Native proteins in organic chemistry. Recent achievements in the use of non hydrolytic enzymes for the synthesis of pharmaceuticals

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Abstract: Industrial chemical companies have taken advantage of enzymes for the production of pharmaceuticals by means of nonselective and selective transformations. This review covers recently published chemoenzymatic synthesis of important drugs, focusing on the production of the desired targets with high to excellent yields. Remarkably, biocatalysts have allowed the development of asymmetric processes through different strategies, providing efficient access to challenging substrates that are difficult to obtain by traditional chemical methods. Thus, the action of different classes of non hydrolytic enzymes has been covered, including a wide number of oxidoreductases, lyases and transferases. The importance of using enzymes with synthetic purposes under mild reaction conditions is fully demonstrated in the production of active pharmaceutical ingredients and other organic compounds with interesting biological profiles.

1. Introduction.

The use of biotransformations for synthetic purposes nowadays plays an important role in synthetic chemistry.[1,2] Enzymes have shown a high level of reactivity and exquisite selectivity catalyzing chemo-, regio- and stereoselective transformations under mild reaction conditions.[3,4] Thus, their application can contribute to existing methods for accessing both non-chiral and optically active chemicals.[5-7] This fact has led to the incorporation of enzymatic catalysis to the synthetic organic expert's toolbox, making the development of highly selective transformations possible.[8,9] Additionally, the advances in protein engineering techniques have broaden the number of enzymes available, and consequently the possibilities of finding the best enzyme for a given process.[10] This is especially relevant when this knowledge is applied at industrial level, and many biotechnology and chemical companies have taken advantage of the activities displayed by enzymes because of their low environmental impact in terms of energy, raw material and waste production.[11-17]

Nowadays the development of catalytic methods inside the pharmaceutical industry is well established, including at large-scale.[18,19] The pursuit of novel drug candidates is feasible through a wide panel of organic reactions,[20] most of which can also be developed using enzymes. In this context, biocatalysis has emerged as a powerful tool for the selective modification of final targets or precursors through different reactions.[21-27]

In this review, the authors aim to provide a broad and representative selection of articles published in the last six years dealing with the chemoenzymatic synthesis of highly valuable organic compounds with applications in medicinal chemistry. The role of native enzymes from the class of oxidoreductases, lyases and transferases for the production of pharmaceuticals and compounds with interesting biological profiles is discussed here. For simplicity, noteworthy achievements made when using evolved enzymes have not been included, an insight into further improvements and a comparison with the use of wild-type enzymes being given in just some cases. The synthetic strategies described have

been categorised according to the class of enzymes employed in the overall process. The strict requirements in terms of the degree of enantiopurity for life-science products have led us to pay special attention towards those steps that facilitate the introduction of chirality or stereodiscrimination for the formation of single enantiomers.[28]

2. Use of oxidoreductases for the synthesis of chiral drugs.

Because of the wide panel of redox enzymes (EC 1)[29] present in nature, this class of biocatalysts offers a myriad of possibilities in synthetic chemistry. The development of oxidation and reduction processes has allowed the preparation of multiple families of compounds by means of whole cells biotransformations or using purified enzymes.[30-32] Oxidoreductases require the presence of the β-nicotinamide adenine dinucleotide as a cofactor. This exists in the oxidized form as a phosphorylated ($NADP^+$) or non phosphorylated structure (NAD^+), as well as in its reduced form (NAD(P)H). This ubiquitous redox cofactor is present in living cells involved in many cellular processes, such as electron transport and oxidative phosphorylation. The high cost of the nicotinamide cofactor makes its *in situ* regeneration necessary in preparative enzymatic synthesis when using the purified enzyme, so alternatively the development of whole cell systems has been extensively explored in the literature.

In this section examples of redox processes for the synthesis of pharmaceuticals will be described, paying special attention to the action of alcohol dehydrogenases (ADHs) and enereductases for bioreduction processes,[33] alcohol oxidases, monoamino oxidases, monoamino acid oxidases and laccases for oxidative processes, and finally oxygenases for the introduction of oxygen in a given molecule.

Most of the examples reported in the literature involve the reduction of carbonyl groups, the oxidation of alcohol functionalities, and the hydroxylation of non activated positions. However, redox enzymes are also valuable catalysts for the production of chiral amines and amino acids, leading to alternative

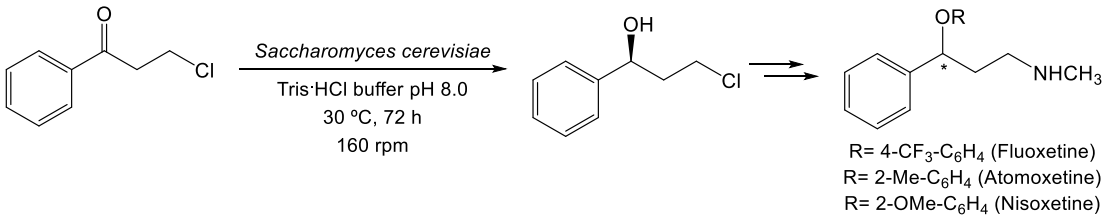
reductive amination reactions where the starting material and final product are identical to those in transaminase-catalyzed reactions. In the case of redix enzymes, amine and amino acid dehydrogenase (AH and AAH) are efficient enzymes for the transformation of ketones into amines or amino acids as occurs with transaminases from the transferase class. As with other types of biocatalysts, molecular biology[34] and directed evolution techniques[35] have led to the development and discovery of more stable and selective dehydrogenases for the synthesis of optically active amines.

2.1. Alcohol dehydrogenases.

Also known as ketoreductases or carbonyl reductases, they are probably the most ubiquitous enzymes in nature. Their ability to stereoselectively reduce carbonyl groups makes them attractive tools for the synthesis of enantiopure alcohols,[36-39] and the pharmaceutical industry has taken clear advantage of this.[40] In addition to the vast number of native ADHs, their substrate specificity and stereochemical outcome can also be modified by the application of a series of advanced techniques involving the production of recombinant species with one or more catalysts in an adequate host,[41-48] or through the development of directed evolution methods.[49] In this section, we summarise the action of ADHs for the bioreduction of ketones in order to produce valuable intermediates and final compounds with interesting applications in medicinal chemistry.

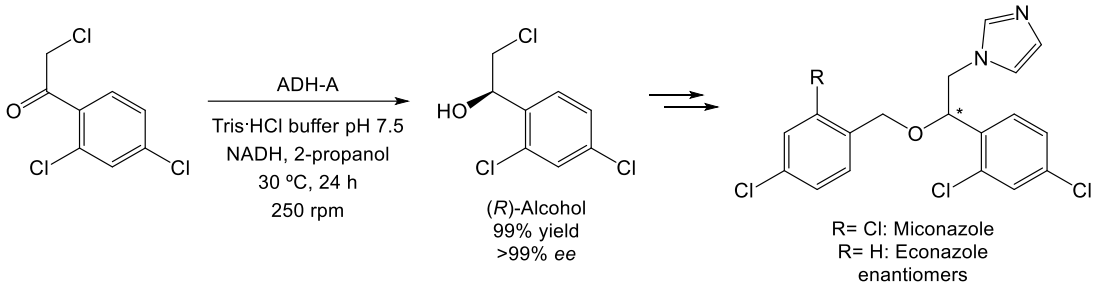
The use of *Saccharomyces cerevisiae* CGMCC 2266 has allowed the asymmetric reduction of 3-chloropriophenone to produce (*S*)-3-chloro-1-phenylpropanol, a versatile intermediate in the synthesis of antidepressant agents such as Fluoxetine, Atomoxetine and Nisoxetine (Scheme 1).[50] The immobilization of the cells in calcium alginate provided benefits in terms of conversion and stereoselectivity, the final product being obtained in 80% conversion and enantiopure form. Significantly, after being reused three times, the immobilized enzyme retained approximately 60% of its catalytic activity with complete stereopreference. González and co-workers reported the use of five

endophytic yeast strains isolated from edible plants for the bioreduction of the same chlorinated intermediate or alternatively the 3-azido-1-phenylpropanone, finding modest levels of selectivity and generally low yields due to the formation of side-reaction products because of the presence of the catalytic activities in the enzyme preparations.[51] Other enzymes such as ADH from *Rhodococcus ruber* (ADH-A) and ADH from *Thermonoanaerobacter* sp. (ADH-T), available from different commercial sources, have also served for the production of Fluoxetine intermediates as described by Gotor[52] and Berkowitz,[53] who also described the use of horse liver alcohol dehydrogenase (HLADH) or *Candida parapsilosis* alcohol dehydrogenase (CP-ADH) for the quantitative production of an enantiopure Aprepitant intermediate, of a human neurokin-1 antagonist, and of Talampanel, used for the treatment of epilepsy.



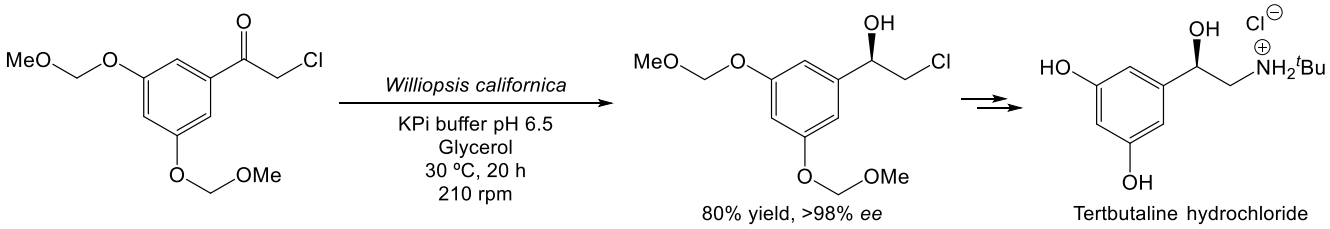
Scheme 1. Asymmetric bioreduction of 3-chloropropiophenone for the production of antidepressant intermediates.

