoleucine (HIL), an insulinotropic agent (Figure 51). Remarkably, given the difference with the usual 3C donors of the pyruvate aldolases (pyruvate or PEP), the use of 2-ketobutyrate as donor opens up new biocatalytic opportunities since two stereocenters are formed in the reaction.[344,345]

Chiral amino alcohols are important intermediates in organic synthesis. Glycine-dependent enzymes can be used to prepare amino alcohols of type 2-amino-1-arylethanol (see Figure 48) or 2-amino-1,3-diol (by formal reduction of the corresponding β-hydroxy-α-amino acid). Recently, a methodology to access to

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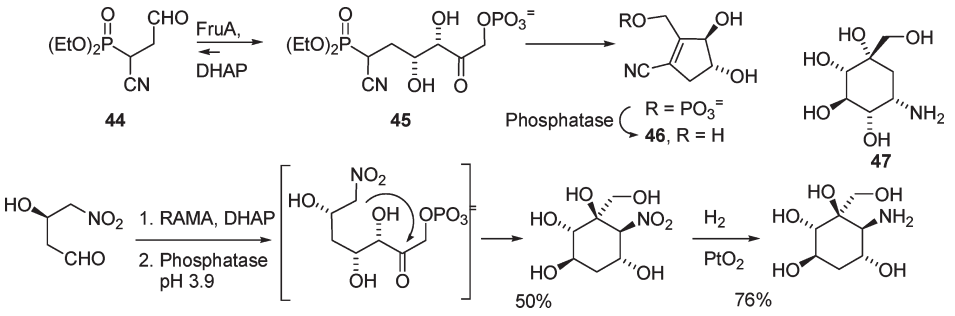

Figure 45. RAMA-catalyzed synthesis of carbocycles.

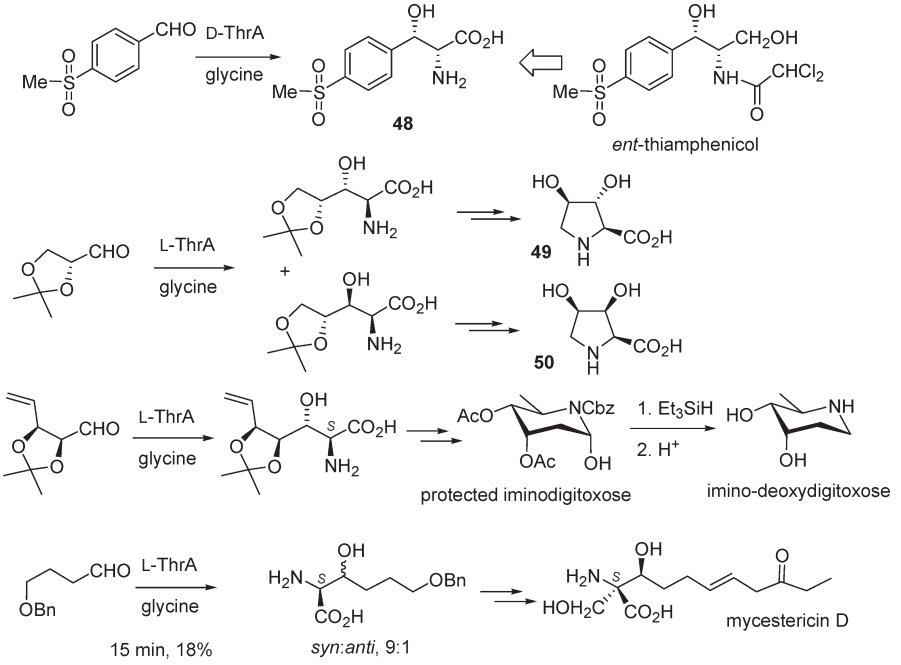

Figure 46. Synthetic applications of β-hydroxy-α-amino acids prepared by ThrA-mediated aldolization.

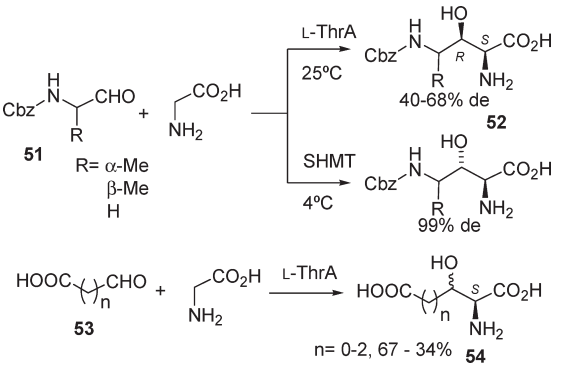

Figure 47. Enzymatic production of functionalized L-β-hydroxy-α-amino acids.

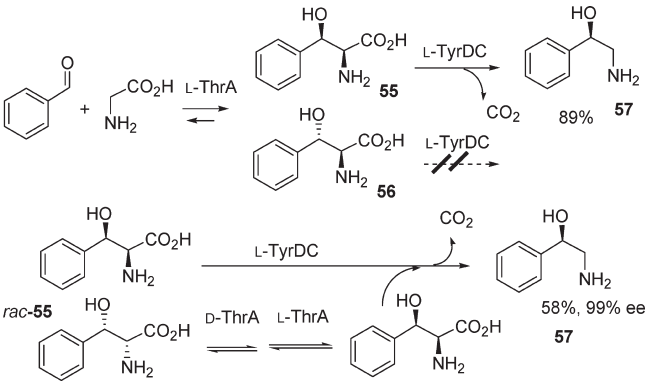

Figure 48. Multienzyme system for dynamic kinetic discrimination of diastereomers. L-TyrDC: Tyrosine decarboxylase.

chiral 2-amino-1,3-diols through a TK-mediated reaction was described, using (2*R*,3*R*)-2-amino-1,3,4-butanetriol[346] and (2*S*,3*S*)-2-aminopentane-1,3-diol[347] as specific examples.

The designed biocatalytic strategy was previously simulated to provide guidance for further processes and biocatalytic developments.[348] The synthetic route takes advantage of the *E. coli* TK mutant (Asp469Thr), which is able to catalyze the reaction of hydroxypyruvate with propanal to give (3*S*)-1,3-dihydroxypentan-2-one (DHP) in good yield. Then, a ω-transaminase-catalyzed step converts the product into the desired enantiomerically pure diol, using isopropylamine as the donor amine (Figure 52).

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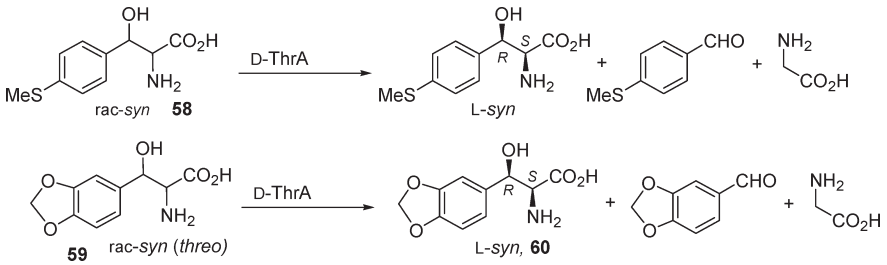

Figure 49. Kinetic resolution of aromatic β-hydroxy-α-amino acids.

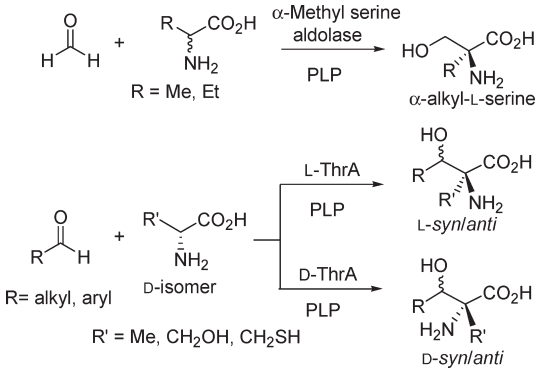

Figure 50. ThrA-mediated production of α-branched aminoacids.

2.2.9. Preparation of Natural Products. Aldolases have been used mainly in the carbohydrate field, but their ability to catalyze highly stereoselective aldol reactions has prompted their utilization in the synthesis of other types of natural products.

In this regard DHAP aldolases have participated in the preparation of macrocycles, macrolide antibiotics, pheromones, terpenoids, and alkaloids.

Representative examples of the use of DHAP-dependent aldolases in natural products synthesis are shown in (Figure 52). The C3−C9 hydroxylated fragment of the macrolactone (+)-aspicillin was prepared in 10% overall yield from γ-benzyloxybutanal through a FruA catalyzed aldol reaction, followed by selective reduction and protection.[349] Similarly, a functionalized hexanal was used as acceptor for FruA to prepare the C9−C16 fragment of the macrolide antibiotic pentamycin.[350]

The aggregation pheromone (+)-*exo*-brevicomin, **62**, was obtained through FruA mediated stereoselective addition of 5-oxopentanal to DHAP. Dephosphorylation and further acidic treatment afforded the bicyclic skeleton, which was then deoxygenated to the final compound.[351]

In the preparation of the microbial elicitor (−)-syringolide 2, the FruA mediated stereoselective aldol addition of protected glycolaldehyde to DHAP (**63**, 65% yield) established the configuration of the only two independent stereogenic centers of the molecule, the others were set according to the kinetic preference in the cyclization.[352]

The first total synthesis of sphydrofuran, a secondary metabolite produced by a variety of *Streptomycetes* strains, was based in a RAMA-catalyzed aldol addition of chloroacetaldehyde to DHAP, providing two of the three chiral centers of the target molecule. The third quaternary center was introduced via a highly diastereoselective Grignard addition of allylmagnesium bromide (Figure 53).[285]

In a concise chemoenzymatic approach, analogs of the cytotoxic alkaloid pancratistatin were prepared by selective RhuA-catalyzed aldolization of a hydroxylated dialdehyde (obtained from microbial dioxygenation of a naphthalene derivative) followed by oxidation to the final lactone.[353] In the reaction catalyzed by the RhuA, both the desired pyranoid isomer and an equal fraction of the furanoid isomer were formed. However, in the reaction catalyzed by FruA, only the undesired furanoid product was found (Figure 53).

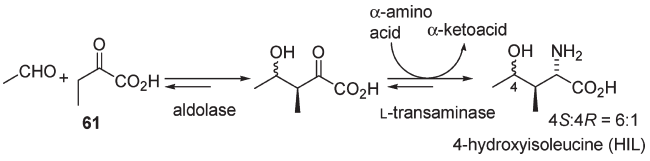

Figure 51. Amino acid production through a coupled aldolase-transaminase system.

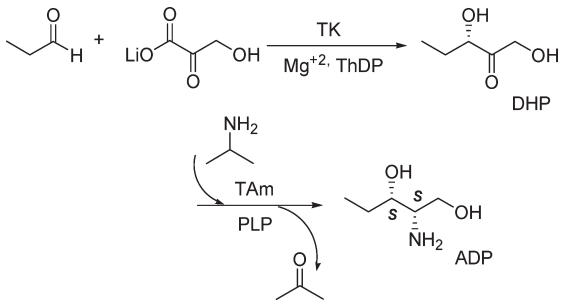

Figure 52. Two-step biocatalytic synthesis of 2-amino-1,3-diols using TK Asp469Thr and transaminase (TAm). PLP: pyridoxal-5′-phosphate; DHP: 1,3-dihydroxypentan-2-one; ADP: (2*S*,3*S*)-2-aminopentane-1,3-diol.

An advanced precursor to the pyranosic portion of the macrolide antibiotic amphotericin B was prepared in good yield and selectivity by NeuA-catalyzed aldol reaction of the branched chain *manno*-configured substrate (**64**) with pyruvate.[354]

Nikkomycins (e.g., Nikkomycin K) are a group of peptidyl nucleoside antibiotics regarded as promising antifungal agents in agriculture and human therapy. The *N*-terminal amino acid moiety was prepared in a two-step enzymatic synthesis using a native KDPG aldolase-catalyzed aldol reaction to give exclusively the (*S*)-enantiomer, followed by a phenylalanine dehydrogenase-catalyzed reductive amination with cofactor cycling. The whole side chain was thus obtained in 76% overall yield (Figure 54).[133]

DERA-catalyzed aldolization was used in the preparation of lactols (**65**) and (**66**), which are useful intermediates for the synthesis of potent antitumor agents epothilone A and C.[355,356] Interestingly, whereas the aldehyde (**67**) reacted with acetaldehyde with good yield, the enantiomer of aldehyde (**67**) was not substrate for DERA (Figure 55).

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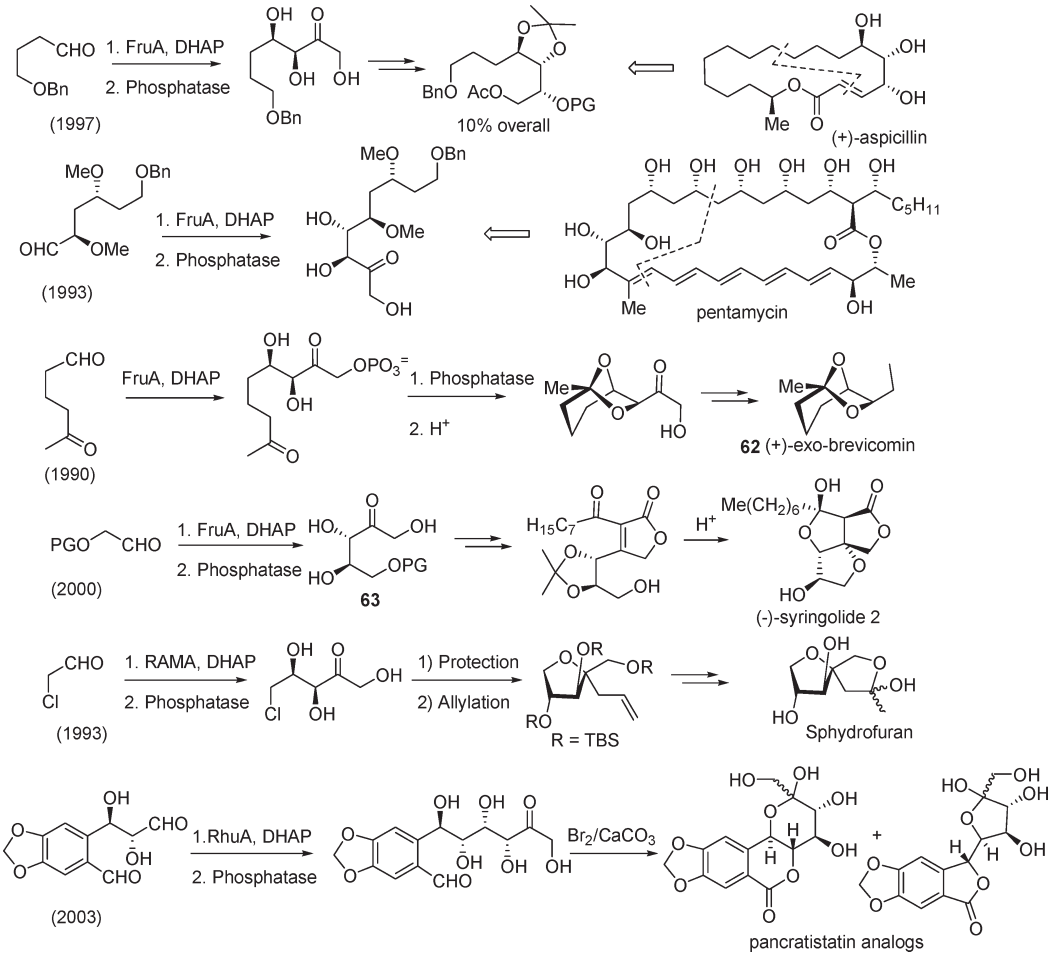

Figure 53. DHAP-dependent aldolases in natural products synthesis. PG: Protective group.

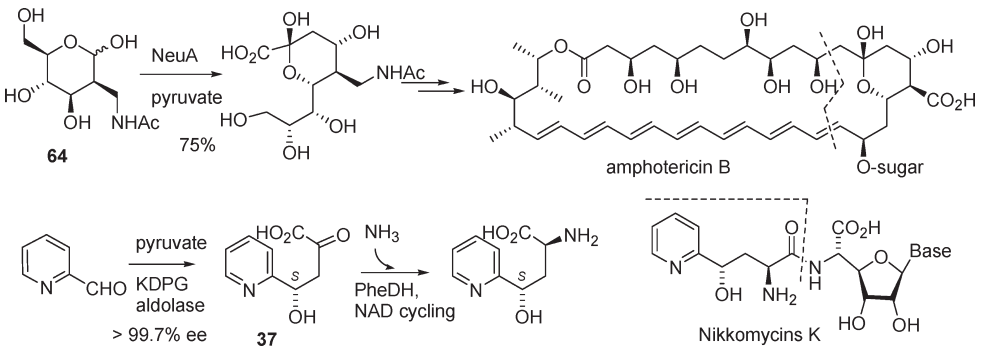

Figure 54. Pyruvate-dependent aldolases in natural product synthesis.

2.2.10. Pharmaceutical Intermediates. In the pharmaceutical industry, DERA is used mainly for the production of the side chain of the statin drugs and consequently,[259] several efforts to improve the catalyst performance were undertaken.