Miconazole and Econazole are two potent antifungal agents that are administrated in patients as racemates. The chemoenzymatic asymmetric synthesis of all four of their enantiomers was studied by means of lipase-catalyzed acylation of the corresponding racemic alcohols and the ADH-mediated reduction of 2-halogen-1-(2,4-dichlorophenyl)ethanones, the best results being found for the purified enzymes ADH-T and ADH-A in the formation at 30 ºC after 24 h of enantiopure (*R*)-2-chloro-1-(2,4-dichlorophenyl)ethanol, a versatile precursor of the drugs with (*R*)-configuration (Scheme 2).[54] Unfortunately, the (*S*)-alcohol was isolated in low conversions with other ADHs tested, so for the synthesis of (*S*)-Miconazole and (*S*)-Econazole a chemical Mitsunobu inversion-deprotection sequence was required.



Scheme 2. Asymmetric bioreduction of 2-chloro-1-(2,4-dichlorophenyl)ethanone for the synthesis of Miconazole and Econazole enantiomers.

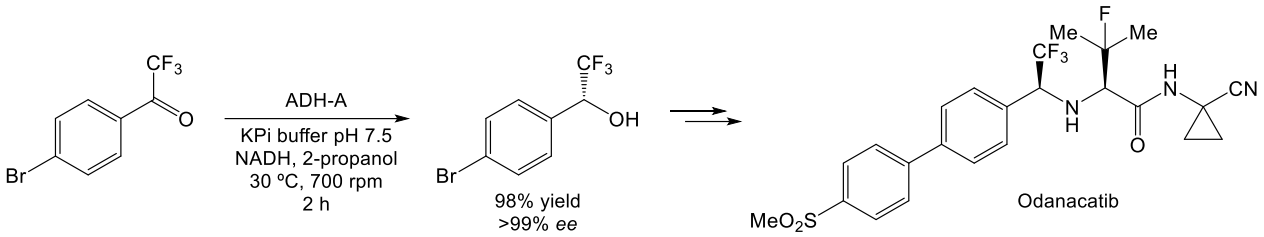
The bioreduction of other α-chloroacetophenone derivatives also gave excellent results in this case of the synthesis of an (*R*)-tertbutaline hydrochloride intermediate, a potent β_2-adrenoceptor-stimulating agent (Scheme 3).[55] The yeast *Williopisis californica* JCM 3600 in whole cell form stereoselectively reduced the carbonyl group to obtain the desired (*R*)-alcohol in 98% *ee* and 80% isolated yield, the cofactor regeneration system being improved by adding glycerol.



Scheme 3. Asymmetric bioreduction of a substituted α-chloroacetophenone for the synthesis of (*R*)-tertbutaline hydrochloride.

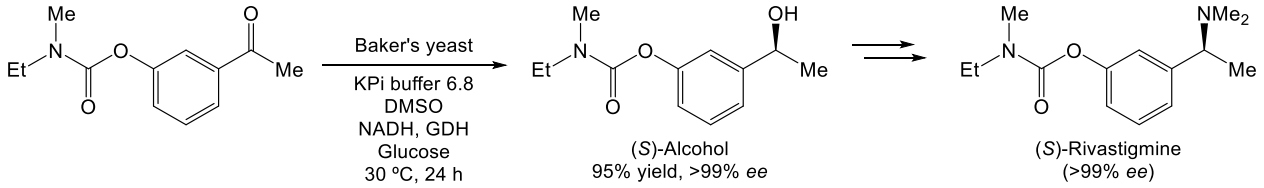
Odanacatib is an active and selective cathepsin K inhibitor used in the treatment of postmenopausal osteoporosis. Recently, Souza and co-workers described the development of a chemoenzymatic process for the preparation of an Odanacatib precursor under continuous flow conditions, once the overexpressed ADH-A in *E. coli* had been found as a selective biocatalyst for the reduction of the key intermediate 1-(4-bromophenyl)-2,2,2-trifluoroethanone (Scheme 4).[56] The desired enantiopure (*R*)-1-

(4-bromophenyl)-2,2,2-trifluoroethanol was recovered with 98% yield after 2 h in the supernatant, and subsequently subjected to a Suzuki-Miyaura palladium-catalyzed coupling.



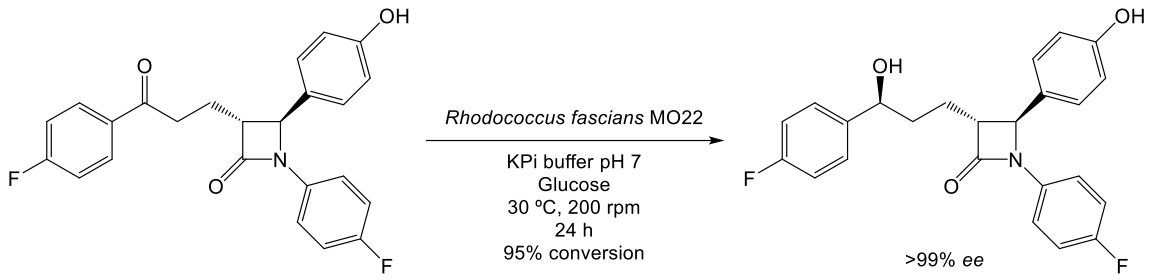
Scheme 4. Chemoenzymatic synthesis of Odanacatib involving the ADH-catalyzed bioreduction of 1-(4-bromophenyl)-2,2,2-trifluoroethanone.

Rivastigmine is an acetylcholinesterase inhibitor that acts selectively in the brain. It possesses a carbamate functionality, the (*S*)-enantiomer having applications in dementia caused by Parkinson's disease. A crude ADH from Baker's yeast has been found to catalyze the stereoselective reduction of *N*-ethyl-*N*-methyl carbamoyl acetophenone at 50-g scale, affording the enantiopure (*S*)-alcohol precursor in 95% isolated yield at 30 ºC (Scheme 5).[57]



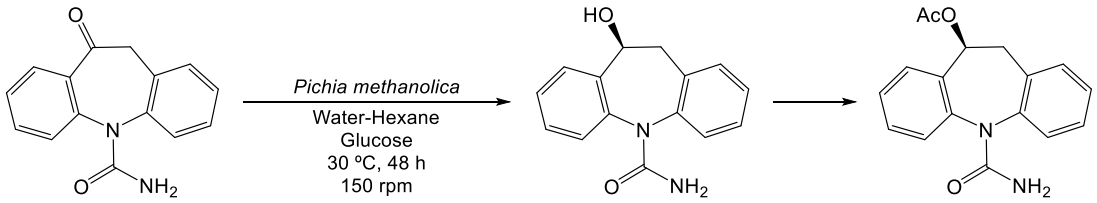
Scheme 5. Synthesis of a (*S*)-Rivastigmine intermediate through a bioreduction process.

Ezetimibe marketed as *Zetia* or *Ezetrol* is employed in the treatment of patients with coronary diseases, inhibiting the adsorption of cholesterol and related sterols. Its enzymatic synthesis was achieved by diastereoselective bioreduction of 1-(4-fluorophenyl)-(3*R*)-[3-oxo-3-(4-fluorophenyl)-propyl]-(4*S*)-(4-hydroxyphenyl)azetidin-2-one using the *Rhodococcus fascians* MO22 microorganism in whole cell form (Scheme 6).[58] After optimization of the reaction conditions, a 95% conversion into the desired diastereoisomer was attained after 24 h at 30 ºC.



Scheme 6. Enzymatic synthesis of Ezetimbe.

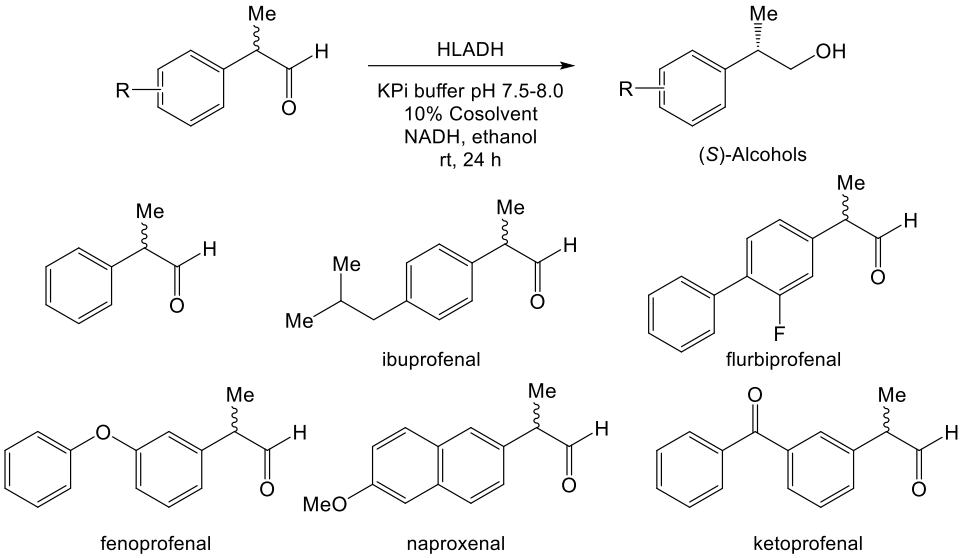
Eslicarbazepine acetate, also known as *Aptiom*, *Zebinix* or *Exalief*, is a third-generation drug belonging to the class of carbamazepines with application in the treatment of epilepsy, neuralgia and other brain disorders. Resting cells of an ADH from *Pichia methanolica* were able to stereoselectively reduce the ketone intermediate (Scheme 7), providing the immediate (*S*)-alcohol precursor.[59] For this purpose, an exhaustive optimization was performed including reaction parameters such as the glucose present (1-7%), substrate concentration (0.5-3.0 g/L), temperature (20-40 ºC), orbital shaking (100-200 rpm) and reaction medium. Remarkably, hexane was found as a suitable immiscible organic cosolvent for the isolation of the desired enantiopure alcohol in >85% conversion using a 2.5-g scale reaction.



Scheme 7. Bioreduction of an Eslicarbazepine acetate precursor using an ADH from *Pichia methanolica.*

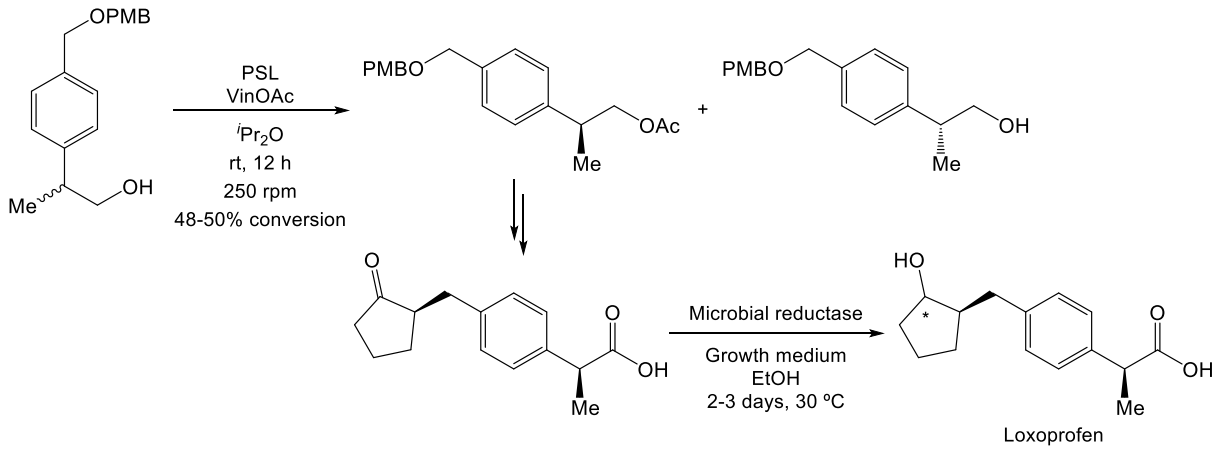
The production of enantiopure profens such as Ibuprofen or Naproxen is highly appealing as they possess different biological profiles. The development of dynamic reductive kinetic resolutions for the production of these drugs has been possible starting from the corresponding racemic aldehydes using archeal thermophiles from *Sulfolobus solfataricus*[60] or HLADH.[61] (*S*)-2-Arylpropanols were obtained

in excellent yields and selectivities through ADH-catalyzed dynamic kinetic resolutions (DKRs) of the corresponding 2-arylpropanals (Scheme 8).



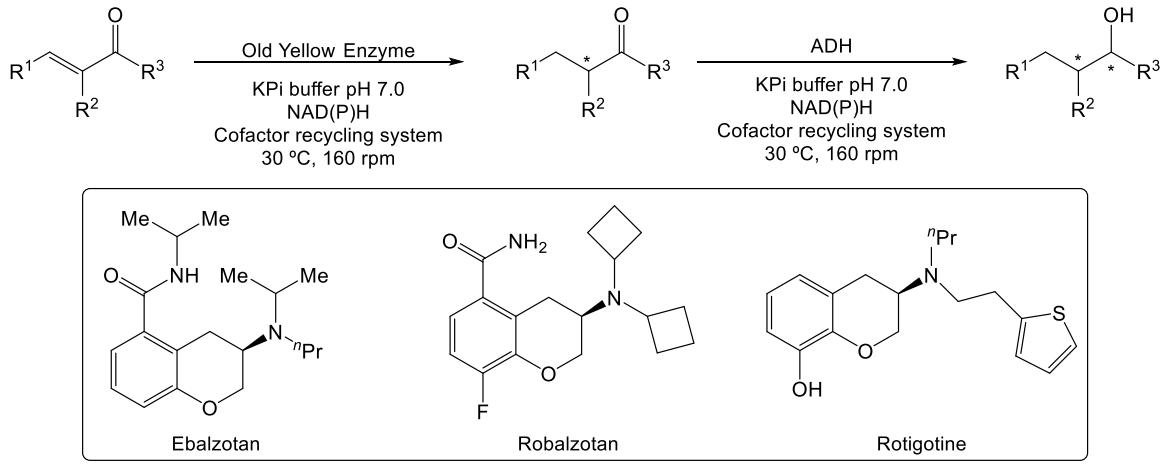
Scheme 8. Dynamic reductive kinetic resolutions of 2-arylpropanals for the synthesis of profen derivatives.

Loxoprofen belongs to the profen class and possesses non-steroidal anti-inflammatory properties. Its synthesis has been possible by using two independent enzymatic steps (Scheme 9). Firstly, the enzymatic kinetic resolution of the corresponding racemic alcohol was achieved using 8 equiv. of vinyl acetate and PSL as biocatalyst in $^{i}Pr_2O$.[62] After convenient chemical modification, a series of microbial ketoreductases were tested in the diasteroselective reduction of a ketoacid intermediate, excellent discrimination being found for the formation not only of the desired *trans*-active Loxoprofen form with growing cells from *Geotrichum candidum* NBRC 4597 (20:1 dr in 78% isolated yield), but also of the *cis*-diasteroisomer using *Torulaspora delbrueckii* NBRC 10921 (1:20 dr in 76% isolated yield).



Scheme 9. Chemoenzymatic synthesis of Loxoprofen.

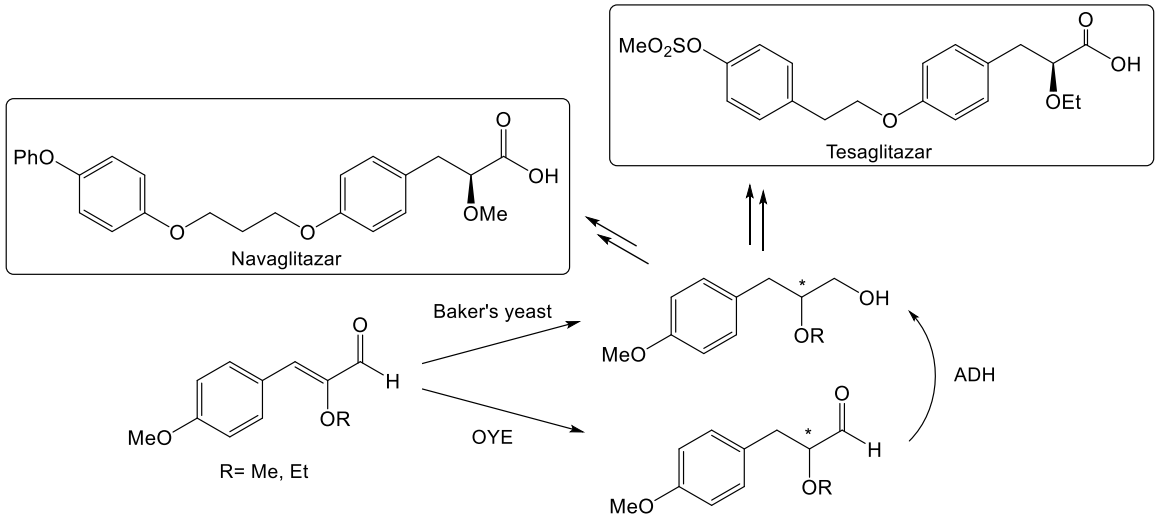
Interestingly, the combination of an ADH such as HLADH and enoate reductases from the family of Old Yellow Enzymes (OYEs) has been applied for the reduction of α,β-unsaturated aldehydes and ketones with excellent selectivities,[63] providing access to optically active alcohols with different configurations. These are precursors in the synthesis of pharmaceuticals such as Ebalzotan, Robalzotan and Rotigotine (Scheme 10).



Scheme 10. Combination of enoatereductases and ADHs in the bioreduction of α,β-unsaturated aldehydes and esters.