A directed evolution approach, aiming at increasing the affinity of the *E. coli* aldolase for chloroacetaldehyde and the stability against high acetaldehyde concentrations, rendered a variant with 10-fold improved productivity under industrially relevant conditions.[357]

Directed evolution was also applied to expand substrate specificity, using site-directed mutagenesis. In this case, the variant Ser238Asp exhibited a 2.5-fold improvement over the wild type enzyme and also could use 3-azidopropanal as an acceptor (which is not a substrate for the native enzyme) (Figure 56).[358] Sequential aldol addition of 3-azidopropanal to acetaldehyde gives a deoxy-azidoethyl pyranose which, upon oxidation to lactone (**68**), is also a precursor of the side chain of statin drugs.[260] Either the nitrile (**69**) or the azido compound (**68**) can be used to furnish the side chain of Atorvastatin. In addition, other versatile pyranoid building blocks (of types **70** and **71**) can be prepared in large scale by the same strategy.[262]

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In other approach to catalyst improvement, an increase of almost 400-fold in volumetric productivity relative to the published enzymatic reaction conditions was reported, obtaining 31 g L^{-1} h^{-1} of lactol (**8**) with 96.6% de and >99.9% ee. These improvements were achieved by a combination of discovery from environmental DNA of DERAs with reaction optimization to overcome substrate inhibition.[261]

Also, DERA enzymes from two hyperthermophilic microorganisms showed increased resistance to high concentrations of acetaldehyde while performing efficiently tandem aldol addition at ambient temperature.[359]

3. ACYLOIN CONDENSATION

3.1. Enzymes Involved. Classification

Another group of ThDP-dependent enzymes with an interesting synthetic potential for C−C bond formation are those related to the acyloin condensation. This group of lyases catalyze the formal acyloin condensation between an aldehyde and an adequate donor, generating enantiomerically enriched α-hydroxyketones, usually from inexpensive aldehydes.[168,197,360−363]

The key step in the catalytic mechanism is the reaction of the thiamine diphosphate cofactor with the "donor" aldehyde, forming the active zwitterion. Then, the attack of the "active species" to the acceptor aldehyde, generates the (*R*)-α-hydroxyketone upon releasing of the cofactor (Figure 57).[162,361,364−366]

Several ThDP-dependent lyases, such as benzaldehyde lyase (BAL, EC 4.1.2.38),[367,368] pyruvate decarboxylase (PDC, EC 4.1.1.1),[369−372] benzoylformate decarboxylase (BFD, EC 4.1.1.7),[373] phenylpyruvate decarboxylase (PhDC, EC 4.1.1.43),[374,375] and indole-3-pyruvate decarboxylase (InPDC, EC 4.1.1.74)[376,377] were characterized and some of them have been extensively used as powerful biocatalysts.[168,197,198,360,362,378]

3.1.1. Pyruvate Decarboxylase (PDC). PDC is an important enzyme in glycolysis and ethanol fermentation, the first enzyme of the branched glycolytic pathway, catalyzing the nonoxidative decarboxylation −under anaerobic conditions− of pyruvic acid to acetaldehyde, and it is widely used in the field of biocatalysis.[362,378] Genes from PDC have been isolated from yeasts or filamentous fungi such as *S. cerevisiae*, *Kluyveromyces marxianus*, *Candida utilis*, *Neurospora crassa*, *Zymomonas mobilis*, and *Rhizopus javanicus*, and plants such as maize, tomato, rice, and pea.[378−381] PDC from *S. cerevisiae* and *Z. mobilis* are the most intensively studied enzymes regarding reaction mechanism[382,383] and substrate specificity,[363,379,384] thus they are also the most widely used in organic synthesis. The crystal structure for PDC from *Z. mobilis* was determined by Dobritzsch et al., consisting of three domains, all of the α/β type.[385] In yeast, PDC and alcohol dehydrogenase (EC 1.1.1.1), are responsible for the conversion of pyruvate into ethanol.

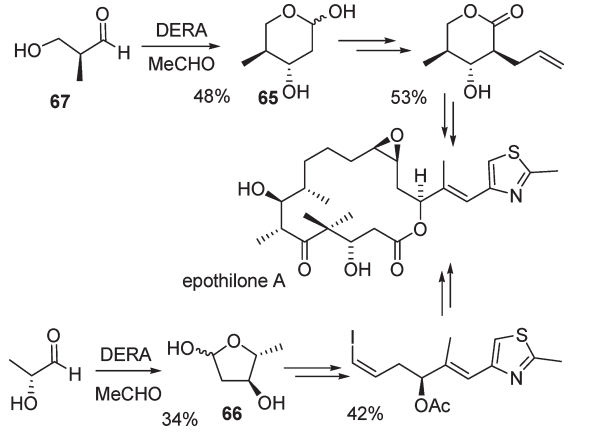

Figure 55. DERA-catalyzed reactions in natural product synthesis.

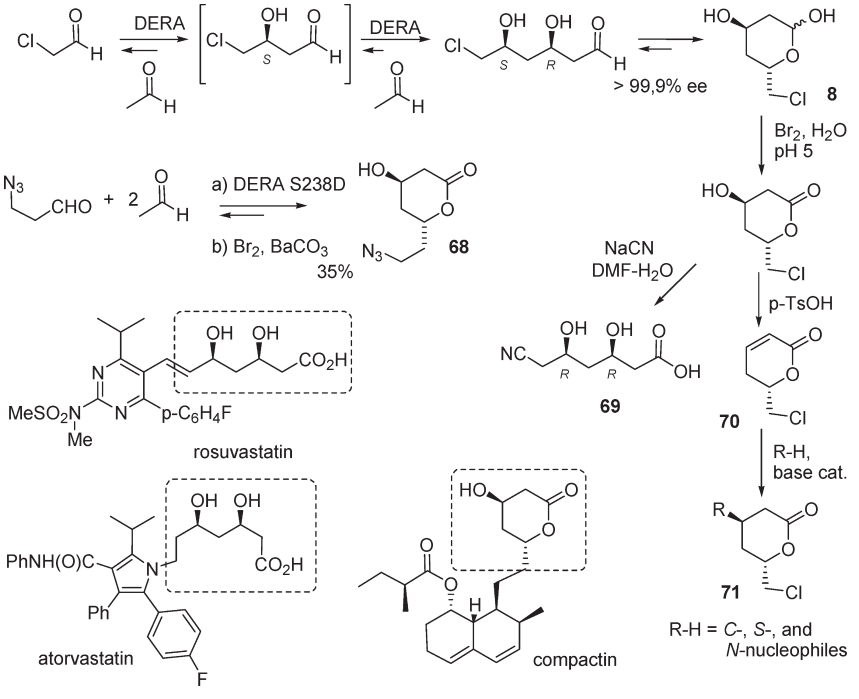

Figure 56. Use of DERA for the preparation of statin intermediates.

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The reaction mechanism, leading to acetaldehyde (decarboxylation) or (*R*)-phenylacetylcarbinol [(*R*)-PAC] (carboligation), is illustrated in Figure 58 (R_1 = Me, R_2 = Ph).

A number of decarboxylations of α-keto acids, together with the formation of chiral α-hydroxyketones, have been described with PDC, involving different aldehydes as substrates, either with whole cell systems or isolated enzymes.[363,379,386] The enzyme tolerates a broad range of substrates for the decarboxylation reaction, including α-keto acids such as α-keto butyric and α-keto valeric acids, and branched substrates such as α-ketoisocaproic acid or α-ketoisovaleric acid. Regarding the origin of the PDC, it was stated that bacterial enzymes show lower affinities for longer-chain-aliphatic α-keto acids than those of yeast.[380]

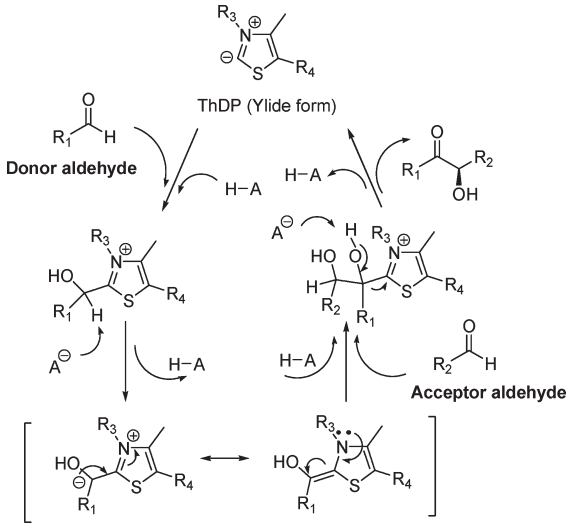

Figure 57. General mechanism proposed for ThDP-dependent lyases catalyzing the umpolung carboligation of aldehydes.

3.1.2. Benzoylformate Decarboxylase (BFD). Benzoylformate decarboxylases have been found in bacteria such as *Pseudomonas putida*, *Pseudomonas aeruginosa*, and *Acinetobacter calcoaceticus*, but only BFD from *P. putida* has been investigated for its potential use in biocatalysis.[387] The encoding genes were cloned by Gerlt and colleagues,[373] and the crystal structure was determined as well,[388] showing four subunits, similar to PDC from *Z. mobilis*.[385]

The enzyme plays an important role in the mandelate pathway, and its main reaction is the nonoxidative decarboxylation of benzoylformate to benzaldehyde,[373,389] through a mechanism similar to that of PDC.[390−393] The enzyme also catalyzes the enantioselective formation of C−C bonds[394] (see Figure 58, R_1 = Ph) as a side reaction with a yet unknown physiological role.[395]

Fortunately for synthetic purposes, the enzyme accepts also aldehydes as donors, instead of α-ketoacids, to give the corresponding acyloins with (*S*)-configuration.[396]

The enzyme works well within a broad range of pH (5−8) and temperature (20−40 °C), catalyzing the enantioselective acyloin and benzoin condensations.[394,397] Ward and co-workers reported for the first time the carboligase activity of BFD from *P. putida*, introducing the catalyst as an efficient alternative for the enantioselective synthesis of (*S*)-2-hydroxyketones. The native enzyme does not accept *o*-substituted benzaldehyde derivatives, but its range of substrates is broad preferring aromatic aldehydes, including *m*-substituted aromatic aldehydes, heteroaromatic aldehydes, and also acyclic and α,β-unsaturated ones,[387,395,398−402] and its stereoselectivity is highly dependent on the structure of the substrate aldehydes.[364] The substrate specificity was broaden through genetic engineering techniques, and therefore mutants accepting *o*-substituted benzaldehydes as donors, and even aromatic aldehydes as acceptors, are available.[401] When the acceptor aldehyde is aromatic, the native enzymes as well as the mutants, give the acyloin with (*R*)-configuration, with high enantiomeric excess.

3.1.3. Phenylpyruvate Decarboxylase (PhDC). This ThDP-dependent α-ketoacid decarboxylase was described by

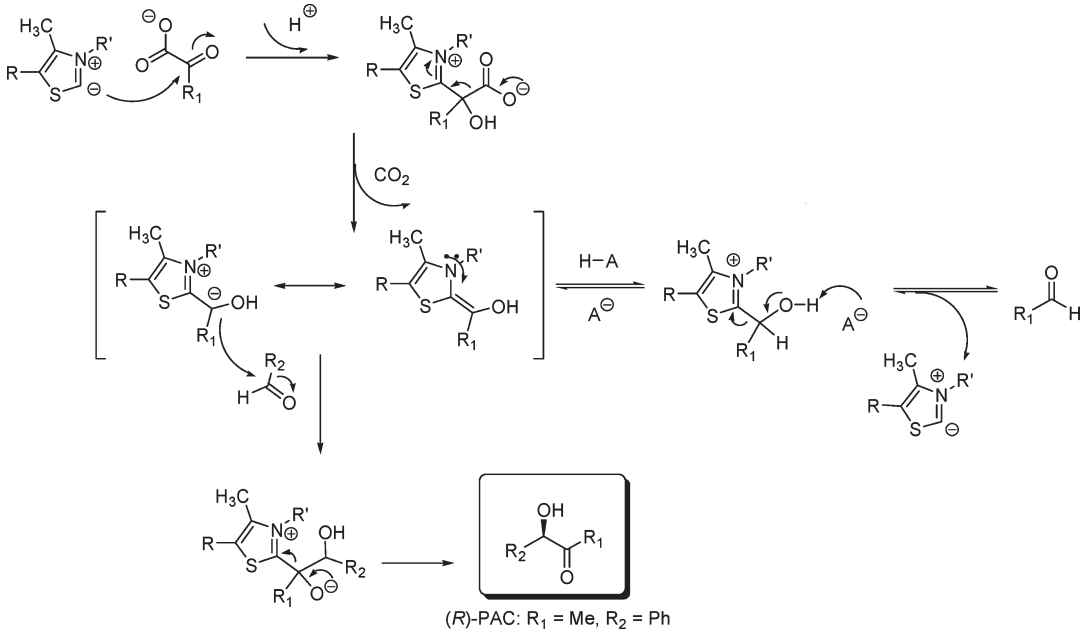

Figure 58. ThDP-mediated mechanism of PDC catalysis. Preparation of (R)-PAC: R_1 = Me, R_2 = Ph.

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Guo et al. as a suitable biocatalyst for the acyloin condensation of phenylpyruvic acid with acetaldehyde.[403] The enzyme is part of the catabolic pathway of aromatic compounds in *Achromobacter*, *Acinetobacter*, and *Thaurea* spp., being produced by induction with L-phenylalanine, tryptophan, or mandelate.[404,405]

Regarding the substrate specificity, PhDC differs substantially from PDC. Linear aliphatic aldehydes are accepted by PhDC as acyl acceptors, while aromatic ones are not substrates of the enzyme. In addition, moderate activity was found when using indolepyruvate as donor substrate, and no reaction was observed with benzoylformate.[406]

The proposed catalytic mechanism of PhDC is analogue to PDC and BFD, involving a first step in which decarboxylation of phenylpyruvic acid occurs, followed by the reaction of the thiamine-enzyme bound complex with the acceptor aldehyde, to provide the acyloin as product.[403]

3.1.4. Benzaldehyde Lyase (BAL). BAL from *Pseudomonas fluorescens* Biovar I was described for the first time in 1989 by Vicuña et al., being part of the group of ThDP-dependent enzymes that also uses divalent cations as cofactors.[367] The gene (*bzl*) encoding BAL from *P. fluorescens* was subsequently cloned, characterized, and overexpressed in *E. coli*.[368] The catalytic activity of the enzyme in C−C bond forming reactions using generally acetaldehyde as acceptor, affords (*R*)-benzoin and (*R*)-2-hydroxypropiophenone derivatives. The reaction was first described by Müller and co-workers,[407] and to date, its carboligation activity has been extensively studied with a wide range of substrates.[364,386,396,400,408−417] BAL and BFD are homologous enzymes, that catalyze both carboligase and carbolyase reactions, and both allow the formation of 2-hydroxyketones from aldehydes. However, the reverse reaction has only been observed with BAL.[364] Opposite to BFD, in similar conditions the stereospecificity of BAL is strictly specific for *R* configurations. Pohl and co-workers used molecular modeling studies to explain the experimentally observed differences in activity, stereo- and enantioselectivity, as well as substrate specificity, for both enzymes.[364]

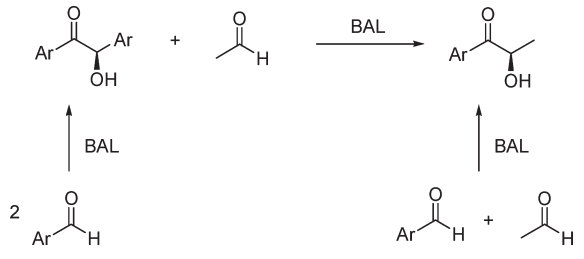

Figure 59. General scheme for BAL-catalyzed carboligations.

The general scheme for BAL-catalyzed carboligation is shown in Figure 59.

The catalytic mechanism of BAL is in accordance with other ThDP-dependent enzymes, and it has been widely studied.[393,407,418−421] The first step of the cycle is the attack of the ylide form of ThDP to the carbonyl carbon of the (*R*)-benzoin, producing the corresponding adduct. The electrophilic nitrogen atom of ThDP promotes a rearrangement leading to the formation of the enamine intermediate, which reacts then with the acceptor aldehyde, and undergoes the carboligation reaction. The release of the acyloin and the regeneration of the ylide, completes the catalytic cycle. In the absence of an acceptor aldehyde, the protonation of the enamine intermediate results in the release of the bound benzaldehyde and the regeneration of the ylide. In addition, the formation of the enamine-carbanion intermediate can also take place by the attack of the ylide form of ThDP to the carbonyl group in the released benzaldehyde in case of absence of the acceptor aldehydes (Figure 60).[362]

Besides being stereochemically complementary to BFD, BAL shows broader substrate specificity. *o*-Substituted aromatic aldehydes, heteroaromatic aldehydes, simple aliphatic ones such as propionaldehyde, mono- and dimetoxyaldehyde, and also glyceraldehyde and butyraldehyde derivatives are accepted; even though aromatic substrates are preferred.[396,400,407,409,410,412,422,423] However, pyridine derivatives, as well as sterically hindered aldehydes, are not easily accepted by the enzyme. Aliphatic aldehydes are both suitable donors and acceptors, as long as they are not highly sterically hindered.[413] Moreover, heteroaromatic benzoins and acyloins are accepted as substrates for kinetic resolution of racemates, via C−C bond cleavage.[407]

Structural differences between BAL and BFD show that the reason of the different enantioselectivities of both enzymes, might be the shape of the binding site.[364] The 3D-structures of the enzymes are highly similar,[420,424] despite their sequence identity is only around 24%.[364] The active sites are located at

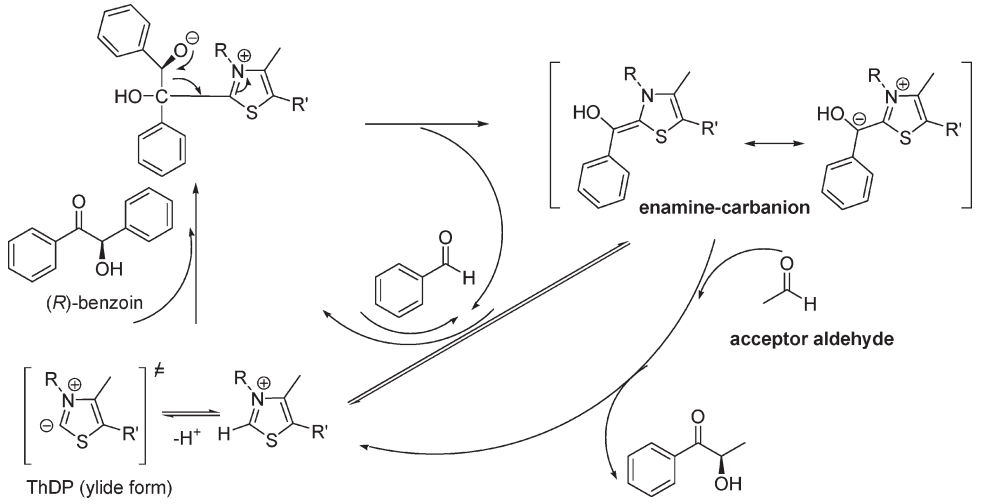

Figure 60. Catalytic mechanism of BAL-catalyzed carboligation reactions.

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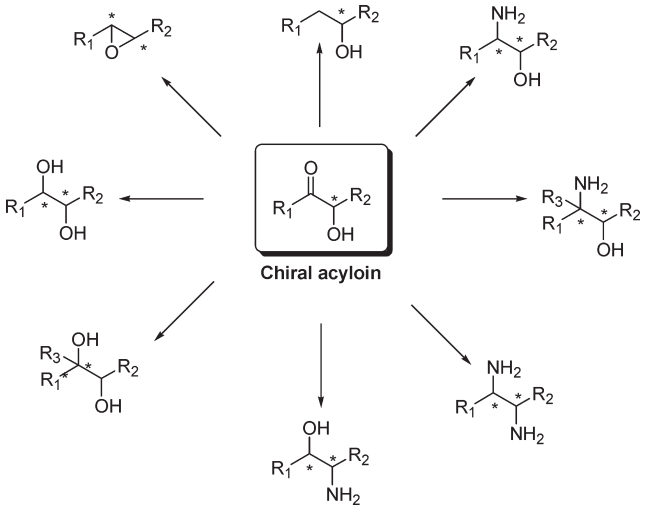


Figure 61. Synthetic potential of acyloins as chiral building blocks.

the interfaces of two monomers that form one dimeric half of the enzyme. The main difference between the structure of BAL and BFD is the wider binding site in BAL compared to BFD, and a positional shift of the substrate channel entrance on the protein surface. Thus, steric restrains are conditioning both active sites, and make the difference in substrate specificities.[364]

3.2. Synthetic Applications

Because of their versatile functional groups, optically active α-oxyfunctionalized compounds, namely, carboxylic acids, aldehydes and ketones, are key building blocks for asymmetric synthesis, since they can be easily transformed into other functionalities such as diols, halo- and amino-derivatives, or epoxides.[425] Among these α-oxyfunctionalized compounds, α-hydroxyketones, known as acyloins, plays an especially important role (Figure 61).