2.2. Enereductases.

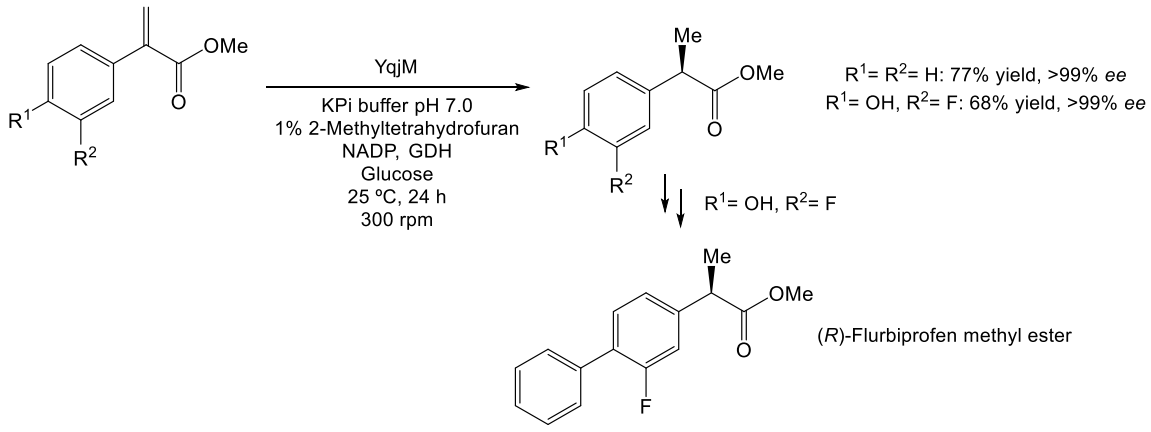
Also known as enoate reductases (ERs), this class of redox enzymes is responsible for the bioreduction of C=C bonds of activated organic compounds such as prochiral α,β-unsaturated ketones, aldehydes, carboxylic acid derivatives, and nitroalkenes,[64-66] and possesses the same restrictions of cofactor dependency as previously seen with ADHs. The synthesis of anti-diabetic Tesaglitazar and Navaglitazar intermediates has been described by Gatti and co-workers through bioreduction of α,β-unsaturated aldehydes using native Baker's yeast (BY)[67] or the nicotinamide-dependent flavoproteins OYEs.[68] BY led to the optically active hydroxy ethers, while the cloned, overexpressed and finally purified OYEs allowed the preparation of the optically active aldehydes that were reduced to alcohols using an ADH (Scheme 11).[69]



Scheme 11. Use of enoate reductases for the synthesis of Tesaglitazar and Navaglitazar.

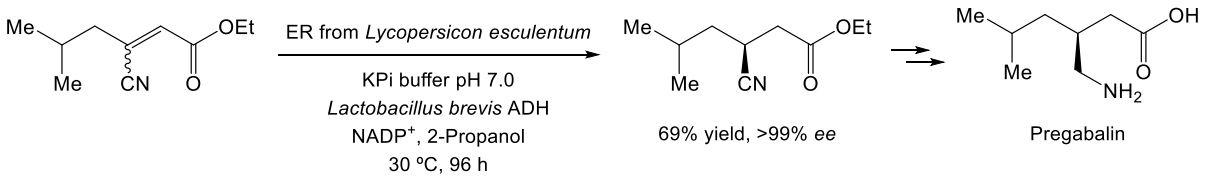
The enereductase YqjM from *Bacillus subtilis* in lyophilized form has been applied in the bioreduction of methyl-2-phenyl acrylate.[70] After an exhaustive optimization of the reaction conditions in terms of type of buffer and cosolvent, a system composed of a 20 mM phosphate buffer pH 7.0 and 1% of 2-methyltetrahydrofuran was found to produce the desired ester in excellent conversion, 77% isolated yield and enantiopure form (Scheme 12). These conditions were later applied to a panel of substrates

including the synthesis of (*R*)-Flurbiprofen methyl ester, so the methyl 2-(3-fluoro-4-hydroxyphenyl)acrylate was stereoselectively reduced in moderate yield (68%).



Scheme 12. Synthesis of (*R*)-profen derivatives using enereductase YqjM from *Bacillus subtilis*.

The synthesis of Pregabalin, a lipophilic γ-aminobutyric acid (GABA) has been developed by means of the bioreduction of (*E*)-ethyl 5-methyl-3-cyano-2-hexanoate using an ER from *Lycopersicon esculentum* overexpressed in *E. coli* (Scheme 13).[71] The preparative reaction proceeded smoothly in the formation of the enantiopure alkane after 96 h. The evolution of native enzymes towards more active and selective mutants has allowed the efficient synthesis of other Pregabalin intermediates.[72,73]



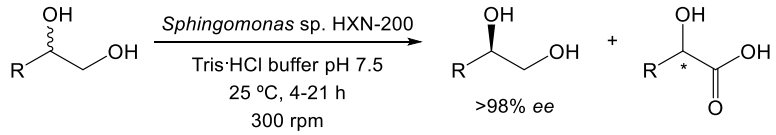
Scheme 13. Chemoenzymatic synthesis of Pregabalin using an enoate reductase.

2.3. Alcohol oxidases, monoamino oxidases, monoamino acid oxidases and berberines.

Enzymatic oxidation reactions have been traditionally explored less than their reverse reduction processes because of a series of inconveniences such as the destruction of chiral centers, unfavoured

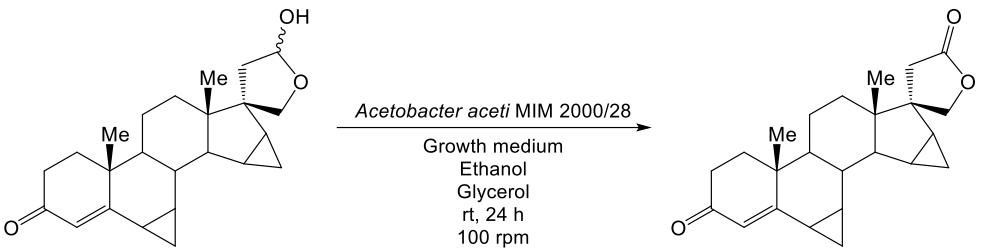
thermodynamic equilibria, and high pHs for the development of efficient transformations. Nevertheless, oxidases provide an elegant regio-, chemo- and stereoselective access to broad families of organic substrates including, among others, ketones, aldehydes and imines, by means of single or multi-step transformations.[74,75] Some examples are presented here in the search for sustainable routes to biologically active compounds.

Enantiopure vicinal diols are useful building blocks for drug synthesis. In this context, Li and co-workers developed an efficient methodology for the oxidative resolution of a series of 1,2-diols including 3-*O*-benzylglycerol, 1-(4-chlorophenyl)-1,2-ethanediol, 1-(4-methylphenyl)-1,2-ethanediol and 1-(4-phenyl)-1,2-ethanediol, all of them being key fragments of important pharmaceuticals. The regio- and stereoselective oxidation was achieved using *Sphingomonas* sp. HXN-200 microbial cells, yielding the corresponding optically active diols in excellent optical purity (>98% *ee*), and the hydroxy carboxylic acids as overoxidized products through the formation of the aldehydes as intermediates (Scheme 14).[76]



Scheme 14. Regio- and stereoselective oxidation of racemic 1,2-diols using *Sphingomonas* sp.

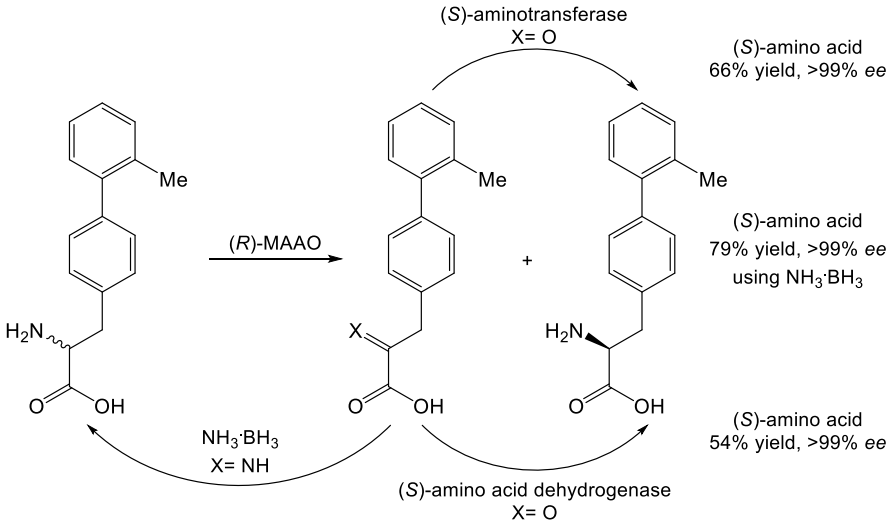
Functionalized lactones are also important intermediates in the synthesis of biologically active compounds, and their preparation is favoured starting from 1,4- and 1,5-diols. Molinari and co-workers reported the oxidation of 1-alkyl-1,4-butanediols into γ-lactones with moderate selectivities but good yields using *Acetobacter aceti* MIM 2000/28 as growing cells or with cells centrifuged and resuspended in phosphate buffer.[77] The formation of the corresponding lactols was observed and validated by the synthesis of Drospirenone, a synthetic steroid used as a hormonal contraceptive, starting from 6β,7β;15β,16β-dimethylene-3-oxo-17α-pregn-4-en-21,17-carbolactol (Scheme 15).



Scheme 15. Synthesis of Drospirenone by *Acetobacter aceti*-catalyzed oxidation.

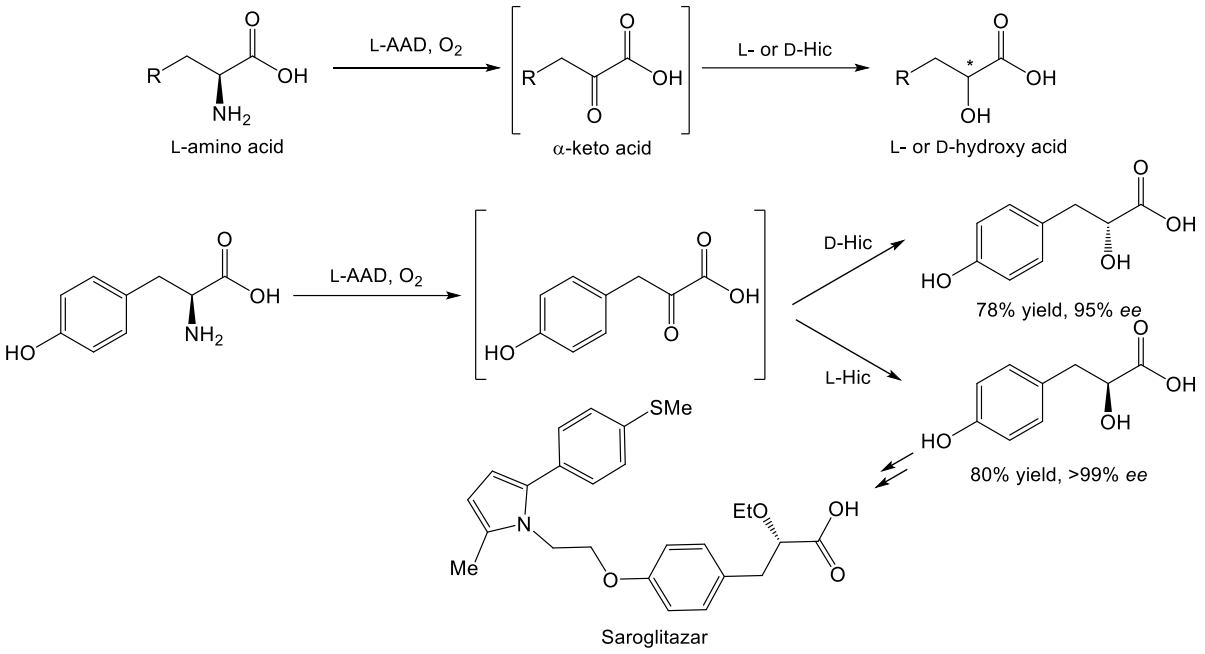
Monoamino oxidases (MAO) and monoamino acid oxidases (MAAO) are two types of versatile oxidative enzymes which act towards amino groups in amines and amino acids, respectively, and open new possibilities for subsequent *in situ* chemical or enzymatic reduction leading to efficient deracemization processes. Thus, the isolation of an enantiopure compound is possible, theoretically, in 100% yield. The modification of their amino acid sequence through directed evolution techniques has allowed a spectacular advance in this field, especially for MAO enzymes, leading to the preparation of pharmacologically active compounds or amine-based intermediates such as prolyl peptides,[78,79] Telaprevir,[80] Crispine A,[81] Boceprevir intermediates,[82] tetrahydro-β-carbolines[83] and different alkaloid natural products.[84] Regarding the use of wild-type enzymes, Patel and co-workers described three independent strategies for the biocatalytic production of (*S*)-2-amino-3-(6-*o*-tolylpyridin-3-yl)propanoic acid, a key intermediate of an anti-diabetic drug candidate, using a monoamino acid oxidase from *Trigonopsis variabilis* expressed in *Escherichia coli* (Scheme 16).[85] On one hand, the authors reported the selective oxidation of the racemic amine to the α-keto acid using (*R*)-MAAO, which reacts exclusively with the (*R*)-amino acid. This resulting α-keto acid was later converted to its (*S*)-antipode with an (*S*)-aminotransferase and aspartate as the amine donor, yielding the enantiopure product in 66% isolated yield. On the other hand, the deracemization of the racemic amino acid was achieved using (*R*)-MAAO in combination with a chemical reduction of the imine intermediate using the ammonia borane complex that regenerates the racemic amino acid whilst the enantiopure (*S*)-amino

acid accumulates (79% isolated yield). Finally, the combination of this (*R*)-MAAO and an (*S*)-amino acid dehydrogenase/ammonium formate/NADH system was used to obtain the enantiopure (*S*)-amine in 68% conversion and 54% isolated yield.



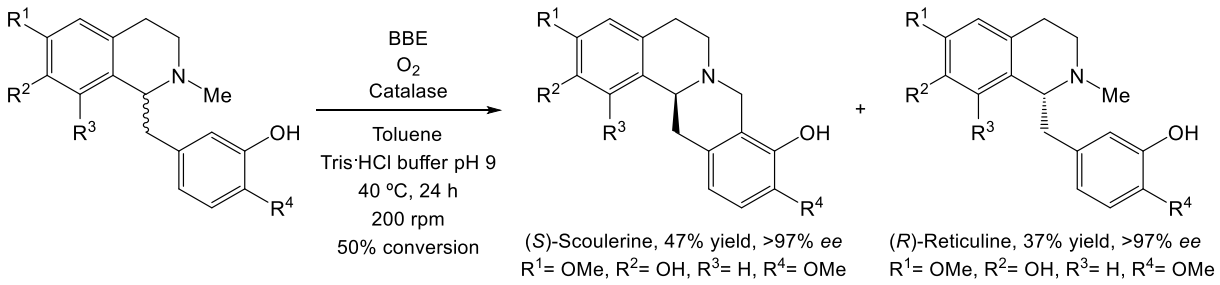
Scheme 16. Deracemization strategies for the synthesis of a valuable (*S*)-amino acid using a monoamino acid oxidase from *Trigonopsis variabilis*.

Interestingly, Kroutil and co-workers recently reported the combined use of freeze-dried *E. coli* cells containing overexpressed L-amino acid oxidases or L-amino acid deaminases (L-AAD) with selective reductases to transform prochiral α-keto acids into optically active α-hydroxy acids (Scheme 17).[86] The best results were found with the L-AAD from *Proteus myxofaciens* providing six different α-keto acids, which were later stereoselectively reduced with L- or D-isocaproate reductases (HicDHs) from *Lactobacillus paracasei* DSM 20008 (L-Hic) and *Lactobacillus confuses* DSM 20196 (D-Hic). Twelve α-hydroxy acids were obtained with excellent selectivities and conversions, including enantiopure 4-hydroxyphenyl lactic acid, an interesting building block for the preparation of compounds with medicinal interest such as anti-diabetic Saroglitazar, Aeruginosins 298A, peroxime proliferator activated α/γ agonists, Microcin SF608, and others.



Scheme 17. Multi-step enzymatic synthesis of α-hydroxy acids including enantiopure 4-hydroxyphenyl lactic acid.

Berberine bridge enzymes (BBEs) are redox enzymes that catalyze the anaerobic oxidative carbon-carbon bond formation, transforming benzylisoquinolines to berberines. The action of BBE from *Eschscholzia californica* heterologously expressed in *E. coli.* for the asymmetric chemoenzymatic synthesis of interesting alkaloids such as (*S*)-Scoulerine and (*R*)-Reticuline was reported by Kroutil and co-workers.[87] (*S*)-Scoulerine belongs to the family of berbines, a group of compounds with diverse biological profiles such as analgesic, anti-inflammatory, hypnotic or sedative properties. (*R*)-Reticuline is inside the 1-benzyl-1,2,3,4-tetrahydroisoquinoline group and has interesting anti-spasmodic and hypotensive properties. The oxidative KRs of racemic Reticuline and other substituted 1-benzyl-1,2,3,4-tetrahydroisoquinolines were possible with complete selectivity at 40 ºC for 24 h by using crude catalase for the consumption of any hydrogen peroxide generated (Scheme 18).

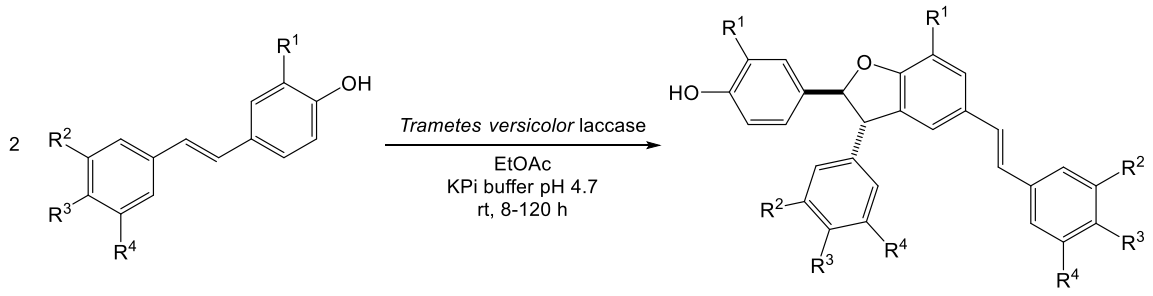


Scheme 18. BBE-catalyzed oxidative KRs of racemic 1-benzyl-1,2,3,4-tetrahydroisoquinolines including the synthesis of (*S*)-Scoulerine and (*R*)-Reticuline.

2.4. Laccases.

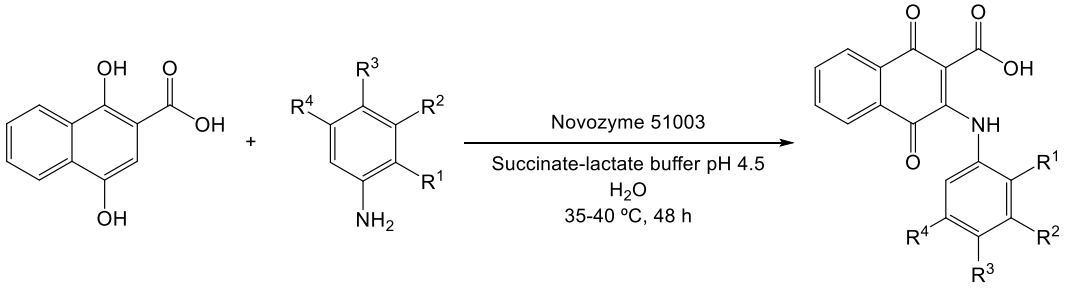
Laccases are blue multi-copper oxidases able to catalyze mild oxidative transformations. The actions of laccases are usually accomplished by combination with a laccase mediator system (LMS) in the presence of molecular oxygen that is reduced to water, providing a green access to different families of compounds.[88I] Ideal redox mediators are good laccase substrates whose oxidized and reduced forms are stable without inhibiting the enzymatic reaction.