Different biocatalytic approaches can be developed for the synthesis of acyloins,[361] and among them, the use of ThDP-dependent enzymes is outstanding.[168] ThDP-dependent lyases catalyze the umpolung carboligation of aldehydes, obtaining chiral α-hydroxyketones even from inexpensive substrates. There have been characterized a number or lyases with a broad substrate spectrum. Through the use of genetic engineering (recombinant whole cells overexpressing lyases), as well as reaction engineering (biphasic media, enzyme immobilization, or preparative scale reactors), these enzymes have became available in considerable amounts, and high productivities (80−100 g/L) and enantiomeric excesses up to >99% can be achieved using them as biocatalysts.[168,197,360−362,386,409,411,413]

Varying the donor or acceptor aldehydes, the production of a wide variety of structurally different building blocks can be achieved, so the identification of lyases able to accept a broad range of substrates is always a challenge for biocatalysis.

Among α-keto acid decarboxylases, the most widely used in organic synthesis are PDC and BFD, while PhDC and InPDC have been less studied.

The first report on a biotechnological process for the acyloin condensation was described by Neuberg in the early 1920s, by using *S. cerevisiae* as whole cell biocatalyst.[426,427] In this work, optically active 1-phenyl-1-hydroxypropan-2-one [(*R*)-phenylacetylcarbinol, (*R*)-PAC] was prepared from benzaldehyde, with the acetaldehyde formed during fermentation. This reaction

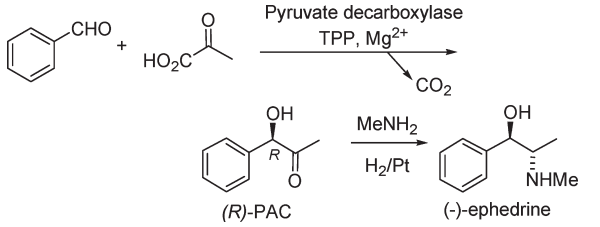


Figure 62. Yeast-mediated acyloin formation, as the initial step in (−)-ephedrine synthesis.

represents one of the first uses of biotransformations in industrial processes, and can be carried out either using free whole cells or immobilized ones.[384] The use of benzaldehyde acyloin obtained through this biocatalytic route for preparing (−)-ephedrine via reductive amination was patented,[428] and currently this biocatalytic process is used for the preparation of (*R*)-PAC as ephedrine precursor (Figure 62).[379,380,429,430] The enzyme responsible of the formation of acyloins in yeasts is Pyruvate decarboxylase. PDC catalyzes the non oxidative decarboxylation of pyruvate to acetaldehyde during yeast fermentation, and the C−C bond formation by addition of an anionic acetyl equivalent to an aldehyde acceptor.

Synthetic applications of PDC can involve then two different reactions: the nonoxidative decarboxylation of α-ketoacids to the corresponding aldehydes, and the carboligation side reaction forming α-hydroxy ketones.[363,378,402,431] The enzyme prefers short aliphatic acceptor aldehydes as substrates, and this preference is reflected in its carboligation reaction, where PDC catalyzes the conversion of benzaldehyde and acetaldehyde to (*R*)-PAC and predominantly (*S*)-acetoin (Figure 63).[402]

While the enzyme from *S. cerevisiae* accepts a broad range of α-ketoacids as substrates, the one from *Z. mobilis* is limited to unbranched aliphatic ones, such as α-keto-butanoic and α-keto-pentanoic acids.[378] The enzyme from *S. cerevisiae* is also capable to catalyze the acyloin-type condensation of acetaldehyde with different aromatic aldehydes, yielding a number of L-acetyl aromatic carbinols.[432] Aldehydes substituted in the *o*-position are poor substrates, whereas those having $-CH_3$, $-CF_3$, or -Cl substituents located in *p*-position produce higher yields than their *m*-counterparts (Figure 64).

The efficiency of the hydroxyketone formation can be improved by controlling the concentration of the cofactors ThDP and Mg^{2+} during the transformation,[433] and also toxic effects on cells of benzaldehydes can be counteracted by progressive addition of the substrates,[434] strain selection,[430] using immobilized cells,[435] or even carrying out the biotransformation using biphasic systems involving organic solvents in order to decrease the substrate concentration in the aqueous phase.[436] Additionally, a number of mutations in the active site of PDC have been developed and examined for their effects on both, the decarboxylation and carboligation reactions, leading to a widened substrate range regarding either donor or acceptor aldehydes.[437−442]

Benzoylformate can be converted to benzaldehyde using both free whole-cell BFD from *P. putida*, and the whole-cell immobilized in calcium alginate liquid-core capsules,[443] or in sepabeads.[444] However, the synthetic potential of BFD as catalyst emerges in the C−C carboligation reactions, that were described for the first time by Wilcocks and co-workers.[394,397] The authors reported the formation of (*S*)-2-hydroxy-1-phenylpropanone (2-HPP) by decarboxylation of benzoylformate in the presence of acetaldehyde, and analyzed the formation of other

dx.doi.org/10.1021/cr100299p |*Chem. Rev.* 2011, 111, 4346–4403

2-hydroxyketones from benzaldehyde and acetaldehyde as substrates, yielding the (*S*)-stereoisomer with enantiomeric excesses in the range of 91−92%. These findings appear as an advantage, allowing the possibility of using aldehydes directly, without the previous decarboxylation step, instead of the corresponding α-keto acids as substrates of BFD for the preparation of chiral α-hydroxy ketones.[387,422,430] With this new starting point, benzaldehyde derivatives have been used as substrates in acyloin condensations with acetaldehyde as acceptor, mediated by wild-type BFD from *P. putida*, yielding (*S*)-α-hydroxyketones in high enantiomeric excesses (Table 6).[387]

Systematic studies were then undertaken to characterize the substrate range of BFD in terms of steric and electronic influences of substituents in both the aromatic ring of the donor aldehyde, this effect is highly pronounced in *m*-substituted benzaldehydes,[399] as well as on acetaldehyde acting as acyl acceptor. The best substrates in terms of optical purity of the product seemed to be *m*-substituted benzaldehydes, reaching enantiomeric excesses up to 99% (entry 3, Table 6). On the other hand, *p*-substituted benzaldehydes led to significant decrease in conversion rates, and in most cases, in the enantioselectivity of the process (entry 4, Table 6). Although BFD was reported to have no activity with *o*-substituted benzaldehyde derivatives,[389−391] these authors found that decarboxylase activity with Cl, Br, methoxy and methyl substituents in *o*-position of the aromatic ring was rather low, but *o*-fluorobenzaldehyde is well accepted by the enzyme (entry 2, Table 6). It was also demonstrated that steric features affect conversion rates, as well

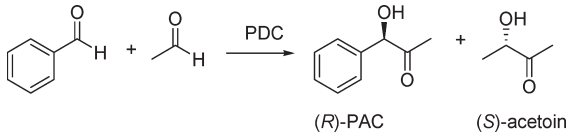


Figure 63. Carboligation products of acetaldehyde and benzaldehyde via PDC catalysis.

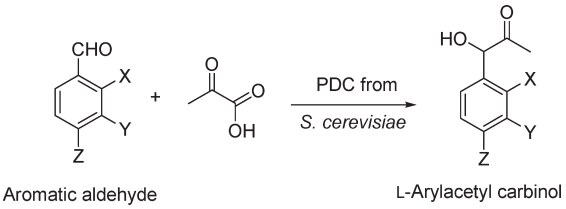
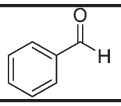
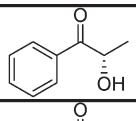
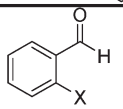
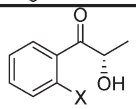
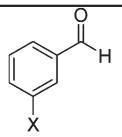
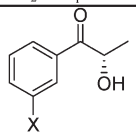
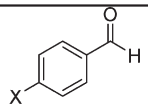
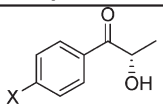
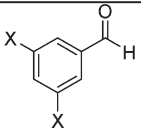
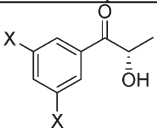


Figure 64. Synthesis of L-arylacetyl carbinols by PDC from *S. cerevisiae*.

Table 6. Benzaldehyde Derivatives as Donor Substrates in Wild-Type BFD from *P. putida* Catalyzed Benzoin Condensations with Acetaldehyde as Acceptor

Entry	Acyl donor	2-hydroxy ketone	Conversion (%)[a]	ee[b] (%)
1			99	92
2	 X = Me		4	n.d.
	X = F		91	89
	X = Cl, Br, OMe, OH, NO_2: No product formation observed[387,399]			
3	 X = F		100	87
	X = Cl		94	94
	X = Br		68	96
	X = Me		99	97
	X = OMe		94	96
	X = OEt		91	97
	X = O*i*Pr		62	>99
	X = OPh		12	>99
	X = OMOM		88	>99
	X = OAc		80	>99
	X = OH		62	92
	X = CN		95	92
	X = NO_2: No product formation observed[399]			
4	 X = F		69	87
	X = Cl		85	82
	X = Br		42	83
	X = Me		65	88
	X = OMe		23	92
	X = OH		11	86
	X = CN		89	74
	X = NO_2, NMe_3, NHAc: No product formation observed[399]			
5	 X = F		93	81
	X = OMe		11	>99

[a] Reactions were performed in buffer pH 7.0 at 25 °C during 20 h. [b] n.d.: Not determined.

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as enantioselectivities, while *ee* increases with bulky groups in the aromatic ring, and simultaneously decreases the conversion rate (see *o*- and *m*-substituted aldehydes, entries 2 and 3, Table 6).

Regarding the acyl acceptor aldehyde, acetaldehyde proved to be the most efficient in the series tested by Pohl and co-workers.[387] BFD showed low activity with benzaldehyde to form (*R*)-benzoin, and in the synthesis of acetoin (entry 1, Table 6) the weak decarboxylase activity toward pyruvate is consistent with proposing that acetaldehyde might bind to the active site of the enzyme. Furthermore, the carboligation of benzaldehyde as donor with propionaldehyde, 2-chloroacetaldehyde, glycolaldehyde, acrolein, or propynal was not catalyzed by BFD.

BFD from *P. putida* was subjected to directed evolution with the aim of generating mutants with enhanced carboligase activity or enzyme stability in nonaqueous solvents.[366,395,400,402,424] Lingen and co-workers reported that after a single round of random mutagenesis, mutants which exhibited a 5-fold increased carboligase activity toward benzaldehyde and acetaldehyde compared to the wild-type enzyme were isolated, obtaining high enantiomeric excesses for (*S*)-HPP.[395] Also mutants with enhanced carboligase activity in water-miscible organic solvents have been isolated from the same library. In the highly active mutants, Leu476, which is not located in the active site of the enzyme, has been mutated. The substrate specificity regarding substituted aromatic aldehydes was also widened though error-prone PCR, identifying Leu476Gln and Met365Leu-Leu461Ser mutants, which accepted *o*-substituted benzaldehyde derivatives as donor substrates.[401] Both variants catalyzed the formation of enantiopure (*S*)-2-hydroxy-1-(2-methyl)propan-1-one with excellent yields, a reaction which is only poorly catalyzed by the wild type enzyme.

Bifunctionalized aldehydes were also evaluated as substrates of BFD, aiming at the enantioselective synthesis of chiral transition metal complexes for catalytic asymmetric processes and chiral auxiliaries (Figure 65).[398]

Carboligation of aliphatic aldehydes as donor substrates with acetaldehyde as acceptors, mediated by BFD was also investigated by Pohl et al. (Table 7).[387] The authors suggested that the lower conversion rate and *ee* in carboligation of cyclohexane carbaldehyde with acetaldehyde, compared to cyclohexene carbaldehyde with the same acceptor, might be a consequence of the occurrence of more unstable interactions in the active site of the enzyme. Furthermore, aromatic substrates are preferred than aliphatics due to stabilization by specific supramolecular interactions in the active site of the enzyme. The steric fit of planar aromatic substrates with Phe397 and Phe464 are desired in the catalytic process.

The synthetic potential of benzaldehyde lyase for both, C−C bond cleavage and formation was demonstrated for the first time by Müller et al., with the formation of benzaldehyde from (*R*)-benzoin, and the acyloin condensation of benzaldehyde in aqueous buffer, for the almost quantitative synthesis of (*R*)-benzoin in an enantiomerically pure form (ee >99%). However, the reaction furnished low to moderate yields, which was attributed to the low solubility of aromatic substrates in aqueous buffer solutions.[407] Regarding this drawback, various strategies were investigated. The addition of cosolvents, such as DMSO,

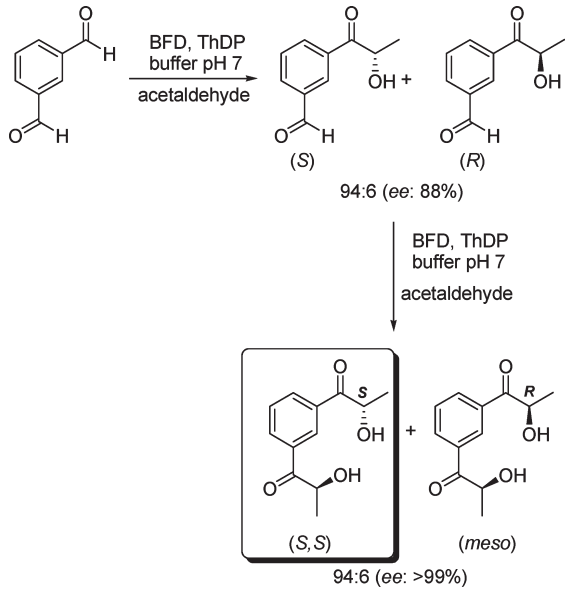


Figure 65. Selective formation of *bis*-(α-hydroxyketones) *via* BFD-catalyzed carboligation, as key step for the preparation of chiral auxiliaries for asymmetric synthesis.

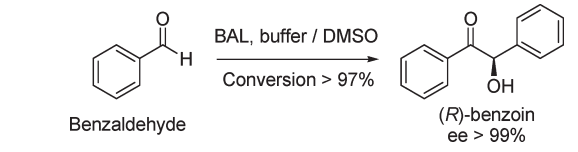
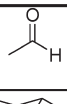
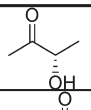
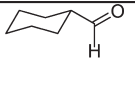
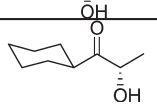
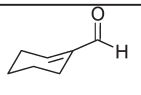
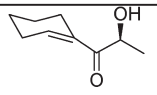


Figure 66. BAL-catalyzed acyloin self-condensation of benzaldehyde.

Table 7. Aliphatic Aldehydes as Donor Substrates in Wild-Type BFD from *P. putida* Catalyzed Benzoin Condensations with Acetaldehyde as Acceptor

Entry	Acyl donor	2-hydroxy ketone	Conversion (%)[a]	ee (%)
1			n.d.	13
2			21	61
3			50	94

[a] n.d.: Not determined.

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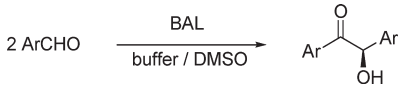
Table 8. BAL-Catalyzed Carboligation of Aromatic Aldehydes to the Corresponding (*R*)-Benzoins



entry	Ar	yield (%)[a]	ee (%)[b]
1	2-FC_6H_4	68	96
2	2-ClC_6H_4	80	97
3	2-BrC_6H_4	90	>99
4	2-$MeOC_6H_4$	87	>99
5	3-FC_6H_4	80	97
6	3-ClC_6H_4	94	>99
7	3-BrC_6H_4	94	>99
8	3-$MeOC_6H_4$	93	>99
9	3-HOC_6H_4	84	n.d.
10	4-FC_6H_4	89	>99
11	4-ClC_6H_4	95	>99
12	4-BrC_6H_4	83	>99
13	4-$MeOC_6H_4$	95	>99
14	4-MeC_6H_4	94	>99
15	2,4-$F_2C_6H_3$	87	>99
16	2-naphtalenyl	98	>99
17	2-furanyl	88	92
18	2-thienyl	73	91

[a] Reactions were performed in potassium phosphate buffer [(50 mmol/L, pH 7.0) containing $MgSO_4$ (2.5 mmol/L) and ThDP (0.15 mmol/L) at 21 °C during 6 h. [b] n.d.: Not determined

appears as adequate to facilitate the formation of acyloins in higher yields. Accordingly, the addition of 20% (v/v) of DMSO to the aqueous solution containing BAL resulted in the quantitative formation of (*R*)-benzoin (Figure 66).

Detailed studies of the reaction parameters on the enzymatic activity and stability in BAL-catalyzed carboligation with the recombinant enzyme as well as some enzyme variants have been also reported.[412,414,445,446] The reaction was optimized by Liese and colleagues exploring the influence of the cosolvents such as DMSO, the role of the cofactor ThDP, the optimum pH for the reaction medium, and the substrate ratio in cross condensations.[412] Continuous processes were also developed, based on kinetic studies of the reaction,[447] including membrane reactors that combines the advantages of high conversion and excellent selectivities with high space-time yields and high total turnover numbers for gram-scale reactions.[448,449]

Enzyme immobilization is another alternative that allows the improvement of the process in terms of productivity.[423,450−455] BAL-catalyzed benzoin condensations were performed in gel-stabilized two-phase systems, where the enzyme in the aqueous phase was entrapped with polyvinyl alcohol (PVA), while the organic solvent acted as a reservoir for substrates and products.[423] Self-condensation of 3-furaldehyde could also be conducted with BAL immobilized in PVA criogels, enabling the synthetic application of the biocatalyst in unfavorable nonaqueous media, so providing a key for successful application of complex multistep biocatalyzed synthesis.[451,452] The application of immobilized enzymes by metal ion affinity binding[453,456] or on superparamagnetic solid supports[455] was also tested in repetitive

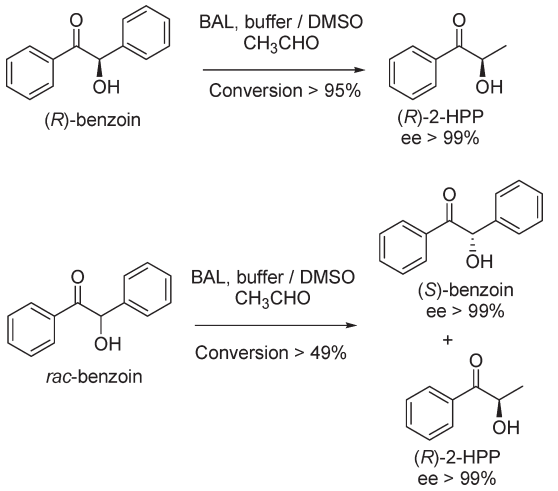


Figure 67. Different types of reactions catalyzed by BAL.