Because of their high stability and selectivity for phenolic substrates, their applications in sustainable synthetic routes have received great attention in recent years.[89] For instance, seven Resveratrol oligomers with anti-proliferative activities have been produced by oxidative coupling of their monomeric forms using the laccase from *Trametes versicolor*.[90] These stilbenoids were converted into the corresponding dehydrodimers incorporating a dihydrobenzofuran moiety as major products with regio- and diasteroselectivity but no enantioselectivity (Scheme 19). The reactions were carried out in a biphasic system composed of ethyl acetate (EtOAc) and a phosphate buffer pH 4.7 at room temperature, leading to the racemates which were later separated in their enantiomers by repeating chiral HPLC protocols until enough product had been recovered to develop circular dichroism experiments.



Scheme 19. *Trametes versicolor* laccase-catalyzed dimerization of Resveratrol monomers.

The synthesis of aminonaphtoquinones with anti-cancer activity has been possible by laccase-catalyzed C-N bond formation.[91] Nuclear monoamination of a 1,4-naphthohydroquinone using 2 equiv. of primary aromatic amines with commercially available Novozyme 51003 laccase from the thermophilic ascomycete *Myceliophthora thermophile*, led to the formation of the final products in low to high yields (Scheme 20). Limited improvements were found at pH 6 and with DMF as cosolvent to favor the solubility of the starting materials.



Scheme 20. Laccase-catalyzed synthesis of aminonaphthoquinones by nuclear amination of 1,4-naphthohydroquinones.

Together with other oxidases and oxygenases, laccases have also allowed the development of selective modifications in steroids.[92] For instance, Riva and co-workers reported the unexpected C-4 hydroxylation of ergot alkaloids.[93] In addition, laccases can be coupled to the action of

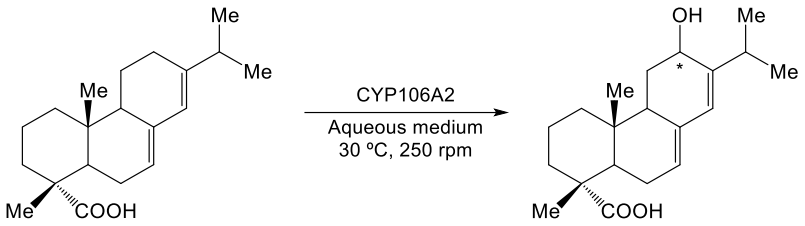
dehydrogenases in order to find effective conditions for the nicotinamide cofactor recycling. This is the case of the purified *Toxicodendron pubescens* laccase using the redox mediator Meldola's blue (7-dimethylamino-1,2-benzophenoxazine). This has been used in combination with the 7α-hydroxystereoid dehydrogenase for the gram scale oxidation of cholic acid and its corresponding methyl ester to the 7-keto derivatives in up to 70% yield, using a phosphate buffer pH 6.5 or a biphasic system with isopropanol as cosolvent, and oxygen as mild oxidant.[94]

2.5. Oxygenases.

This type of enzyme catalyzes the incorporation of molecular oxygen into a molecule, giving access to oxygenated compounds in a sustainable and safe manner in comparison with traditional chemical methods.[95,96] Oxygenases usually act with excellent regio- and stereoselectivity, the functionalization of non activated substrates such as alkanes, alkenes and aromatic compounds being possible.[97-99] In this context, the action of some cyctochromes P450 (CYPs),[100-104] Baeyer-Villiger monooxygenases (BVMOs),[105-109] and toluene dioxygenases (TDOs), is remarkable.[110]

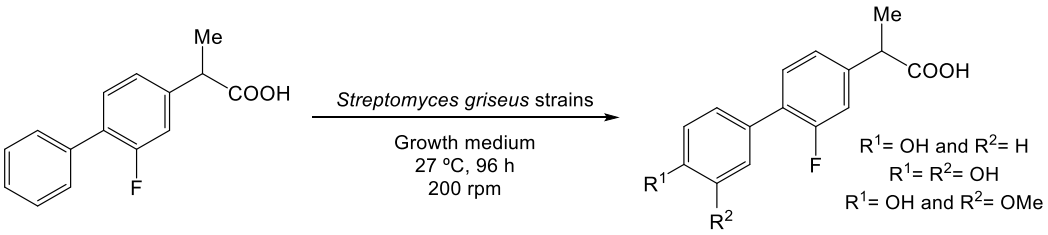
The main limitations in these reactions reside in the low substrate concentration employed, the low catalytic activity, and their limited availability, the addition of an external cofactor and other enzymes being required for the development of an efficient transformation. The development of recombinant and evolved enzymes rather than the use of native proteins has provided better access to organic compounds with interesting biological profiles. This is the case, for instance, in the synthesis of different drugs using CYPs,[111-113] Flavin MOs[114] and TDOs.[115-118] Nevertheless, in some cases whole cell or purified oxygenases have been used for synthetic purposes aimed at the production of compounds with interesting biological activities. Bernhardt and co-workers described the use of the cytochrome P450 monooxygenase CYP106A2 from *Bacillus megaterium* ATCC 13368 in whole-cell form for the hydroxylation of variety of 3-oxo-Δ^4-steroids such as progesterone and

deoxycorticosterone at the 15β-position, and the use of abietic acid, which displays interesting anti-allergic, anti-inflammatory and anti-convulsant properties, at the 12-position to obtain an α/β mixture with moderate stereodiscrimination (Scheme 21).[119]



Scheme 21. Regio- and setereoselective hydroxylation of abietic acid at the 12-position.

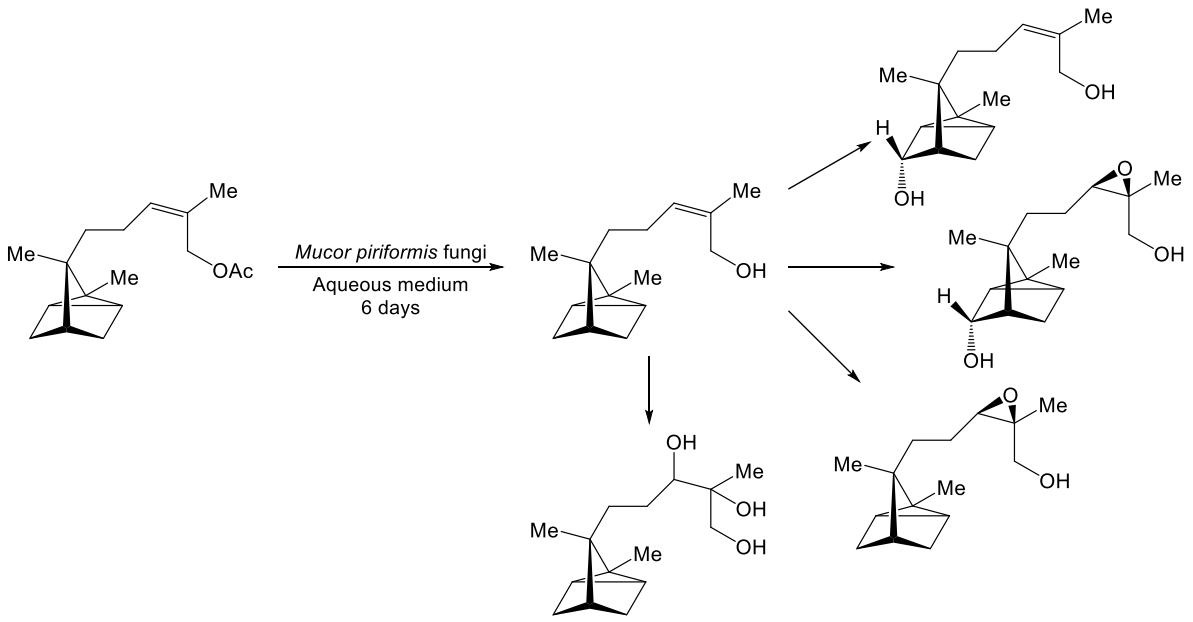
Streptomyces griseus ATCC13273 and DSM40236 were found to be active microorganisms for the production of hydroxylated Flurbiprofen derivatives, which possess potential anti-inflammatory activities, the presence of methyl transferase activity also being found due to the formation of a methoxy analogue (Scheme 22).[120] In addition, two new fluorometabolites, flurbiprofenamide and 7-hydroxy-flurbiprofenamide, formed via the action of an amidotransferase, were detected in culture extracts of *Streptomyces lavenduligreseus* and *Streptomyces rimosus* used as resting cells in the presence of different nitrogen sources.



Scheme 22. Regioselective hydroxylation of Flurbiprofen by using *Streptomyces griseus* strains.

A fungal strain from *Mucor piroformis* has catalyzed the regio- and stereoselective functionalization of (*Z*)-α-santalyl acetate into four novel metabolites identified as 10,11-*cis*-β-epoxy-α-santalol, 5α-hydroxy-(*Z*)-α-santalol, 10,11-dihydroxy-α-santalol, and 5-α-hydroxy-10,11-*cis*-β-epoxy-α-santalol

(Scheme 23).[121] The transformations initially failed using (*Z*)-α-santalol as staring material due to its potent anti-fungal activity, so its acetate was synthesized and then successfully tested in biotransformations with a series of fungi, involving the same alcohol in the medium as intermediate.



Scheme 23. Oxygenation of (*Z*)-α-santalyl acetate using a fungal strain from *Mucor piroformis*.