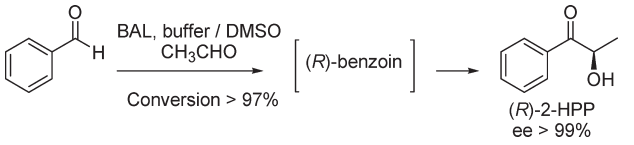


Figure 68. BAL-mediated acyloin condensation between benzaldehyde and acetaldehyde.

batch reaction, and in continuously operated plug flow reactor, with hexahistidine-tagged benzaldehyde lyase, improving the cost effectiveness of the catalyst. Gas-phase carboligation was investigated by immobilizing the catalyst, namely BAL or BFD, via deposition on nonporous supports, showing the gas−solid system as an interesting tool for conducting reactions involving volatile products.[454]

The reactions involving BAL-catalysis was scaled up to a semipreparative scale and different (*R*)-benzoins were obtained in high yield and almost in an enantiomerically pure form from different aromatic and heteroaromatic aldehydes (Table 8).[396]

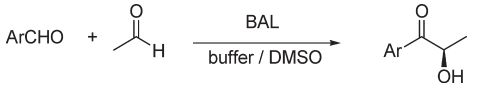
Widening the substrate spectrum of BFD, BAL also accepts aromatic aldehydes substituted at the *ortho*-position (entries 1−4, Table 8). On the other hand, a few aromatic aldehydes such as pyridine-3- and −4-carbaldehyde, 4-hydroxybenzaldehyde and pyrrole-2-carbaldehyde, as well as sterically demanding aldehydes like vanillin, isovanillin and 3,4-dimethoxybenzaldehyde gave either very low yields or no benzoin condensation.[396]

Additionally, when (*R*)-benzoin reacted with BAL in the presence of acetaldehyde, (*R*)-2-HPP was formed quantitatively in an optically pure form, while the same reaction starting from (*S*)-benzoin failed. When the reaction was performed with *rac*-benzoin as substrate, both (*R*)-2-HPP and (*S*)-benzoin were obtained with high enantiomeric excesses (ee >99%) (Figure 67).

Other aromatic and heteroaromatic benzoin-like acyloins were accepted as substrates for the kinetic resolution of racemic mixtures via C−C bond cleavage.[407]

BAL also catalyzed the condensation of benzaldehyde with acetaldehyde, showing complementary stereochemistry with BFD, giving (*R*)-2-HPP in high yield (95%) and enantiomeric excesses (>99%) (Figure 68).

Table 9. BAL-Catalyzed Carboligation of Aromatic Aldehydes and Acetaldehyde, to the Corresponding (*R*)-2-Hydroxypropiophenone Derivatives



entry	Ar	yield (%)[a]	ee (%)[b]
1	2-FC_6H_4	64	>99
2	2-$MeOC_6H_4$	63	97
3	3-FC_6H_4	85	>99
4	3-ClC_6H_4	94	95
5	3-BrC_6H_4	88	>99
6	3-$MeOC_6H_4$	80	93
7	4-ClC_6H_4	88	>99
8	4-BrC_6H_4	86	>99
9	4-$MeOC_6H_4$	64	>99
10	2,4-FC_6H_3	65	>99
11	3,5-FC_6H_3	67	97
12	3-OH-4-$MeOC_6H_3$	80	>99
13	3-MeO-4-HOC_6H_3	90	n.d.
14	3,4,5-$(MeO)_3$-C_6H_2	92	n.d.
15	2-furanyl	61	>99

[a] Reactions were performed in potassium phosphate buffer [(50 mmol/L, pH 7.0) containing $MgSO_4$ (2.5 mmol/L) and ThDP (0.15 mmol/L)] at 21 or 25 °C during 6 or 48 h. [b] n.d.: Not determined

Because of the "catalytic promiscuity" of BAL, a wide range or aromatic aldehydes substituted at ortho-, meta-, or para-positions with electron-releasing as well as electron-withdrawing properties (i.e., chloro, fluor, and methoxy groups) are also accepted as substrates (Table 9).[396,407]

It can be observed that *o*-substituted aromatic aldehydes afforded the corresponding (*R*)-2-HPP derivatives in lower yields than *m*- and *p*-substituted aldehydes. Furthermore, 2-chloro-and 2-methylbenzaldehyde gave no acyloin formation. Comparing the catalytic behavior of BAL in benzoin condensation of 2-methoxyacetaldehyde (87% yield, Table 8) with the corresponding acyloin condensation of 2-methoxybenzaldehyde and acetaldehyde (63% yield, Table 9), it is noteworthy the preference of the enzyme for aromatic aldehydes as substrates. Sterically demanding aromatic aldehydes were also accepted by BAL as donor substrates in the presence of acetaldehyde (entries 12−14, Table 9). It is important to highlight that BAL and BFD are enantiocomplementary enzymes with regard to the formation of 2-HPP derivatives, as well as aliphatic acyloins such as propioin. Thus, most of this class of valuable synthons, except for *o*-substituted (*S*)-2-HPP derivatives, can be synthesized in either enantiomeric form using these two enzymes.[396,415,416,422] Exploiting this complementary behavior, interesting building blocks for asymmetric synthesis, such as optically active vicinal diols, can be obtained in enantiopure forms. All four possible 1-phenylpropane-1,2-diol stereoisomers were synthesized separately employing a combination of the enantioselective acyloin condensation of benzaldehyde and acetaldehyde in BAL-or BFD-catalyzed reactions, followed by a diastereoselective alcohol dehydrogenase-catalyzed reaction.[408] The corresponding diols were obtained in all cases in high yields (81−90%) and excellent diastereomeric excesses (>98% de).

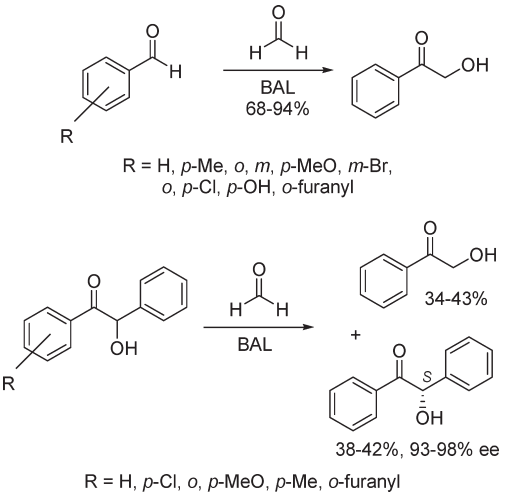


Figure 69. BAL-mediated synthesis of 2-hydroxy-1-arylethan-1-one derivatives.

Also in contrast to BFD, BAL can accept longer chain aliphatic aldehydes such as propionaldehyde or butyraldehyde, as well as olefinic aromatic aldehydes (i.e., cinnamaldehyde) as acceptors.[407] Additionally, neither BFD nor PDC can catalyze the carboligation of aromatic aldehydes with acetaldehyde derivatives, however BAL can accept mono- and dimethoxyacetaldehyde as substrates reacting with a diverse range or aromatic aldehydes,[409] and leading to the formation of important chiral polyoxigenated compounds such as HPP derivatives.

Hydroxyacetophenone (HAP) derivatives can also be prepared either through BAL-assisted carboligation of aromatic aldehydes with formaldehyde or kinetic resolution of benzoin derivatives (Figure 69).[411]

A broad range of aromatic and heteroaromatic aldehydes were used by Demir and colleagues as substrates for carboligation with formaldehyde, providing the corresponding HAP derivatives in different yields, depending on the structure of the aldehyde. Thus, steric and electronic demands of the substituents play important roles in the reaction yield. Fluorine substitution on 2- and 2,4-positions of the aromatic ring decreases the overall yield, and also does pyridine carboxaldehyde. Accordingly, when (*R*)-benzoin reacted with formaldehyde in the presence of BAL, 2-hydroxy-1-phenylethan-1-one was obtained in high yield, while the same reaction starting from (*S*)-benzoin failed. The reaction using *rac*-benzoin as starting material resulted in the kinetic resolution of the racemic mixture, remaining (*S*)-benzoin in an enantiomerically pure form.

Optically pure (*R*)-3,3-dimethoxybenzoin was synthesized in preparative scale (3 g scale) in 93% yield, starting from 3-methoxybenzaldehyde, with BAL at room temperature, showing the usefulness of the enzyme for synthetic applications (Figure 70).[407]

The enzyme-catalyzed asymmetric cross-benzoin condensation was also described with the alternate use of BAL and BFD His281Ala,[424] leading to a donor−acceptor concept for the enantioselective synthesis of mixed benzoins, showing the same absolute configuration in the final compound, for both enzymes (Figure 71).[400]

The results showed a donor−acceptor selectivity of the enzymes. The initial experiments (Table 10) were performed as a substrate-enzyme screening, with 2-chlorobenzaldehyde (entry 1), 2-methylbenzaldehyde (entry 2), 2-methoxybenzaldehyde (entry 3),

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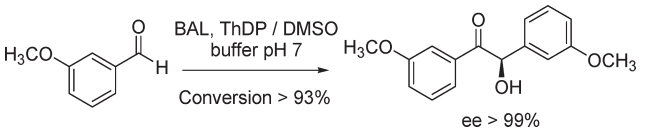


Figure 70. Preparative synthesis of (*R*)-3,3-dimethoxybenzoin through BAL-mediated acyloin condensation.

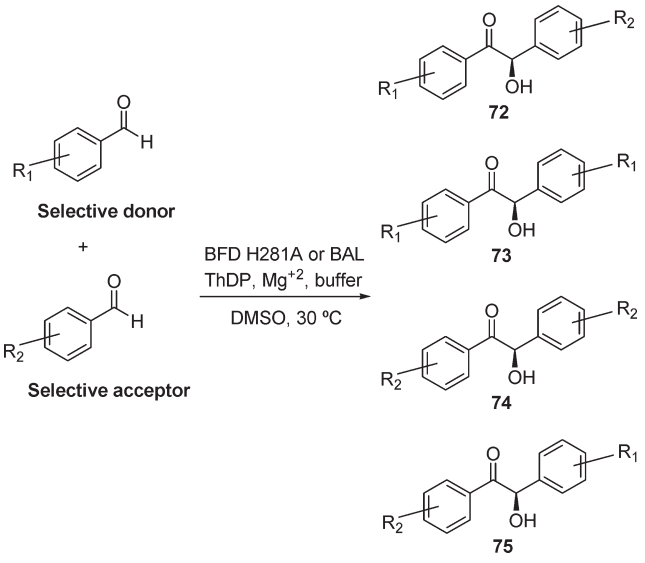


Figure 71. Enzyme-catalyzed cross-benzoin condensation of substituted aldehydes. Substituents R_1 and R_2: See Table 10.

Table 10. Combined Enzyme-Substrate Screening for the Asymmetric Synthesis of Mixed Benzoins

entry	R_1	R_2	enzyme	**72** (%)	**73** (%)	**74** (%)	**75** (%)
1	H	2-Cl	BFD H281A	90	10		
			BAL	70	16	14	
2	H	2-Me	BFD H281A	34	66		
			BAL	39	30	12	19
3	H	2-MeO	BFD H281A	40	60		
			BAL	66	25	10	
4	H	2,6-F_2	BFD H281A				
			BAL	88	12		
5	H	2,3,5-F_3	BFD H281A	53	46	1	
			BAL	67	24	9	
6	H	2,3,4,5,6-F_5	BFD H281A				
			BAL	90	10		

and fluorinated benzaldehydes (entries 4−6) with benzaldehyde as donor, in the presence of both ThDP-dependent enzymes.

As shown in Table 10, 2,2-disubstituted benzoins **74** or mixed benzoins **75** substituted in 2-position (entries 1−3) were not generated by BFD His281Ala, while 2,6- and 2,3,4,5,6-Fluoro substituted dibenzoins **74** and 2,6- and 2,3,4,5,6-Fluoro substituted mixed benzoins **75** (entries 4 and 6) were not formed in the BAL-catalyzed reactions. These results show that the screened acceptors react selectively in the presence of the respective biocatalyst.

Additional experiments were performed in preparative scale, looking for selective donors substrates in the presence of BAL

Table 11. Mixed Benzoins Synthesized Chemoselectively and Asymmetrically on a Preparative Scale

entry	donor aldehyde	enzyme	conversion (%)	selectivity[a] (%)	ee (%)
1	3-CN	BFD H281A	>99	>99	90
2	4-Br	BFD H281A	90	95	95
3	4-CF_3	BFD H281A	75	>99	93
4	3,4-CH_2O_2	BAL	98	83	>99
5	3,4,5-$(CH_3O)_3$	BAL	82	97	>99
6	3,5-$(CH_3O)_2$	BAL	>99	95	>99

[a] Selectivity is defined as the percent ratio of product in relation to the sum of all benzoin obtained.

and BFD His281Ala, and a broad variety of reactive benzaldehyde derivatives was identified by reacting with 2-chlorobenzaldehyde as acceptor aldehyde (Table 11).

Unsurprisingly, BAL has a much broader substrate range concerning aromatic aldehydes with sterically demanding substituents, while BFD His281Ala shows higher selectivity with aromatic aldehydes with small substituents. Thus, the complementary substrate range of the enzymes enables the synthesis of a large diversity of mixed benzoins.

The identified donor aldehydes were then evaluated with selective fluorinated acceptors, in the presence of BAL, and the mixed benzoins type **76** were obtained in most cases with high-to-excellent selectivities.

Enzymatic condensation of benzaldehydes and phenylacetaldehydes could lead to chiral α-hydroxychalcones. The first step was the BAL-catalyzed carboligation of methoxybenzaldehydes with phenylacetaldehyde to the corresponding α-hydroxydihydrochalcones (**76**) (Figure 72),[410] which upon subsequent chemical demethylation and dehydrogenation, afforded the final products. According to this strategy, phenylacetaldehyde was examined as acceptor substrate in the biocatalyzed formation of mixed acyloins with a recombinant BAL cloned in *E. coli* JM109 and partially purified, using methoxybenzaldehydes as donors.

BAL reactions between phenylacetaldehyde and several mono- and dimethoxybenzaldehydes (**a**−**e**) gave mixtures of products **76**, **77**, and **78** (Figure 72). Interestingly, phenylacetaldehyde self-condensation products were not observed, while different proportions of **76**, **77**, and **78** were obtained in moderate yields depending on the structures of benzaldehyde reactants. All possible products were produced with 2-methoxybenzaldehyde as donor, while with dimethoxy substituted aldehydes compounds **78** was not obtained.

Also α,β-unsaturated aldehydes were evaluated as substrates for asymmetric C−C bond forming reactions with aliphatic aldehydes, mediated by ThDP-dependent enzymes, namely, PDC, BAL, and BFD.[386] These substrates might act as both donors and acceptors for the 1,2- and 1,4-addition. According to each reaction, α,β-unsaturated aldehydes or aliphatic aldehydes can bind to ThDP, and react as donors resulting in the formation of different hydroxyketones or hydroxyenones. Different α,β-unsaturated aldehydes were evaluated in BAL-, wild-type BFD- (BFDwt), and variant BFD-His281Ala-catalyzed C−C coupling reactions using acetaldehyde as acceptor substrate (Figure 73).

BAL-catalyzed the reaction of acetaldehyde and several α,β-unsaturated aldehydes as donor substrates, giving the corresponding (*R*)-α,β-unsaturated acyloins, in yields from poor to

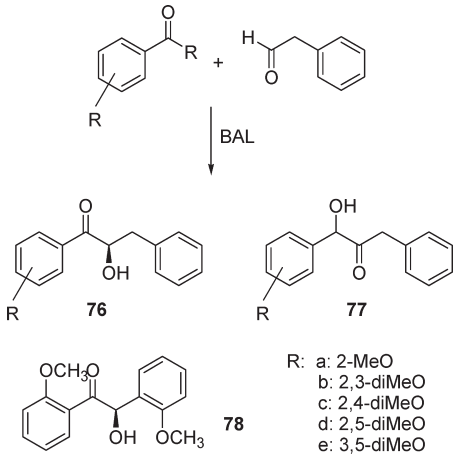


Figure 72. BAL-catalyzed phenylacetaldehyde and benzaldehyde condensations.

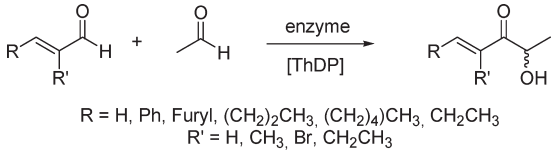


Figure 73. Acyloin condensation of α,β-unsaturated aldehydes with acetaldehyde, catalyzed by BAL, BFDwt, or BFD His281Ala.

very good, and moderate to excellent enantioselectivities. On the other hand, hexenal and octenal were donor substrates for BFDwt and only hexenal was substrate in the ligation with acetaldehyde in the reaction catalyzed by BFD His281Ala, with moderate conversion and poor enantioselectivity. The scope of acceptor substrates was extended in BAL-catalyzed reactions, to formaldehyde, aromatic aldehydes, and α-heteroatom-substituted aldehydes, such as methoxy- and dimethoxyacetaldehyde. These substituted aldehydes behave different depending on the donor substrate, giving from poor to moderate yields when using α-methylcinnamaldehyde, furylacrolein and 2-methylenebutanal, whereas carbocyclic aldehydes gave the desired products in good yields with *R*-configuration in the new created stereocenter. The acceptor aldehyde adds in all cases to the carbonyl carbon of the α,β-unsaturated aldehydes (1,2-addition). Additionally, α,β-unsaturated aldehydes reacted with pyruvate in the presence of crude extract of *E.coli* cells overexpressing the PDC gene from *Z. mobilis* (*Zm*PDC), and only 1,2-regioselectivity was observed in the addition reaction. Benzaldehyde, 2-Cl- and 2-F-benzaldehyde were investigated as possible acceptors with pyruvate as donor, and no condensation products were observed.

The activity and substrate specificity of these ThDP-depending enzymes can be modified by site-directed mutagenesis and directed evolution techniques, to interconvert catalytic properties of one enzyme into each other's. Pohl and co-workers reported the exchange of the substrate specificity of PDC from *Z. mobilis* and BFD from *P. putida* through site-directed mutagenesis.[402] Both proteins share about 18% sequence identity and 22% sequence similarity. Two highly conserved residues, Ile472 and Ile476 in the active site of PDC, have been identified as playing a key role in the binding of substrates to the enzyme, and have BFD counterparts in Ala460 and Phe464.[439] To evaluate whether these analogous residues have similar effects in the substrate specificity and enantioselectivity of both enzymes, residues Ile472 and Ile476 in PDC were replaced with alanine and phenylalanine respectively. Similarly, the BFD residues Ala460 and Phe464 were both replaced with isoleucine. Variants of both enzymes were prepared with individual mutations, as well as containing both mutations. With the Ile472Ala mutation, BFD decarboxylase activity was introduced into PDC, and it was stated that Ile472 plays a significant role in substrate specificity or PDC, but has limited role in catalysis. On the other hand, although Ile476 has a role in substrate recognition and binding, its critical role is in positioning the substrate for catalysis. The approach was also successful when introducing PDC activity into BFD. However, pyruvate was bound with low affinity, suggesting that there are other residues also involved in the enzyme specificity toward small aliphatic 2-keto acids. In this way, exchanging structurally equivalent residues in the active sites in PDC and BFD, two effective medium-chain 2-keto acid decarboxylase, PDC-Ile472Ala and BFD-Ala460Ile were obtained. Furthermore, in addition to its decarboxylase activity, PDC-Ile472Ala is also able to stereospecifically catalyze the ligation of C3−C6 aldehydes with benzaldehyde in moderate yields. Similarly, the variant BFD-Ala460Ile/Phe464Ile showed reversal enantioselectivity than wild-type BFD.

On the other hand, BFD and BAL share 24% sequence identity, both acting on aliphatic and aromatic substrates and carrying out carboligation reactions providing products with opposite stereochemistry for aliphatic acceptors, and the same stereochemistry for aromatic ones (compare Figures 65 and 71).[168] Examination of the active sites of both enzymes showed that Ser26 and Ala28 occupy similar positions in BFD and BAL respectively. Furthermore, the replacement of Ala28 by serine provided a BAL variant which K_m value for (*R*)-benzoin was virtually identical to that of the wild-type enzyme, but whose k_{cat} value was reduced 10-fold.[457] The mutation Ala28Ser in BAL allowed the enzyme to decarboxylate benzoylformate, displaying a substrate tolerance similar to that of BFD, albeit with reduced activity. Based on these findings, McLeish et al. demonstrated that this point mutations can convert BAL into a BFD.[417] The authors reported that Ser26 may play a role in removing CO_2 from the active site of BFD.

Phenylpyruvate decarboxylase was described for the first time as catalyst in asymmetric acyloin condensations by Patel and coworkers, in the reaction of phenylpyruvic acid with acetaldehyde, to produce (*R*)-(-)-3-hydroxyphenyl-2-butanone, using enzymes from *Achromobacter eurydice* and *Pseudomonas aromatica* (Figure 74).[403] The acyloin products were obtained with high stereoselectivity (95 and 84% ee, respectively).

The substrate range accepted by PhPDC was subsequently outstretched for both, the donor α-keto acid and the acceptor aldehyde.[406] The substrate range tolerated for the acceptor aldehyde was studied using phenylpyruvate as acyl donor, and the partially purified enzyme (Figure 75).

With straight chain aliphatic aldehydes, the yield of the corresponding acyloin decreased while increasing the chain length (**a**−**d**, entries 1−4, Table 12). When using longer chain aliphatic aldehydes (**h**, **i**, **j**), aromatic aldehydes (**e**, **f**, **k**), hindered aldehydes (**l**, **m**, **n**) as well as bromoacetaldehyde (**o**), bromopropionaldehyde (**p**), and acrolein (**q**) (entry 5, Table 12), negligible amounts (0−3%) of acyloin products were obtained. With chloroacetaldehyde (**r**) and glycolaldehyde (**s**) (entries 6 and 7, Table 12), 13 and 16% conversion were achieved,

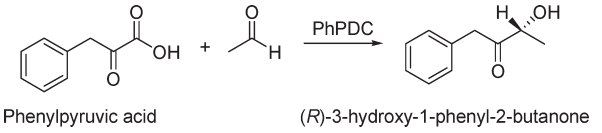
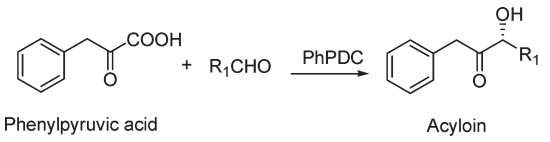


Figure 74. Asymmetric acyloin condensation of phenylpyruvic acid with acetaldehyde, catalyzed by PhPDC.



R_1: a: Me; b: Et; c: *n*-Pr; d: *n*-Bu; e: $PhCH_2$-; f: Ph; g: H;

h: $Me(CH_2)_6$-; i: $Me(CH_2)_8$-; j: $Me(CH_2)_{10}$-; k: PhCH=CH-;

l: Br_3C-; m: Me_2CH-; n: Me_3CCH_2-; o: $BrCH_2$-; p: $BrCH_2CH_2$-;

q: CH_2=CH-; r: $ClCH_2$-; s: $HOCH_2$-

Figure 75. Acceptor aldehydes evaluated as substrates of PhPDC.

Table 12. Substrate Specificity of PhPDC

entry	substituent (R)	yield (%)	enantiomeric excess (%)
1	**a**	76	87
2	**b**	55	98
3	**c**	38	98
4	**d**	24	92
5	**e**−**q**	<3	
6	**r**	13	
7	**s**	16	

respectively, and no sterical discrimination was observed. Formaldehyde (**g**) was not substrate of the enzyme.

Though both PhPDC and BFD accept acetaldehyde as substrate, the substrate specificity of these enzymes is different, since the later accepts aromatic aldehydes but does not accept propionaldehyde, chloroacetaldehyde, and glycolaldehyde.[387,422]

PhPDC-mediated acyloin condensation was also studied varying the donor α-keto acid.[406] Indole-3-pyruvic acid reacted with acetaldehyde as acceptor, giving (*R*)-3-hydroxy-1-(3-indolyl)-2-butanone in 19% yield (Figure 76), while with benzoylformic acid no acyloin product was formed.

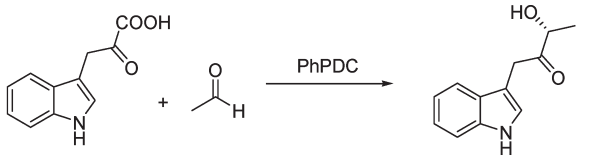


Figure 76. PhPDC-mediated acyloin condensation of Indole-3-pyruvic acid as donor and acetaldehyde as acceptor aldehyde.

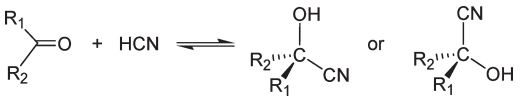


Figure 77. Cyanohydrin formation. R^1= alkyl, cycloalkyl, aryl, heteroaryl; R^2= H, alkyl.

4. CYANOHYDRIN FORMATION

4.1. Enzymes Involved. Classification

Hydroxynitrile lyases (HNLs) or oxynitrilases (EC 4.1.2.x), whose in vivo function is the cleavage of cyanohydrins, belong to the classes of enzymes that are used for biocatalytic carbon−carbon bond formation in synthetic and industrial processes.[10]

The in vitro reversibility of this reaction enables the enantioselective condensation of hydrocyanic acid (HCN) with aldehydes or ketones yielding (*R*)- or (*S*)-α-hydroxy nitriles (Figure 77).

Theses enantiomerically enriched cyanohydrin synthons have significant utility. They are bifunctional molecules because of the ability of both the nitrile and alcohol groups to be effectively derivatized without reducing optical purity. Despite their limited stability, especially at elevated pH values, cyanohydrins can be used as versatile polyfunctional building blocks for a myriad of products, such as α-hydroxyketones,[458] *β*-hydroxyamines,[459−461] α-aminonitriles,[462,463] α-hydroxy carboxylic acids,[464,465] α-sulfonyloxynitriles,[466] α-fluoronitriles,[467] α-hydroxyesteres,[466] aziridines,[468] α-azidonitriles,[469] among other derivatives.[470,471]

Consequently, hydroxynitrile lyases have emerged as potential biocatalysts for the synthesis of a variety of chiral compounds, which find applications in pharmaceutical, agrochemical and cosmetic formulations. So, over the past years, much research efforts on the study of chemical and biological catalysts useful for asymmetric cyanohydrin production have been devoted.[471−478]

4.1.1. Enzymes Involved. Hydroxynitrile lyases are widely distributed in nature, being found in more than 3000 plants species. Their ability to release HCN from the tissue is a process known as cyanogenesis. This phenomenon is widely observed in higher plants, as well as a few nonplant sources (diverse groups of organisms like bacteria,[479−481] fungi, lichens, millipedes (*Apheloria corrugata*), and insects (*Zygaena trifollii*, a moth).[482−484] They have been found to possess two main functions: (i) plant defense and (ii) nitrogen source (Figure 78).[471,474,476,478]

More than ten different HNLs from six different plant families have been isolated. However, preparative applications in organic synthesis have been described only for a few of them. *R*-Selective hydroxynitrile lyases can be found in *Prunus amygdalus* (*Pa*-HNL, EC 4.1.2.10, almonds) and other *Prunus* sp., *Linum usitatissimum* (*Lu*-HNL, flax), and *Phlebodium aureum* (*Pha*-HNL, fern). *S*-Selective HNLs are present in *Hevea brasiliensis* (*Hb*-HNL, EC 4.1.2.39, tropical rubber tree), *Manihot esculenta* (*Me*-HNL, EC 4.1.2.38, cassava), and *Sorghum bicolor* (*Sb*-HNL, EC 4.1.2.11, millet) (Table 13). Accessible amount of the respective HNLs depends on the natural sources they are isolated from. Some of them contain small amounts of HNLs while others contain large amounts of the desired enzyme. So, different strategies have been used to identify potential novel or better performing HNL activities, namely, screening plants for appropriate enzymes,[485−487] directed evolution, rational design, or metagenomic approaches.[488−490] All these strategies need simple and powerful high-throughput screening (HTS) assays. HNL-activity has usually been determined by GC or HPLC analysis,[485] and spectrophotometric assays.[491−494] Recently, a colony assay based on NADH-fluorescence has been developed to detect

HNL-activity,[495] as well as a novel assay in which the HCN liberated by the HNL-catalyzed reaction is detected by a visible colorimetric reaction.[496]

Some new HNLs have been reported by Asano et al. in 2005.[485] A screening of 163 plant species as potential sources of HNLs established that homogenates from *Baliosperman montanum* showed (*S*)-HNL activity, whereas leaves and seeds of *Passiflora edulis*, and seeds from *Eriobotrya japonica*, *Chaenomles sinensis*, *Sorbus aucuparia*, *Prunus mume*, and *Prunus persica* displayed (*R*)-HNL activity.

4.1.2. Classification, Characterization, and Reaction Mechanism. Differences between HNL enzymes rely not only in their biochemical properties and enantiospecificities but also in their substrate specificities.

Phylogenetically, HNLs appear to have resulted from a convergent evolution mechanism. There are HNL enzymes related to FAD-dependent oxidoreductases (*Pa*-HNL), serine carboxypeptidases (*Sb*-HNL), Zn^{2+}-containing alcohol dehydrogenases (*Lu*-HNL), and α/β hydrolases (*Hb*-HNL and *Me*-HNL).[497,498]

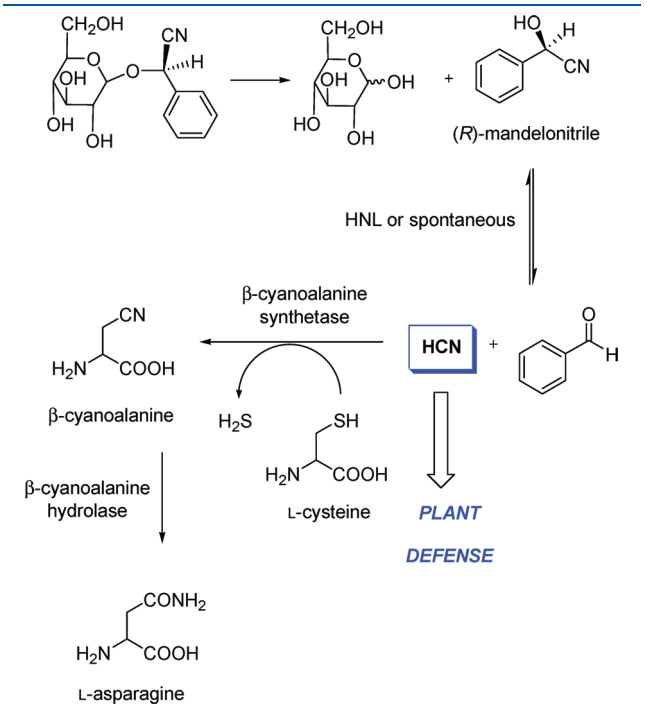


Figure 78. Catabolism of cyanogenic glycosides.

However, a classification of HNLs in two groups based in the presence or absence of FAD was advanced and is still in use.[476,484,499−501]

The FAD-containing enzymes have been isolated exclusively from species of the *Prunoideae* and *Maloideae* subfamilies of the *Rosaceae*. They are single chain glycoproteins, with molecular masses ranging from 50 to 80 kDa. (*R*)-Selective hydroxynitrile lyase, like *P. amygdalus* (*Pa*-HNL) as well as other (*R*)-(+)-mandelonitrile lyases (MDL-EC 4.2.1.10) belong to this group.[501,502]

Sequence and biochemical data are available for FAD-HNLs from the *Prunoidea* subfamily, from almonds (*Prunus amygdalus*, PaHNL) and black cherries (*Prunus serotina*, *Ps*-HNL).[503]

The mechanism of this hydroxynitrile lyase-catalyzed reaction involves general acid−base catalysis, and this would be expected to be the case in the active site of the enzyme. In spite of that, there is no obvious role for FAD in the reaction mechanism.[504] Nevertheless, the flavin cofactor has been shown to be intimately involved in the architecture of the catalytic site. Binding of competitive inhibitors affects the absorption spectrum of the flavin, indicating its proximity to the active site.[501,505] So, it is known that FAD cofactor is not involved in net redox reaction[475] but their removal causes inactivation of the enzyme.[505,506]

Enzymes belonging to the FAD-independent hydroxynitrile lyase group, are found in *S. bicolor*,[497,507,508] *M. esculenta*,[509] *H. brasiliensis*,[507,510,511] *P. aureum*,[512] and *L.usitatissimum*.[513] They are *R* and *S* selective enzymes, some of which belong to the structural class of α/β-hydrolases[514] such as *Hb*-HNL and *Me*-HNL. Others, like *Lu*-HNL have homology to Zn^{2+}-containing alcohol dehydrogenases (Zn^{2+}-ADHs).[498,515]

To date 3D structures are known for four HNLs, from *H. brasiliensis* (*Hb*-HNL),[475,511,516−519] *M. esculenta* (*Me*-HNL),[520,521] *S. bicolor* (*Sb*-HNL),[508,522] and *P. amygdalus* (*Pa*-HNL).[475,503] Investigations on the three-dimensional structure of HNLs from different sources,[523] together with kinetic enzyme studies[520,522,524−526] and genetic studies[527,528] are in progress. Recently, new HNLs have been found, purified and characterized aiming at better application in asymmetric synthesis.[504,529−533]

Wagner et al. reported in 1996,[534] that the active site of HNL (studied for *Hb*-HNL) is located deeply in the protein molecule and connects to the surface of the protein by a narrow channel. The active site cavity is predominantly hydrophobic in nature and the catalytic triad consists of residues Ser80-His235-Asp207 (*H. brasiliensis* numbering). Site-directed mutagenesis

Table 13. Source of Hydroxynitrile Lyases and Enantioselectivity

species (family)	EC no.	enzyme source	*R*/*S* selectivity
P. amygdalusPa-HNL, (Rosaceae)	4.1.2.10	almond bran	*R*
Prunus sp.(Rosaceae)	4.1.2.10	apricot, cherry, plum, peach kernel and leaves	*R*
L. usitatissimum Lu-HNL, (Linaceae)	4.1.2.37	young flax plants	*R*
H. brasiliensis Hb-HNL (Euphorbiaceae)	4.1.2.39	tropical rubber tree leves	*S*
M. esculenta Me-HNL, (Euphorbiaceae)	4.1.2.38	manioc leaves	*S*
S. bicolor Sb-HNL,(Poaceae)	4.1.2.11	millet seedlings	*S*
P. aureum Pha-HNL, (Filitaceae)		leaves of goldfoot fern	*R*
Malus communis Mc-HNL, (Rosaceae)		apple	*R*
Sambucus nigra Sn-HNL, (Caprifoliaceae)		black enderberry	*S*
Ximenia americana Xa-HNL, (Olacaceae)		leaves of plants	*S*
Sorghum vulgare Sv-HNL, (Gramineae)		seedlings of the plant	*S*

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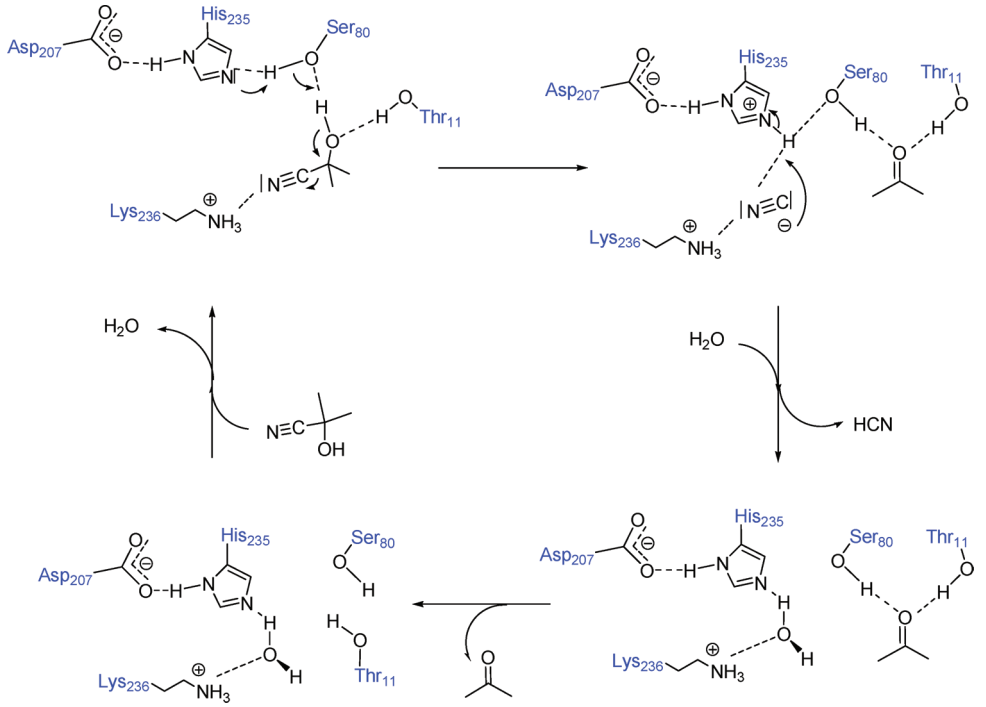