3. Use of lyases for the synthesis of chiral drugs.

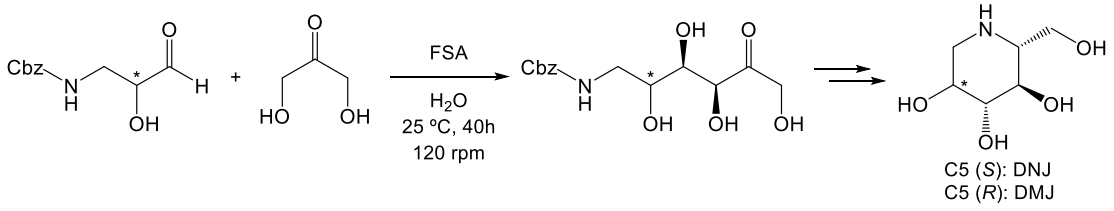
Enzymatic carbon-carbon bond formation reactions have received considerable attention because of the high stereoselectivity displayed by lyases.[122,123] Among this family, aldolases and hydroxynitrile lyases are without doubt the most versatile enzymes for synthetic transformations, although in recent years other biocatalysts have appeared as useful tools.[124,125] The role of aldolases and hydroxynitrile lyases in the synthesis of pharmaceuticals will now be discussed.

3.1. Aldolases.

This class of enzymes catalyze aldol reactions creating carbon-carbon bonds in a highly stereoselective manner, providing access to adducts where a chain elongation of two or three carbon units have

occurred, in addition to the appearance of one or two new stereocenters.[126] Importantly, this enzymatic approach allows the formation of a single diastereoisomer after only one reaction step. Chemists have taken advantage of previously described aldolase-catalyzed reactions to synthesize interesting sugar derivatives with high stereoselectivity, such as Digitoxin, an active glycoside prescribed for patients with cardiac diseases that is composed of a steroid part and a trisaccharide.[127] In recent years, the action of novel aldolases with improved catalytic properties has been described. Advances in enzyme engineering techniques nowadays open a myriad of possibilities for the creation of mutants with broader substrate specificity, facilitating the development of more efficient transformations.[128]

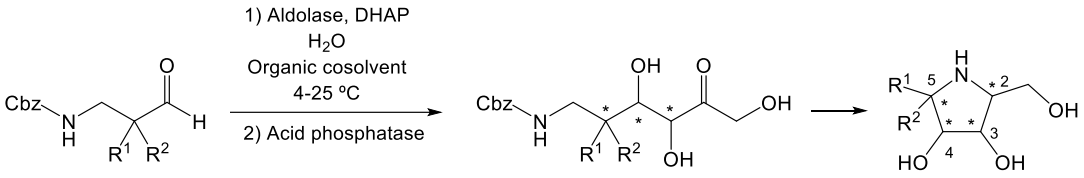
The D-Fructose-6-phosphate aldolase from *Escherichia coli* (FSA) used as a lyophilized powder is a valuable enzyme that catalyzes the addition of dihydroxyacetone and hydroxyacetone to aldehydes, giving access to a large family of sugar derivatives with important applications in medicinal chemistry, such as 1-deoxynojirimycin (DNJ), 1-deoxymannojirimicyn (DMJ), 1,4-dideoxy-1,4-imino-D-arabinitol, 1,4,5-trideoxy-1,4-imino-D-arabinitol and 1-deoxy-D-xylulose, among others.[129] For instance, DNJ and DMJ were obtained by FSA-catalyzed reaction of dihydroxyacetone with (*N*-Cbz-amino)-2-hydroxypropanal enantiomers, leading to DNJ in 49% yield and DMJ in 14% after 40 h and the addition in portions of the aldehyde at 25 ºC (Scheme 24).



Scheme 24. Synthesis of polyhydroxylated sugars DNJ and DMJ by using D-Fructose-6-phosphate aldolase in the addition of dihydroxyacetone to (*N*-Cbz-amino)-2-hydroxypropanal.

The same research group has reported the synthesis of other polyhydroxylated families of compounds. For instance, an optimization study of the aldolase-catalyzed reaction was performed for the subsequent

production of pyrrolidine derivatives that were evaluated as glycosidase inhibitors.[130] The reaction between *N*-Cbz aminoaldehydes and dihydroxyacetone phosphate (DHAP) occurred with variable yields and selectivity values, leading to different diastereoisomers depending on the aldolases employed in the process. In all cases, D-fructose-1,6-biphosphate aldolase from rabbit muscle (RAMA), L-rhamnulose-1-phosphate aldolase (RhuA) and L-fuculose-1-phosphate aldolase (FucA), led to the final products with an excellent stereocontrol (Scheme 25).

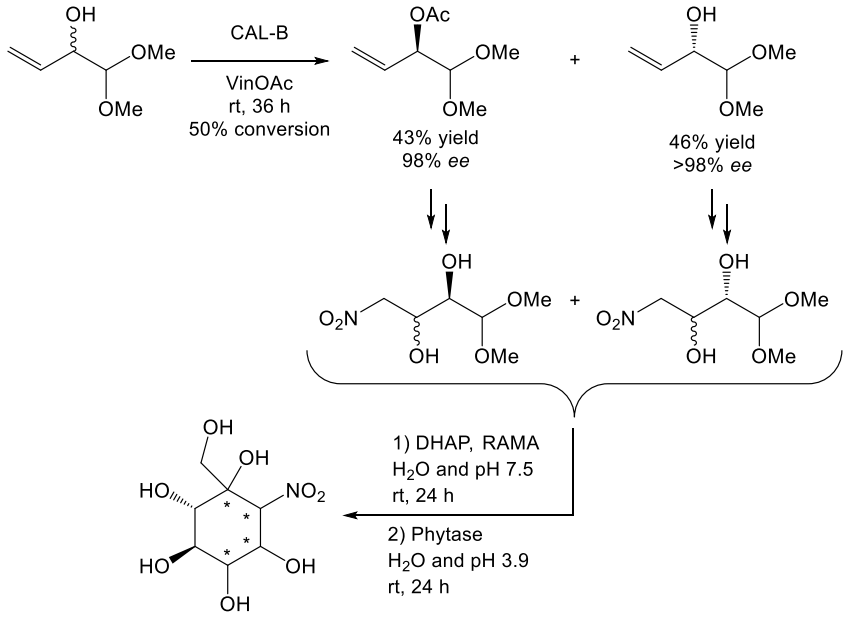


Scheme 25. Synthesis of polyhydroxylated pyrrolidine derivatives using aldolase-catalyzed additions of DHAP to *N*-Cbz aminoaldehydes.

The chemoenzymatic syntheses of pyrrolizidine derivatives such as Casuarine stereoisomers have been described through a two-step route. These nitrogenous compounds possess interesting biological profiles, acting as α-glucosidase inhibitors. The approach consisted in the aldolase-catalyzed addition of DHAP to *N*-Cbz-aminoethanal, furnishing 1,4-dideoxy-1,4-imino-D-arabinitol (DAB) and its enantiomer LAB, followed by subsequent one-pot chemical deprotection-reductive amination.[131] In the first step, a FucA mutant was required, while the wild type from RhuA displayed excellent results for the synthesis of LAB. The use of a FSA mutant also allowed the preparation of other pyrrolizidine derivatives with good yields and selectivities.[132] Indolizidines and quinolizidines are also conformationally restricted iminocyclitols, some of them acting as glycosidase inhibitors and possessing interesting therapeutic applications as anti-cancer, anti-diabetic, anti-metastatic, anti-viral and immunoregulating agents. Clapés and co-workers also reported the synthesis of these families of compounds through stereoselective carbon-carbon bond formation reactions. RhuA and FucA were identified as possible catalysts for aldol additions, although the use of evolved mutants led to better

results.[133]

Lemaire and co-workers have efficiently obtained aminocyclitols that are also potent glycosidase inhibitors. The chemoenzymatic route involves two key enzymatic transformations. Firstly, *Candida antarctica* lipase type B (CAL-B) catalyzed the kinetic resolution of 1,1-dimethoxybut-3-en-2-ol in combination with vinyl acetate at room temperature, leading to the (*S*)-alcohol and the (*R*)-acetate in good optical purity. These were later transformed to the 1,1-dimethoxy-4-nitrobutan-2,3-diol enantiomers (Scheme 26).[134] These building blocks were then reacted with DHAP in the presence of RAMA to produce the corresponding nitrocyclitols, the immediate precursors of the target aminocyclitols, in low yields but with excellent selectivity.



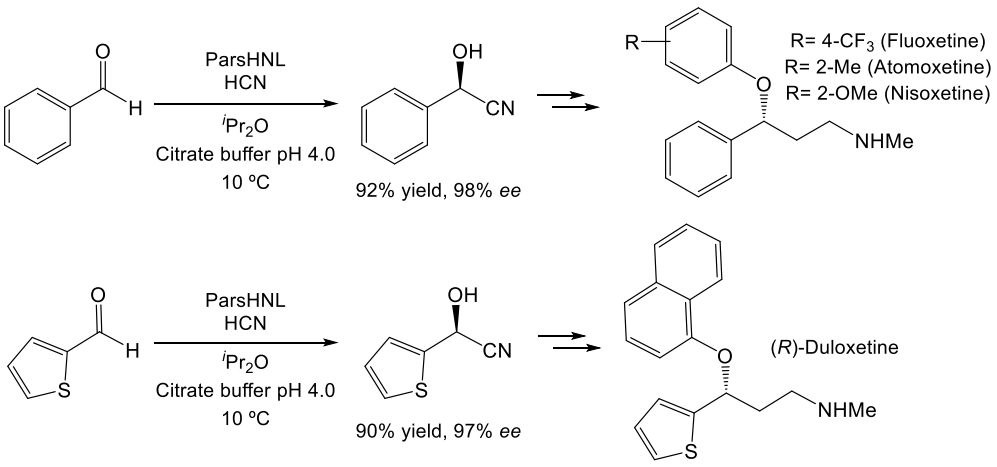
Scheme 26. Synthesis of aminocyclitols using CAL-B and RAMA.