Figure 79. Catalytic mechanism of *Hb*-HNL formulated for the cyanohydrin cleveage direction.

identified Ser80 and His235 as essential elements for the catalytic function of the enzymes from *H. brasiliensis*.[475,511,534−540] and *M. esculenta*.[509,520,538,541] The schematic representation of a mechanism for the *Hb*-HNL-catalyzed cleavage of acetone cyanohydrin was proposed by Zuegg in 1999,[511] and further supporting evidence has been published.[475,484,519,527,536−540,542] Key aspects of this mechanism are (1) deprotonation of the OH-Ser80 by His235 and concomitant abstraction of a proton from the substrate hydroxyl by Ser80; (2) the C−C bond cleveage of the cyanohydrin compound; (3) protonation of the cleaved cyanide by His235 (Figure 79). The cleveage of the C−C bound is the rate-limiting step and it is a general acid/base catalytic mechanism, with the catalytic triad of amino acid residues Ser80, His235, and Asp207.[540]

Extensive details of reaction mechanism of many HNLs were reported.[475,484,527,537,540] However, the question of wether a common mechanism for all Hydroxynitrile Lyases exists is still valid.[504] The amino acid residues at the active site of these enzymes differ significantly but share the common cyanogenetic function.[475] Despite catalyzing the same reaction they differ in substrate specificity and do not share any significant homology on either sequence or structural level. Because of these substantial differences, HNLs from different species are believed to have evolved from unrelated precursor proteins by convergent evolution.[476,500]

Knowledge of the three-dimensional structure is a prerequisite for understanding the enzyme mechanism, as well as for any attempt to alter enzyme properties by chemical or genetic means. Therefore, many efforts to crystallize HNLs with the goal of determining their three-dimensional structures by X-ray crystallography have been undertaken. To date 3D structures are known for four HNLs, from *H. brasiliensis* (*Hb*-HNL),[475,511,516−519] *M. esculenta* (*Me*-HNL),[520,521] *S. bicolor* (*Sb*-HNL),[508,522] and *P. amygdalus* (*Pa*-HNL).[475,503] Investigations on the three-dimensional structure of HNLs from different sources,[523] together with kinetic enzyme studies[520,522,524−526] and genetic studies[527,528] are in progress. Recently, new HNLs have been found, purified and characterized aiming at better application in asymmetric synthesis.[504,529−533]

4.1.3. Operational Parameters. The selectivity of HNL-catalyzed reactions can be altered in different ways: (i) by changing the reaction conditions, such as reaction medium, water content, cyanide source, buffer pH, and temperature;[543] or ii) by changing the enzyme, either by screening for novel catalysts or by protein engineering of the existing catalyst.[544−546]

4.1.3.1. Solvent System. The influence of water content on the activity and enantioselectivity of an enzymatic reaction in organic media is not clearly understood, and much effort has been devoted trying to predict this effect.[543,547] Water is believed to increase the internal flexibility of the enzyme and thereby increases the catalytic activity.[548] It is also known that water is important for the stability of the enzyme since it is involved in many of the mechanisms in enzyme inactivation.[549] But concerning the influence of water content/water activity on the selectivity of the enzymatic reaction recent studies demonstrated that the concentration of water in an organic medium should not be ignored, as it can affect both activity and selectivity. In general, higher activity was observed at relatively low water levels. However, thorough drying of the enzyme prior to the reaction lead to reversible deactivation. So for the better understanding of the enzymatic reaction in organic solvent, the water contents of enzyme preparations and reagents should always be taken into account.[543,547]

Also, numerous studies describing the possibility of modifying the selectivity of an enzyme-catalyzed reaction by changing the solvent[543,550−552] or using a biphasic system have been reported.[543,550,551,553]

For biphasic systems, Hickel et al. first reported that changes in the conformational nature of an enzyme (unfolding, which can

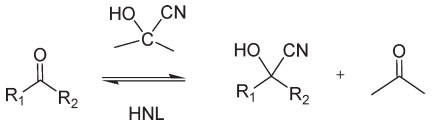

Figure 80. Transhydrocyanation reaction.

cause the loss of enzyme activity) lead to a change in the interfacial tension of the system, which could be followed by dynamic interfacial tension measurements, explaining the observed catalysis.[554−556] Later, it was reported by Straathof that the catalytic activity of enzymes acting in water/organic solvent biphasic systems may be influenced by a number of factors, such as the organic substrate concentration, the initial enzyme concentration, and the volume-specific interfacial area.[553]

As an example catalytic activity and adsorption of *Pa*-HNL was investigated at various organic solvent/water interfaces. First, different solvents were studied in order to observe possible changes in its structure and to delineate where the enzymatic reaction occurs. For hydrophobic solvents large changes in the interfacial pressure were found, while for nonhydrophobic solvents like ethyl acetate (EtOAc), diisopropyl ether (DIPE) and methyl *tert*-butyl ether (MTBE), no changes in the interfacial pressure were observed.[556,557] So usual solvents used in biphasic reactions are EtOAc, DIPE, and other ethers.[558−570]

Enzymes in the DIPE/water interface are more active than in EtOAc.[571,572] As enzyme activity in biphasic systems takes place at the interface rather than in one phase of the solvent system,[555] it is possible to remove the enzyme present in the aqueous phase by a simple phase separation step, so the enzyme could be reused.[493,536,555]

Another advantage of performing reactions in biphasic systems is that the organic phase is beneficial for solubilizing organic compounds and thereby it serves as a reservoir for starting materials and products. It was noted that the partitioning of the substrates between the organic and aqueous phases has a pronounced influence on the enantiomeric purity of the product. Substrates with high solubility in the aqueous phase yield products with significantly decreased optical purity. On the other hand, substrates with low water solubility yield products that are almost enantiomerically pure.[478,573]

Furthermore, chemical background reaction can be minimized if there is low concentrations of the reaction components in the aqueous phase.[471] Vigorous stirring is essential to effect a highly productive reaction[574] by providing optimal contact between the phases in the aqueous−organic emulsion system, but the enzymes have to withstand the shear stress caused by this measure.

In 2004 Gaisberger et al. reported the first HNL-catalyzed cyanohydrin formation in ionic liquids.[575] Room temperature Ionic liquids (ILs) are generally considered to be highly polar and have been used to a large extent as solvents for chemical transformations and for enzyme-catalyzed reactions as well.[576,577] Reactions performed in pure ILs gave racemic cyanohydrins. However, with in an IL/aqueous buffer system, the application of this methodology to enzyme-catalyzed cyanohydrin reactions, using both *Pa*-HNL and *Hb*-HNL, was successful.[575] Since that, different studies and results have been described using IL systems.[578]

In a recent study, the use of an "organic solvent free" system allowed the equilibrium for the conversion to cyanohydrins to be shifted significantly toward the products for substrates with known unfavorable results, such as ketones.[579]

4.1.3.2. Nitrile Donor. Cyanide source is an indispensable component for cyanohydrin formation. Hydrogen cyanide is the most used cyanide source in cyanohydrin synthesis.[558,559,562,580−582]

Besides HCN (generated by addition of acid to an aqueous solution of an alkaline cyanide), several different cyanide sources such as metal cyanides, acetone cyanohydrin, trialkylsilyl cyanides, acylcyanides, and alkyl cyanoformates can be used.[471] They act as an effective source of cyanide at low pH values.[583−585] Furthermore, as hydrogen cyanide and cyanides are highly toxic, a safer alternative is the transhydrocyanation methodology, which employs acetone cyanohydrin as a HCN carrier.[478,586] This methodology produces sufficiently low levels of HCN so the reaction would be directed away from nonenzymatic addition and toward the enzymatic pathway, resulting in high enantiomeric purity and yield (Figure 80).[565,566,587]

Acetone cyanohydrin was selected for this purpose as it has been previously employed in the transhydrocyanation of carbonyl compounds.[478] This donor proved to be advantageous (compared to mandelonitrile)[588] for three main reasons: (i) it is miscible with the buffer used in the system, (ii) it is commercially available, and (iii) its byproduct (acetone) is volatile and has a favorable equilibrium constant.

Using this method, more than ten aldehydes were evaluated and the cyanohydrin products were generally obtained in high optical purity (always greater than 88% ee).[478,589,590]

4.1.3.3. Optimizing Conditions (pH and Temperature). Other important parameter in hydroxynitrile lyase reactions is the medium pH. An increase in enantiomeric excess of the product can be obtained by carrying out the reaction in aqueous medium at low pH. In these conditions the nonenzymatic (spontaneous) addition of HCN to the carbonyl substrate is suppressed. In an aqueous system this suppression can be achieved by lowering the pH (to 3.5−4.0), although working at such low pH values has the disadvantage of significantly reducing the enzyme activity. For example, at pH 4.0 the *Hb*-HNL only retains 20% of its optimal (approximately at pH 6.0) catalytic activity.[474,478,493,524,591,592] This problem can be circumvented by using excess of enzyme in the reaction, at low pH. Recently, unusually high pH-values (above pH 6) in enzymatic cyanohydrins synthesis have been reported, using the highly enantioselective *S* selective *Me*-HNL. In these unusual reaction conditions, also unfavorable substrates can be converted to cyanohydrins with excellent yields and enantiomeric excesses.[593]

Temperature also has effects on the course of the reaction.[525,543] Enzyme stability as well as enantiomeric purity are enhanced at low temperature, usually within the range −5 to 4 °C.[484,493]

4.1.3.4. HNL Source. Availability of HNLs is an important factor to take into account. For (*R*)-hydroxynitrile lyase, almond is the most common source. Defatted almond meal is used as an inexpensive catalyst and does not require immobilization as the enzyme is naturally immobilized in the meal matrix that also provide stability to it.[458,580,594] Some (*S*)-HNL are not easily available and thus can be cloned and overexpressed in microbial expression systems.[510,515,595−600] Enzymes with improved properties have been obtained through genetic modification.[545,546,601−603] Considerable success with the *S* selective *Me*-HNL and *Hb*-HNL has been achieved with this technique by employing *E. coli*,[599,604,605] *S. cerevisiae*, and *P. pastoris*[595] as host organisms.[474,478,598,599] *Lu*-HNL has been cloned and expressed in *E. coli* and *P. pastoris*.[502,515,606] Although clones of

the *S* selective *Sb*-HNL and the *R* selective *Pa*-HNL are available,[478] overexpression in the aforementioned systems has not been successful. Microbial systems have been chosen to perform the cloning of HNLs because they can be easily transformed and cultured in a short time for large-scale production.[484,607]

For *Pa*-HNL isoenzyme 5, recombinant production even enhances the enzyme quality.

In comparison to the mixture of isoenzymes isolated from almonds, it shows increased stability at relatively low pH values, which is especially important for the enantioselective synthesis of cyanohydrins.[471,608]

The immobilization of hydroxynitrile lyases was also studied on different supports. This methodology allows easier handling, together with the possibility of recycling of the enzymes, as well as enabled the application of HNLs in monophasic organic solvents.[609−613]

Supports, like ECTEOLA cellulose (ion exchanger),[610,613−617] DEAE cellulose, sepharose 4B, pore glass beads,[614] silica gel,[614,618] microcrystalline cellulose (Avicel),[561,614] eupergil C, nitrocellulose,[562] Celite, sol−gel matrix,[619,620] liquid crystals and PVAL hydrogels,[621,622] have been used. Recently, CLEA (cross-linked enzyme aggregate)[470,620,623−627] and CLEC (cross-linked enzyme crystals)[628] technologies have emerged for immobilization of hydroxynitrile lyases. CLEAs of HNLs, especially have shown a high degree of stability and activity, even after several runs. One advantage of the immobilization as CLEA is that it combines purification and immobilization in a single step. Thus, a highly pure enzyme is not necessary, in contrast to other enzyme immobilization processes.

CLEAs of the *S* selective *Me*-HNL and *Hb*-HNL, and *R* selective *Pa*-HNL were prepared. They proved to be highly active in the asymmetric cyanation of various aldehydes. In addition, CLEA made from *Pa*-HNL was recycled ten times without any loss of activity. Another important advantage of this technique is that the aggregates can be used in organic solvents. In this experimental conditions the competing, nonenzymatic cyanation is suppressed, allowing higher enantioselectivities compared to free HNLs.[470,612,620,623−626,629]

In addition to immobilization, the possibility of using crude preparations of HNLs is still a valid resource. The use of extracts from almond,[582] apple,[565] or mamey seeds[630] for *R* selective HNLs, and of sorghum shoots[587] for *S* selective HNLs, instead of isolated enzymes is worth noting.[631]

4.1.3.5. Influence of Stereocenters. Another factor to take into consideration when performing HNL-catalyzed reactions is the influence of a stereocenter already present in the substrate on the selectivity of the enzyme. Riva et al. reported on the influence of α- and *β*-substitution on the selectivity of *Pa*-HNL.[632,633] Also the same authors studied the influence of protecting groups present at α-hydroxyaldehydes with a *R* selective *Pa*-HNL and *S* selective *Me*-HNL. For the *Pa*-HNL, a clear dependence of selectivity on the size of the protecting group was observed; however, this was less pronounced with *Me*-HNL.[634]

In summary, as enzyme-catalyzed reaction, they are multiple conditions that have to be taken into account (Figure 81). The development of enantioselective enzymatic synthesis of cyanohydrin into a method of great importance and applicability was made possible by three achievements: (i) performing the reaction in organic solvents not miscible with water, (ii) using a low pH-range at which the nonenzymatic reaction is suppressed, and

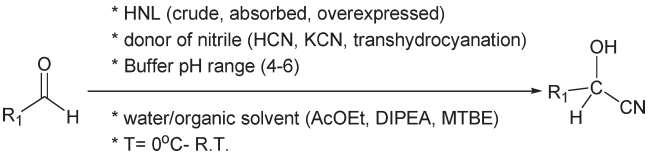

Figure 81. Reaction conditions for the formation of enantiopure cyanohydrins.

(iii) overexpressing of HNLs in sufficient quantities for their large scale application. It was after these discoveries that efficient reaction protocols have been achieved.

Finally, but not less important, the enantiomeric purity of the cyanohydrins and derivatives produced through HNL-mediated reactions is routinely determined by HPLC,[635,636] GC,[561,563,584,637−640] and NMR[458,594,641−643] techniques. Thin layer chromatography using $KMnO_4$ as developer is a convenient technique for detection of cyanohydrins in the reaction mixture.[484,644]

4.2. Synthetic Applications

In 1837, Friedrich Wöhler detected hydroxynitrile lyase activity for the first time in almond.[645] The HNLs from *P. amygdalus* (*Pa*-HNL), *S. bicolor* (*Sb*-HNL), *M. esculenta* (*Me*-HNL), and *L. usitatissimum* (*Lu*-HNL) are all commercially available from the major chemicals suppliers. They are currently the four major HNLs that are being used in asymmetric synthesis. The first example of HNL-catalyzed synthesis was carried out by Rosenthaler in 1908 using hydroxynitrile lyases from almond.[646] Since that, a broad range of aliphatic, as well as aromatic aldehydes, have been converted into their corresponding cyanohydrins with excellent to moderate enantiopurities, being this methodology one of the most versatile tools for the enantioselective synthesis of cyanohydrins.[471,477,484]

As described above, (*R*)-hydroxynitrile lyases come mainly from *Prunus* sp., while (*S*)-hydroxynitrile lyases come from *S. bicolor*, *H. brasiliensis*, and *M. esculenta* (see Table 13). *Pa*-HNL accepts aromatic, as well as aliphatic, unsaturated, and heterocyclic aldehydes.[614,647] *Sb*-HNL accepts only aromatic and heterocyclic aldehydes.[475,558,640] *Hb*-HNL, catalyzes the formation of cyanohydrins from aliphatic, aromatic, heterocyclic, and α,*β*-unsaturated aldehydes.[475,526,529,583,584] *Me*-HNL, which is very similar to the *H. brasiliensis* enzyme, has very similar substrate specificity (Table 14).[475,526,529]

Hydroxynitrile lyases have been extensively used for asymmetric synthesis and for kinetic resolutions to obtain pure enantiomers. A recent review on kinetic resolution using HNLs was reported by Holt et al. in 2009.[477] Herein, we will focus on synthetic transformations of cyanohydrins as useful building blocks in asymmetric organic synthesis.

Enantiopure cyanohydrins, prepared from HNL-catalyzed reactions, are versatile building blocks in asymmetric synthesis. They serve as intermediates for the preparation of multiple biologically active compounds, as they can be converted into a variety of products. There are many transformations using chemical reactions that can be performed, for instance, reactions of the cyano group and the hydroxyl group giving new functionalities (Figure 82).

4.2.1. Cyanohydrins Derived from Different Substrates. The substrates of HNLs are mostly aromatic, as well as aliphatic, unsaturated and heterocyclic aldehydes; despite of that, the reaction with other substrates was studied. The presence

Table 14. Characteristics of Hydroxinitrile Lyases Currently Used in Synthetic Applications

enzyme	accepted aldehydes	cofactor
Pa-HNL	aliphatic, aromatic, heteroaromatic, α,β-unsaturated	FAD
Pr-HNL(*Prunus* sp.)	aliphatic, aromatic, heteroaromatic, α,β-unsaturated	FAD
Lu-HNL	aliphatic, aromatic, α,β-unsaturated	no
Hb-HNL	aliphatic, aromatic, heteroaromatic, α,β-unsaturated	no
Me-HNL	aliphatic, aromatic, heteroaromatic, α,β-unsaturated	no
Sb-HNL	aromatic, heteroaromatic	no
Sn-HNL	aromatic	no

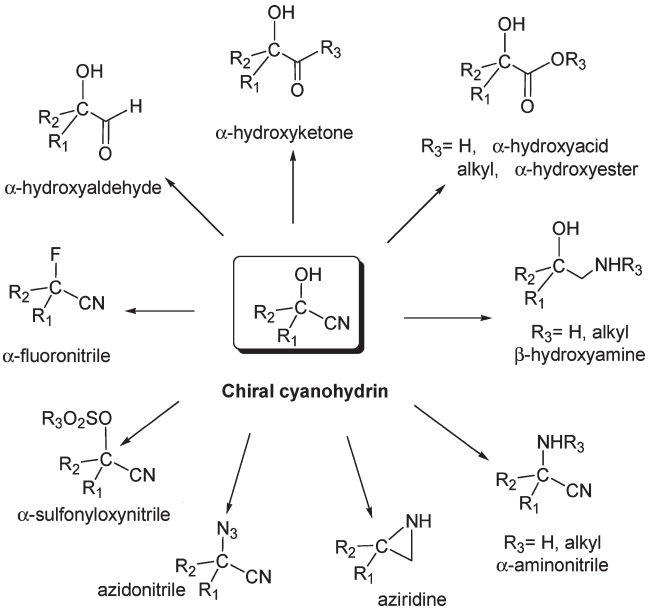

Figure 82. Building blocks from cyanohydrins.

of other functional groups present in the substrate may have important effects in the steroselectivity of the HNL-catalyzed reaction.

4.2.1.1. Cyanohydrin from Ketones. Compared to aldehydes, asymmetric addition of cyanide to ketones has proven to be more difficult because of the higher degree of steric hindrance of this type of compounds.[648] For ketones large excesses of HCN have been used to obtain good yields.[649] The cyanohydrin products of these reactions present quaternary stereocenters, which are of great significance for synthetic biologically active compounds. A variety of reports on the synthesis of enantiopure cyanohydrins derived from aliphatic methyl ketones and aromatic ketones were published (Figure 83).[650−652]

For methyl ketones, very good enantiomeric excesses were obtained when the other group is larger than ethyl. In the case of 2-butanone, the similarity of both substituents directs the substrate with only minor selectivity of spatial orientation and thus the corresponding (*S*)-cianohydrin was prepared in only 54%ee when using *Hb*-HNL.[653] However, the enantiomeric (*R*)-cianohydrin could be obtained with better selectivity using *Pa*-HNL (76% ee) or *Lu*-HNL (87% ee) (Figure 83).[624,653]

Regarding the ketones possessing aromatic rings, Roberge et al. have recently described the asymmetric cyanation (using *Lu*-HNL as catalyst) of this type of ketones, such as acetophenone, phenylacetone, benzylacetone, and propiophenone, with different substitution on the phenyl group. Both reaction yields

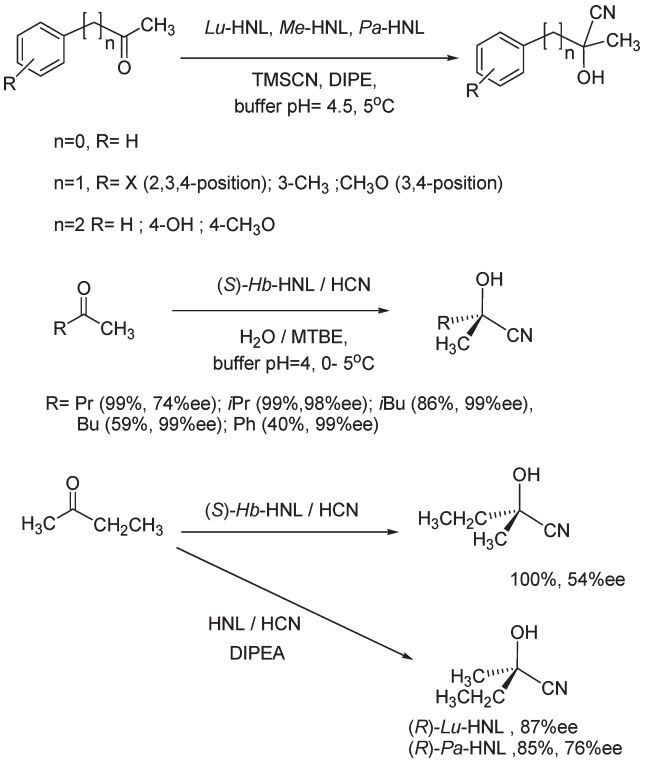

Figure 83. Synthesis of cyanohydrins from aromatic and aliphatic ketones.

and stereoselectivities were shown to be influenced by the length of the carbon chain between the ketone and the phenyl groups. Substrates converted with the greatest degree of productivity and selectivity were phenylacetones with large, electron withdrawing meta-substituents.[652]

Transcyanation of aldehydes with cyanohydrins derived of racemic ketones was studied by Gotor's group in 1995[567] and has proved to be a good methodology for the stereoselective production of cyanohydrins from both aldehydes and ketones in the same process.

Cyanohydrin formation from 2-, 3-, and 4-monosubstituted cyclohexanones were also investigated.[654−656] In this case, the final cyanohydrins contain two stereogenic centers and thus, four stereoisomers could be obtained. Kolber et al. found that with *Pa*-HNL from bitter almonds, *R* selective addition of HCN to 2- as well as 3-substituted cyclohexanones was possible, allowing formation of two epimers, which could be separated by column chromatography after *O*-acylation. Alternatively, with the enzyme (*S*)-*Me*-HNL from cassava, the complementary two diastereomers can be synthesized and separated. It was shown that

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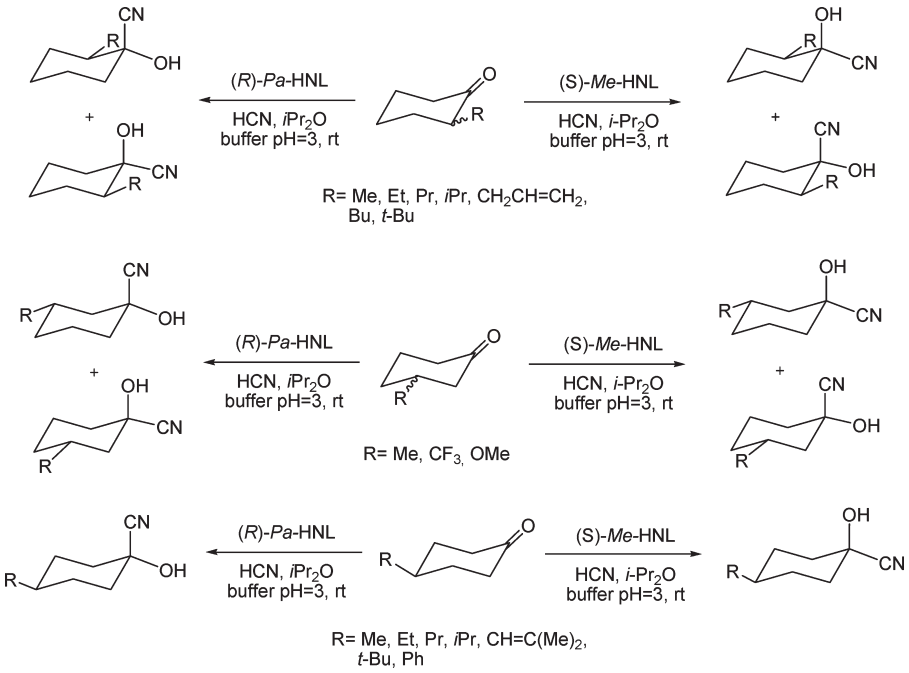

Figure 84. Synthesis of cyanohydrins from substituted cyclohexanones.

increasing bulkiness of the substituents in 2- and 3-position diminishes the catalytic activity of both enzymes (Figure 84).[654−656]

Also Effenberger et al. studied the addition of HCN to 4-substituted cyclohexanones by hydroxynitrile lyase-catalyzed reaction using *Pa*-HNL and *Me*-HNL, toward the stereoselective preparation of tetronic acids. The authors established that selectivity was important, especially for cyclohexanones with larger 4-substituents.[654]

4.2.1.2. Cyanohydrin from Unsaturated Aldehydes. Unsaturated aldehydes in α,β- or β,γ-positions are important substrates for HNLs. The subsequent unsaturated cyanohydrins represent products with an enhanced synthetic potential.[657−660] They allow different kind of transformation such as: oxidative cleavage, dihydroxylation, epoxidation, among other reactions. A myriad of products have been synthesized via cyanohydrins employing these substrates, such as L- and D-sphingosines,[659] pentoses,[657] and nucleosides[660] among others[661] (Figure 85).

4.2.1.3. Cyanohydrin from Heterofunctionalized Aldehydes or Ketones. In the last years, cyanohydrins formation from many novel substrates like heterofunctionalized aldehydes or ketones have been investigated,[529,634,641,653,662,663] (Figure 86). For example, α-alkoxy- and α,β-dialkoxy-substituted aldehydes were subjected to the catalytic action of *Pa*-HNL and *Hb*-HNL to yield the corresponding cyanohydrins.[641] In a similar manner, the bifunctional cyanohydrin of methyl 4-oxobutanoate was prepared in 99% ee.[663] Other substrates, such as 4-hydroxybutanal and 5-hydroxypentanal were transformed through almond-(*R*)-oxynitrilase-catalyzed reaction obtaining optically enriched aliphatic δ-or ω-hydroxycyanohydrins.[662]

Chiral cyanohydrins are suitable starting materials to prepare important building blocks for a large spectrum of compounds, ranging from pharmaceuticals and natural products to those important in the cosmetics, flavor, fragance and agrochemical industries. Alternatively, the transformation can be performed either over the hydroxyl or the cyano moiety.

4.2.1.4. Transformation of O-Protected Cyanohydrins. Chemical reactions over nitrile moiety generally imply protection of hydroxyl groups.

The protective groups provide cyanohydrin stability against transformations to avoid racemization and to minimize side reactions. The most commonly used protective groups are silyl ether, ester, methoxy isopropyl ether, tetrahydrofuryl ether, and tetrahydropyranyl ether.[594,664,665] *O*-Protected cyanohydrins are tolerant to a wide range of cyanide/nitrile transformations.4.2.1.4.1. 1,2-Amino Alcohols. Conversion of the hydroxy functionality to the silyloxy ether[594] allows for a range of 1,2-amino alcohols and hydroxy ketones to be generated, via nucleophilic addition of a Grignard reagent to the nitrile followed by reduction or acidic hydrolysis of the intermediate imine.[474] This 1,2-amino alcohols can give adrenaline-type compounds and adrenergic compounds, among others (Figure 87).[461,581,647]4.2.1.4.2. Hydrolysis of the Nitrile Group. The hydrolysis of the nitrile group of cyanohydrins yields acids or amides. This transformation offers the most interesting general route for the preparation of (*R*)- and (*S*)-2-hydroxycarboxylic acids and derivatives. By treating the crude cyanohydrins in aqueous hydrochloric acid, the corresponding (*R*)- and (*S*)-hydroxycarboxylic acids are obtained in excellent chemical yields and with complete retention of configuration.[647] Under milder conditions (lower temperature, shorter reaction times) cyanohydrins can be hydrolyzed selectively to give the corresponding amides (Figure 88).[666] The use of this methodology was applied in 2000, for the total synthesis of the potent antitumoral agents epothilones A and B.[667]4.2.1.4.3. Reduction of the Nitrile Group. A biocatalytic approach toward enantiopure piperidones, versatile building blocks for the preparation of biologically active compounds, has been reported.[663] The synthetic methodology uses (*S*)-HNL-mediated cyanohydrins formation, followed by hydrogenation. Adjusting the conditions of the latter step, enables the formation of 5-hydroxypiperidinone derived (bicyclic) *N*,*N*-acetals via an unprecedented reductive amination

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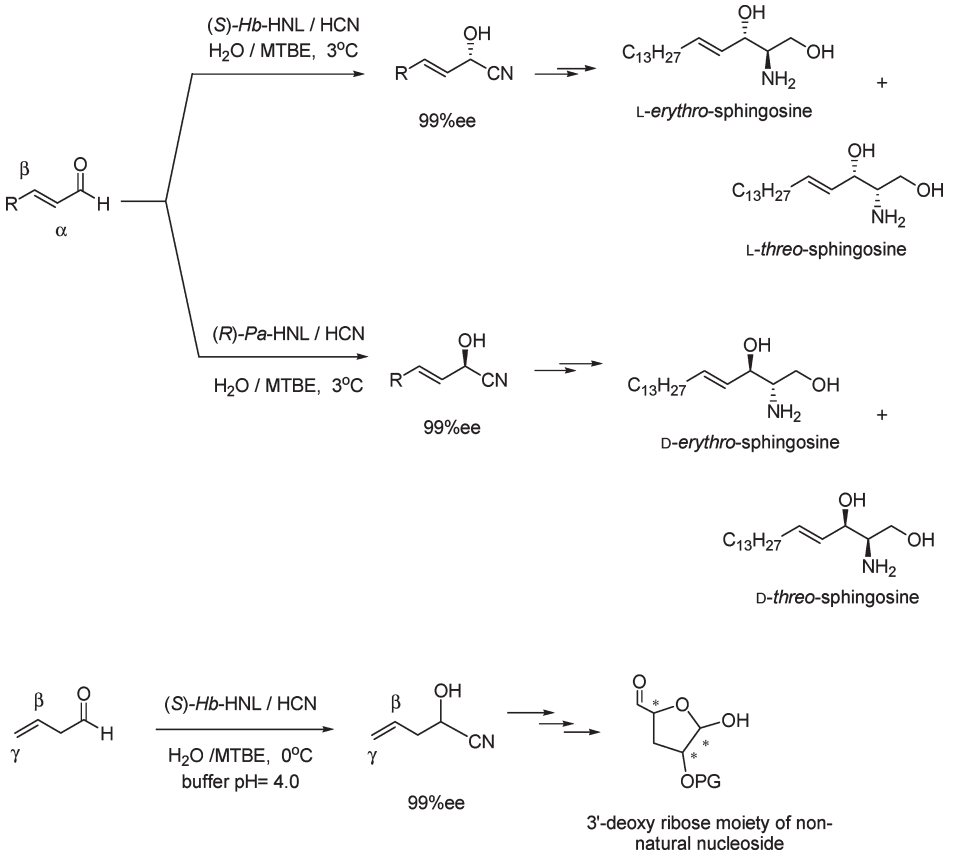

Figure 85. Further transformations of unsaturated cyanohydrins. PG: Protective group.

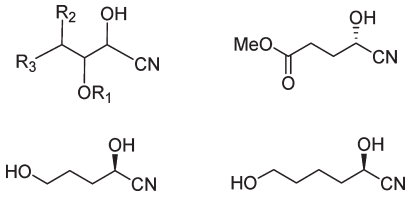

Figure 86. Synthesis of cyanohydrins from heterofunctionalized aldehydes.

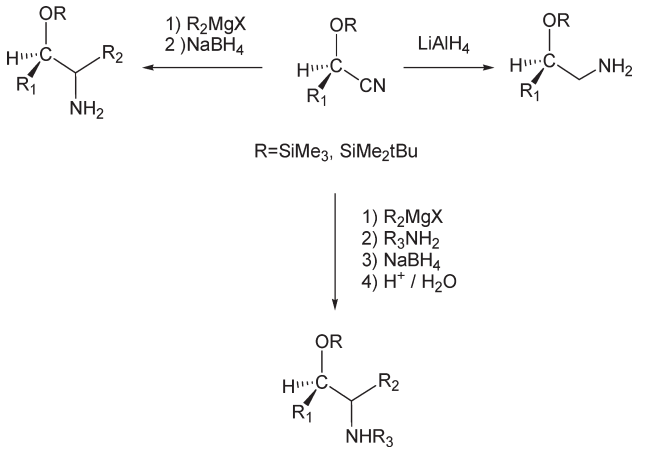

Figure 87. Synthesis of 1,2-amino alcohols from O-protected cyanohydrins.

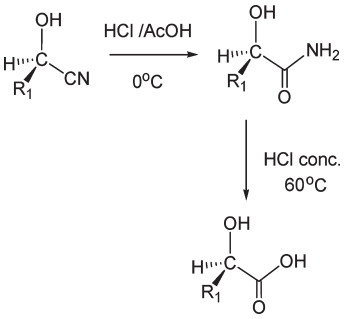

Figure 88. Hydrolysis of the nitrile group in cyanohydrins.

of the nitrile group, as well as form *N*-alkylated 5-hydroxypiperidinone in a single step from the same cyanohydrin intermediate. The stereocenter established in the HNL-catalyzed step induces, together with the diamine, the stereochemistry of the aminal. Because of the stable cyclic aminal, no imine is formed and the reduction cannot proceed to the amine stage. This remarkable catalytic reduction gave access to the final products in good yields (Figure 89).

O-Protected α-hydroxyaldehydes are accessible in high yields by hydrogenation of *O*-protected cyanohydrins with diisobutyl aluminum hydride (DIBALH) followed by mild acidic hydrolysis (Figure 90).[460] Chiral O-protected aldehydes are interesting building blocks for the stereoselective preparation of polyhydroxy compounds.[634] By this route, Effenberger and Roos developed a novel synthesis of *N*-acetyl-L-daunosamine, the glycosidic fragment of the anthracycline antibiotic

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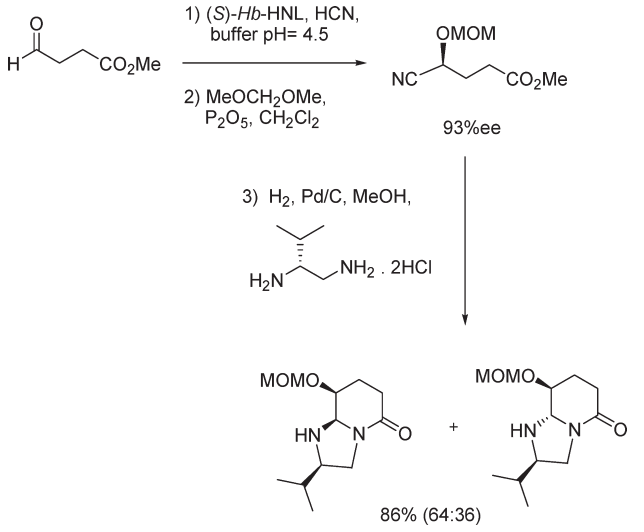

Figure 89. Synthesis of 5-hydroxypiperidinone derivatives by reductive amination of nitrile group. MOM: methoxymethyl.

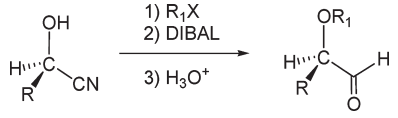

Figure 90. Synthesis of O-protected α-hydroxyaldehydes from cyanohydrins.

daunomycin.[668] 4.2.1.4.4. Transformation of the Hydroxyl Moiety in Cyanohydrins. The hydroxyl group can be converted into a suitable leaving group, followed by nucleophilic displacement with various nucleophiles, such as acetate,[669] *N*-nucleophiles (potassium azide, potassium phthalimide, and amines),[462,469] *S*-nucleophiles,[670] or fluoride.[467] This methodology generates new alternative functionality at the chiral center with stereochemical inversion. The free cyanohydrins are particularly stable under anhydrous or strongly acidic conditions, but treatment with aqueous acid yields α-hydroxy acids.[558]

Enantiopure 2-sulfonyloxynitriles, accessible from chiral cyanohydrins by sulfonylation, have a relatively high configurational stability.[462,669] They have been used for the inversion of configuration of cyanohydrins, as they react with nucleophiles under mild conditions giving the corresponding inverted product. (*R*)-Alkyl sulfonylated cyanohydrins, reacts with sulfur nucleophiles in a typical S_N2 manner,[670] while 2-sulfonyloxynitriles derived from cyanohydrins of aromatic aldehydes react with weak nucleophiles with partial racemization (Figure 91).[462,669]

4.2.2. Expanding the Synthetic Applicability of HNLs. As we have presented here, HNLs have been found to be rather tolerant regarding to the nature of the electrophile and a wide array of aliphatic, aromatic, and heterocyclic carbonyl compounds are accepted. With respect to the nucleophile, no other substrate, except cyanide, was recognized until 2006.[471,671,672]

Trying to expand the synthetic applicability of the HNL methodology, Purkarthofer et al., considered replacing HCN by other nucleophiles to be added to carbonyl compounds catalyzed by these enzymes.[671] On the basis of the mechanism of this biotransformation, as it was described, crucial parameters for such alternative reagents would be (i) molecular size and (ii) pK_a of the CH-acidic portion, (which should be similar to that of

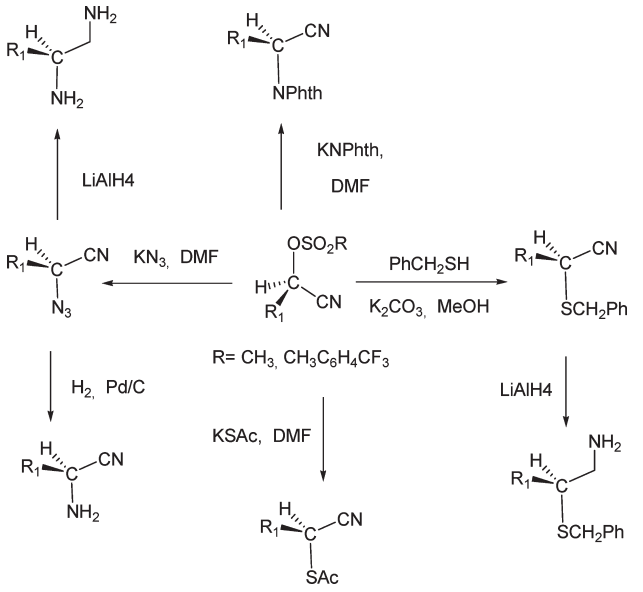

Figure 91. Substitution of the hydroxyl group in O-sulfonylated cyanohydrins.

HCN, $pK_a = 9$). An important substance class that meets these criteria are nitroalkanes. Their reaction with carbonyl compounds, known as the nitroaldol or Henry reaction,[673] constitutes a carboligation process of high synthetic value. Henry reaction furnishes vicinal nitroalcohols, which can easily be transformed to a series of valuable intermediates, such as 1,2-aminoalcohols and α-hydroxycarboxylic acids.[674,675]

Purkarthofer and co-workers found that hydroxynitrile lyase, *H. brasiliensis* (*Hb*-HNL) from the tropical rubber tree, had nitroaldolase activity.[671,672] This enzyme accepted nitroalkanes as donors in a reaction with aldehydes to yield enantiomerically enriched *β*-nitro alcohols. This was the first example of a biocatalytic Henry reaction. Nitroaldol *Hb*-HNL-catalyzed reaction was initially investigated with aromatic, heteroaromatic and aliphatic aldehydes using nitromethane as the nucleophile (Figure 92). Also it was demonstrated that apart from nitromethane, *Hb*-HNL accepted nitroethane as the nucleophile. In this case two stereocenters were generated simultaneously with good diastereo- and enantioselectivity, granting access to substances of the ephedrine family.[471] For an evaluation of the synthetic value of the *Hb*-HNL-catalyzed nitroaldol reaction several aspects were taken into account: (i) besides variability at yields values important enantiopurities are obtained; (ii) access to the enzyme *Hb*-HNL is easy since, it could be overexpressed in *P. pastoris*; (iii) the enzyme can be recycled three times although it losses activity; (iv) enantiopure nitro alcohols (coming from nitromethane or nitroethane) are important synthetic intermediates, as cyanohydrins.

Biocatalytic Henry reaction was also found for HNL from *M. esculenta* but with lower activity and selectivity.[672]

4.2.3. Examples of HNL-Mediated Synthesis of Representative Compounds. Cyanohydrins are valuable intermediates in organic synthesis, enabling an extensive chemistry. Some examples of HNL-mediated synthesis of representative compounds will be presented.

Thiamphenicol and florfenicol can be prepared from an enantiomerically pure cyanohydrin by a chemo-enzymatic approach aiming toward a new stereoselective synthesis. Both compounds have been synthesized stereoselectively from

enantiomerically pure 4-methylsulfanyl mandelonitrile, which was obtained by hydrocyanation reaction of 4-methylsulfanylbenzaldehyde catalyzed by (*R*)-hydroxynitrile lyase of *Badamu* (*Prunus communis* L. var. *dulcis* Borkh, almond from Xinjiang, China) (Figure 93).[676]

Aziridines are an important class of compounds in organic synthesis. A straightforward, five-step procedure for the synthesis of enantiomerically pure 2,3-disubstituted *trans*-aziridines has been developed starting from commercially available aldehydes. Hydroxynitrile lyase-mediated cyanohydrin formation provided cyanohydrins in excellent enantioselectivities and good yields. Subsequent formation of diastereomerically pure *anti*-amino alcohols *via* a one-pot Grignard addition-reduction sequence, CuII- catalyzed diazotransfer, and triphenylphosphine-mediated reductive cyclization provided the corresponding *trans*-aziridines in good yields and excellent diastereoselectivities (Figure 94).[677]

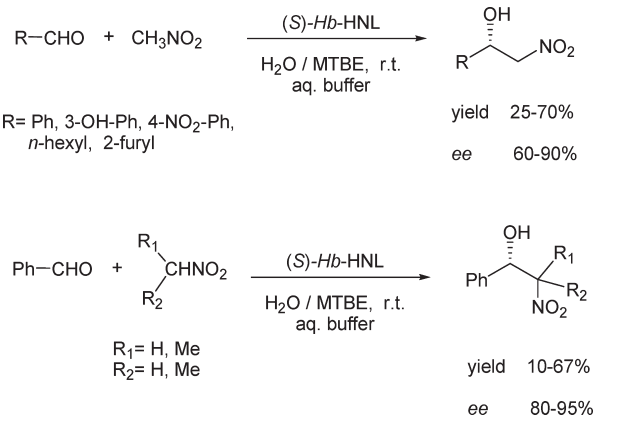

Figure 92. HNL-catalyzed Henry reaction.

Substituted morpholines have attracted considerable interest because of their presence in a vast number of therapeutically and biologically active compounds. A versatile synthesis of enantiomerically pure cis- and trans-2,5-disubstituted morpholines was described. Hydroxynitrile lyase-mediated cyanide addition onto aldehydes provided cyanohydrins in virtually quantitative yield and excellent enantioselectivity. Subsequent formation of diastereomerically pure amino esters via a three-step, one-pot reduction-transimination-reduction sequence, followed by reduction and simultaneous protection provided cyclization precursors. Finally, cyclization and SmI_2-mediated reductive detosylation completed the synthesis of cis- and trans-2,5-disubstituted morpholines in good yields and excellent diastereoselectivities (Figure 94).[678]

Tetronic acid derivatives and their metabolites are widespread in nature, the best known example of which are undoubtedly vitamin C and penicillinic acid. A convenient synthesis of optically active 5,5-disubstitued 4-amino-and 4-hydroxy-2(5*H*)-furanones (frameworks of natural products as vitamin C), from (*S*)-ketone cyanohydrins has been developed (Figure 95).[651]

Chiral ferrocene derivatives have broad applications, such as ligands in asymmetric catalysis, bioelectrochemistry, and in the development of new pharmaceuticals against malaria. A novel route to chiral ferrocene derivatives involving the application of hydroxynitrile lyase from *H. brasiliensis* has been developed.[679]

Also a chemo-enzymatic synthesis of new chiral ferrocenyl-oxazolidinones has been developed. The key step was the addition of HCN to formylferrocene catalyzed by the hydroxynitrile lyase from *H. brasiliensis*, which yielded an enantiomerically pure ferrocenyl-cyanohydrine in excellent yield and ee. The ferrocenyl-oxazolidinones obtained by this strategy were tested as chiral auxiliaries for asymmetric alkylations and aldol

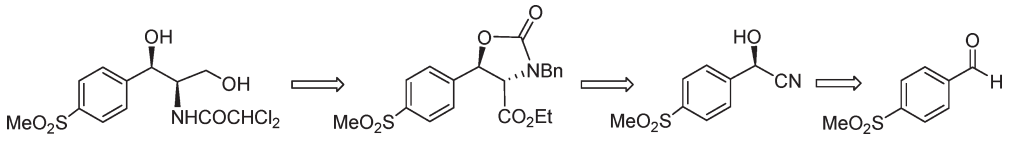

Figure 93. Retrosynthesis of thiamphenicol (hydrocyanation reaction catalyzed by (*R*)-HNL of *Badamu*).

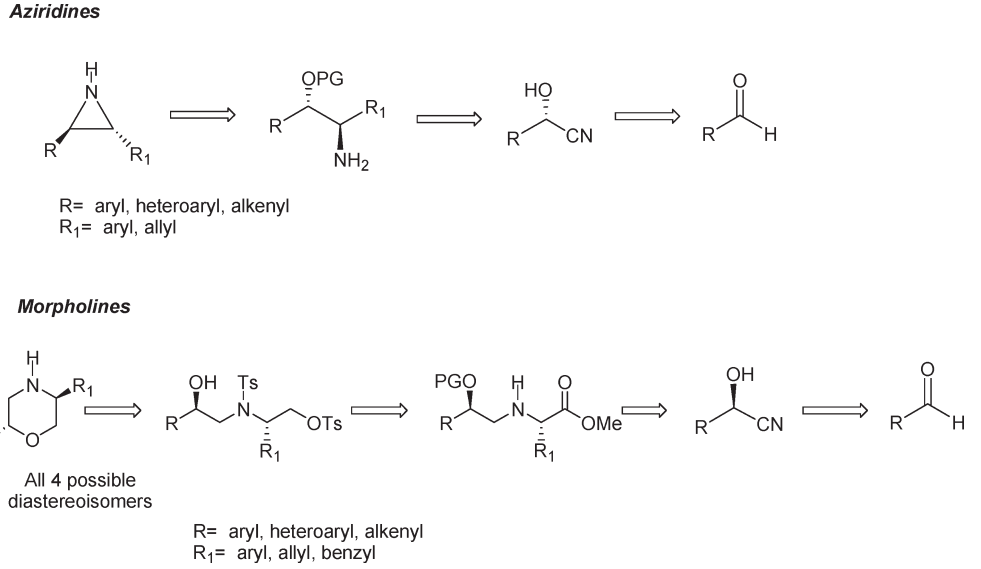

Figure 94. Retrosynthesis of aziridines and morpholines (hydrocyanation reaction catalyzed by *Pa*HNL and *Hb*HNL). PG: protecting group.

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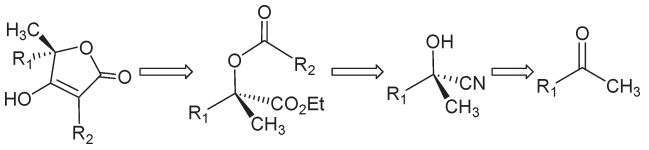

Figure 95. Retrosynthesis of tetronic acids (hydrocyanation reaction catalyzed by *Me*HNL).

reactions and were shown to be effective in terms of yields, stereoselectivities and cleavage conditions (Figure 96).[680]

5. CONCLUDING REMARKS

The use of biocatalytic schemes in organic synthesis is growing steadily over the years. Bioprocesses are becoming attractive for both academic and industrial settings because of their unique stereoselectivity and outstanding potential to meet environmental challenges. Lyase-mediated carboligation, exemplified in this review by the aldol, acyloin condensation, and cyanohydrin formation reactions, is performed under mild conditions, without using heavy-metals, corrosive reagents, chiral auxiliaries or costly protecting groups. During the period under review, new insights on enzymatic mechanism and structure, added to an increasing number of synthetic applications made possible the development of new catalysts and new reactions. The search for new biocatalysts using different approaches, such as protein engineering and screening, was intense. Directed evolution showed a great potential to modify substrate specificity and stereospecificity of aldolases (especially pyruvate- and acetaldehyde-dependent) and other enzymes. Metagenomics, as well as elucidation of new microorganism genomes, were very successful with aldolases (in particular acetaldehyde- and DHA-dependent) and more traditional screening afforded new lyases of synthetic utility, too. Also during this period, the discovery of novel aldolase activity of promiscuous enzymes, or of unexpected relaxed substrate specificity broadened the opportunities to perform enzymatic carboligations to solve synthetic problems.

The synthetic potential of these enzymes was further demonstrated by the development of new reactions. In particular, the ThrA-catalyzed preparation of α-amino acids with an α-quaternary center, the FSA-catalyzed reaction of glycolaldehyde, and the HNL-catalyzed Henry aldol reaction are remarkable examples. Also, several efforts were disclosed to attenuate the need for DHAP of the corresponding aldolases.

A group of interesting new techniques have been described, such as DYKAT, tandem aldol-Henry methods, or buffer trapping, which expand the scope of biocatalytic methods. The high reaction specificity of these enzymes under mild conditions not only eliminates the need for tedious protecting-group manipulations, but also opens up opportunities to work with other enzymes through integrated reaction schemes of various steps of different nature. In particular, aldolases have been used in the production of chimeric enzymes and also in multienzyme systems of up to five steps, allowing for the improvement of practical preparations of known products. Important industrial applications of acetaldehyde and pyruvate-dependent aldolases have appeared in recent years, showing the maturity of the field.

However, to further increase the applicability of these lyases in preparative enantioselective synthesis, some limitations should be addressed. For instance, more flexibility for the donor substrate is desirable for both aldolases and hydroxynitrilases. Improvements in the stereoselectivity of some enzymes are needed, in particular for TagA and ThrA. Additionaly, the stability (of aldolases) and the substrate tolerance could be improved.

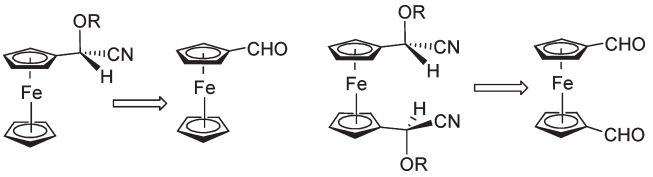

Figure 96. Retrosynthesis.of chiral ferrocenes (hydrocyanation reaction catalyzed by *Hb*HNL).

The examples of applications listed above show some of the diverse possibilities to prepare interesting polyfunctionalized products by aldolases and related C−C bond-forming biocatalysts. The examples further illustrate that the construction of new carbon−carbon bonds is feasible even with substrates that are structurally very distant from the natural functions of these enzymes, which facilitates a rapid access to highly variable and complex structural motifs.

Finally, it is worth to mention that the new insight obtained by the study of the action mechanism and structure of aldolases is useful not only to design and develop new biocatalysts but also to create novel catalysts with aldolase activity. In this regard, several different types of compounds able to catalyze aldol or retroaldol reactions have been developed in the last years, covering from monoclonal antibodies[681] to small organocatalysts,[682] through ribozymes (RNA-based biocatalysts),[683] synthetic peptide amides,[684] small polymers (nanogels),[685] foldamers,[686] and dendrimers.[687] In addition, other C−C bond forming enzymes not specifically covered in this review have recently been used in synthetic applications. Representative examples include the use of methyl transferases to introduce alkyl groups in activated aromatics, representing the first examples of biocatalytic Friedel−Crafts alkylations,[688] and the use of an engineered tyrosine phenol lyase to produce tyrosine derivatives in one step from the corresponding phenols.[689]

In spite of the many advances that have taken place during recent years, the field of lyase-mediated synthesis is likely to continue growing at a steady pace.

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BIOGRAPHIES

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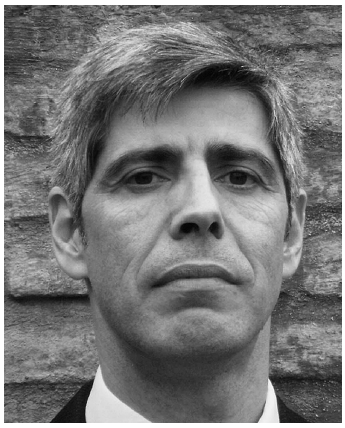
Daniela Gamenara was born in Montevideo, Uruguay, in 1964. She studied chemistry at the Universidad de la República (UdelaR) in Uruguay, and in 1998 she earned her degree. In 2005, she completed her PhD at the same University. After two short postdoctoral internships, she is currently Assistant Professor at the Organic Chemistry Department of the Faculty of Chemistry (UdelaR). Her main scientific interest is the development of enzymatic methodologies for the synthesis of high added value compounds and natural products as well, together with the use of new trends in organocatalysis for the same purposes.

Patricia Saenz Méndez studied at the Universidad de la República (UdelaR) in Uruguay and became a Pharmacist in 1999. She earned her Master degree in Organic Chemistry in 2000 working in Medicinal Chemistry at the same University. Soon afterwards she started a doctoral program in Organic Chemistry and Computational Chemistry, combining organic synthesis, biotransformations, and quantum chemistry to study the oligomerization of cyclitols, and received her PhD in Uruguay in 2006. After one year of postdoctoral studies in Sweden (Biophysical Chemistry, Natural Science Department, Örebro University), she returned to Uruguay, where she is currently an Assistant Professor of Physical Organic Chemistry. Her main scientific interest is the sustainable chemistry, especially the experimental and in silico development of biotechnological and organocatalytic tools for the preparation of high value-added chemicals and natural products from residual biomass.



Gustavo Seoane was born in Montevideo, Uruguay, in 1959. In 1983 he earned a degree in Chemical Engineering at Universidad de la República (UdelaR), Uruguay, and completed his PhD in 1988 at Virginia Tech, USA. After one year of postdoctoral studies, he returned to Uruguay to work in the Faculty of Chemistry of the Universidad de la República, where he was appointed Full Professor in 1997, and is currently Head of the Organic Chemistry Department. His main scientific interest is the use of green procedures for the synthesis of bioactive natural products and analogs. In particular, intensive use of biotransformations to prepare polyoxygenated targets is made.

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