Finally, the possibilities of aldolases to work in one-pot catalytic cascades, as reported by Wever and co-workers for the chemoenzymatic synthesis of D-fagomine, is worthy of comment.[135] The key step involves the orchestral action of four enzymes occurring a phosphorylation, oxidation, carbon-carbon bond formation, and dephosphorylation, using dihydroxyacetone phosphate (DHAP)-dependent RAMA

for the aldol formation to give the desired carbohydrate fragment intermediate.

3.2. Oxynitrilases.

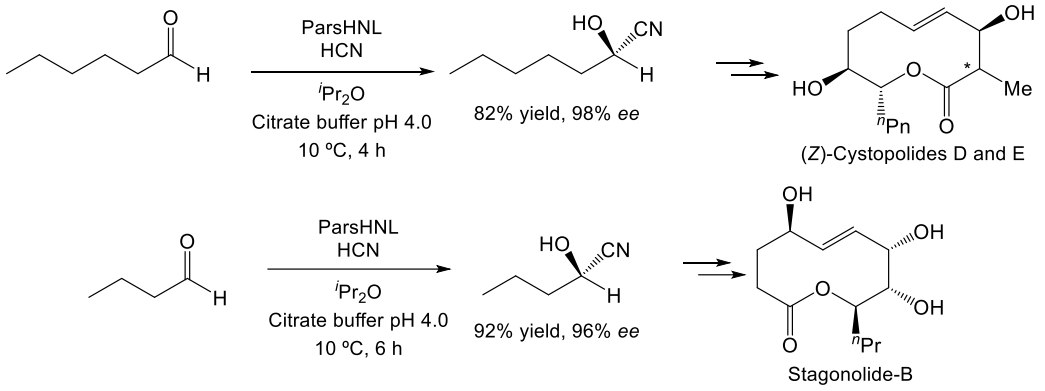
This class of lyases, also known as hydroxynitrile lyases (HNLs), catalyzes the addition of hydrogen cyanide (HCN) to aldehydes or methyl ketones,[136] providing an efficient access to cyanhydrins, which are highly attractive building blocks in synthetic chemistry.[137,138] In this context, the reaction of benzaldehyde with HCN allows the formation of mandelonitrile in optically active form, and this serves as an intermediate in the synthesis of the serotonin re-uptake inhibitors Fluoxetine, Atomoxetine and Nisoxetine. Nanda and co-workers used the crude *Prunus armeniaca* hydroxynitrile lyase (ParsHNL) from Himalayan apricot as biocatalyst and $^{i}Pr_2O$ as solvent for this transformation, affording the (*R*)-mandelonitrile in 92% yield and 98% *ee* (Scheme 63).[139] In addition, the same enzyme catalyzed the addition of HCN to 2-thiophene-carboxaldehyde, yielding (*S*)-2-hydroxy-2-(thiophen-2-yl)acetonitrile in 90% yield and 97% *ee* (Scheme 27). The latter is a valuable precursor in the synthesis of (*R*)-Duloxetine, a serotonin norepinephrine re-uptake inhibitor.



Scheme 27. HNL-catalyzed synthesis of cyanhydrins as valuable precursors of serotonin re-uptake inhibitors.

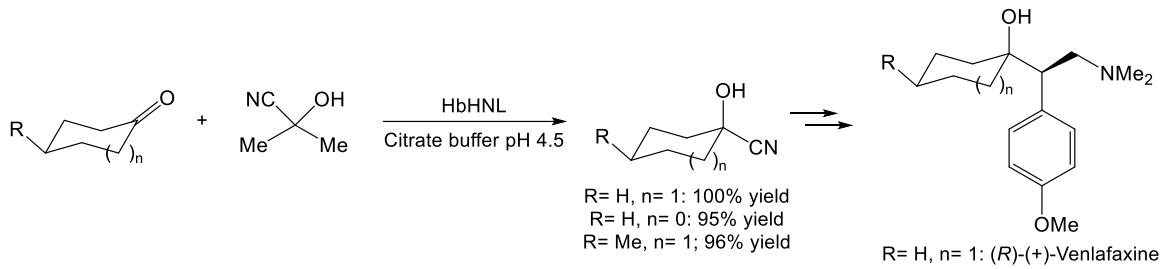
Similarly, the same research group reported the addition of HCN to hexanal also using the ParsHNL,

with 82% yield after 4 h of almost enantiopure (*R*)-2-hydroxyheptanenitrile. This served as an ideal building block in the synthesis of a series of metabolites from the family of nonanolides (Scheme 28).[140] Stagonolides are a class of naturally occurring nonenolides with interesting biological properties. Stagonolide-B has been obtained chemoenzymatically through the ParsHNL-catalyzed addition of HCN to butanal, which occurs in good yield to give (*R*)-hydroxypentanenitrile in the key asymmetric step of the synthesis (Scheme 28).[141]



Scheme 28. HNL-catalyzed synthesis of cyanhydrins for the synthesis of macrolides.

Venlafaxine hydrochloride in an anti-depressant currently commercialized as *EffexorXR*® and used for the treatment of depression and anxiety disorders. Its enantiomers having different profiles, the (*R*)-(+)-enantiomer being a serotonin re-uptake inhibitor, while the (*S*)-(–)-enantiomer is a norepinephrine re-uptake inhibitor, a fact that highlights the importance of their asymmetric synthesis. Nanda and co-workers have reported the synthesis of (*R*)-(+)-Venlafaxine starting from the non asymmetric transcyanation of cyclohexanone in a 10 gram scale process catalyzed by the enzymatic extract of rubber tree HNL (*Hevea brasiliensins*, HbHNL).[142] In this case a mild reagent such as acetone cyanohydrin was used. In addition, the synthesis of two other Venlafaxine analogues was achieved with the HbHNL-catalyzed formal addition of HCN to 2-methylcyclohexanone and cyclopentanone (Scheme 29).



Scheme 29. Chemoenzymatic synthesis of Venlafaxine analogues using *Hevea brasiliensis* hydroxinitrile lyase.

4. Use of transferases for the synthesis of chiral drugs.

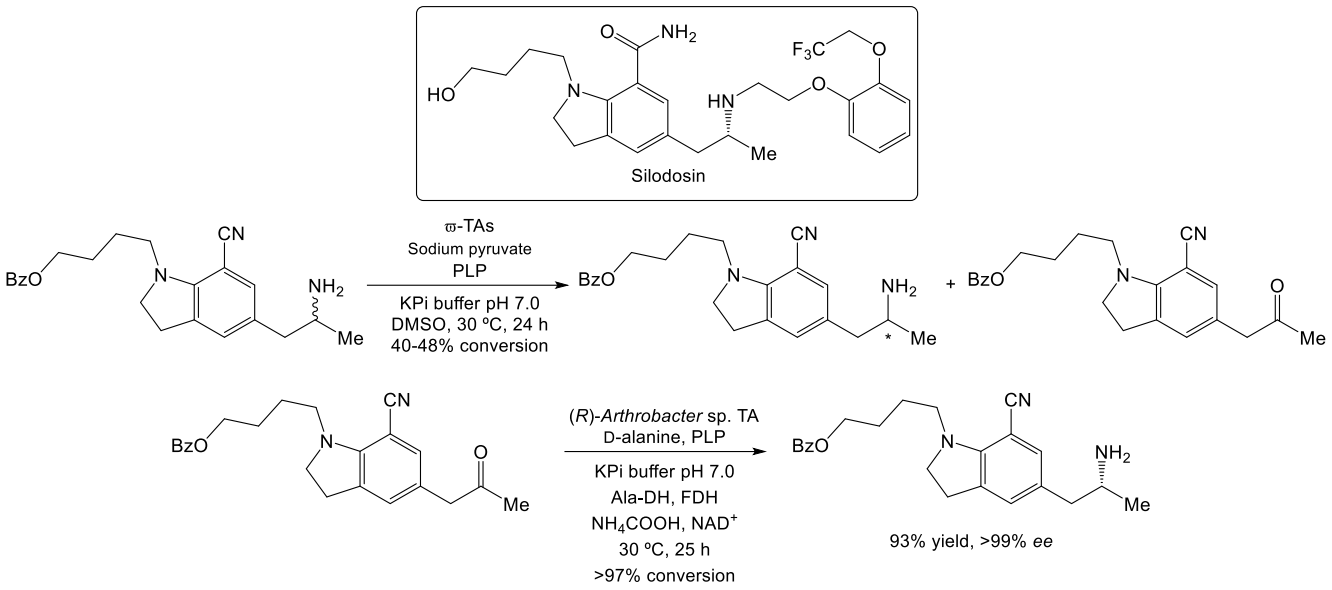
Of the different types of transferases, transaminases have attracted special attention in recent years; they allow the production of chiral amines, which are compounds with multiple biological profiles that serve as building blocks for numerous families of compounds.[143] Because of this, the development of enzymatic methods for this class of nitrogenous compounds is now highly appealing.[144-147] The application of ω-transaminases is having an extraordinary influence on the development of academic and industrial processes, since it allows the oxidative resolution and deracemization of racemic amines.[148] More excitingly, it also allows the transamination of prochiral ketones to afford optically active amines in a single step.[149,150,151] These transformations require the addition of pyridoxal-5'-phosphate (PLP) as cofactor for the effective action of the enzyme. In addition, the action of other enzymes are required to assure proper cofactor recycling and to avoid enzyme inhibition, the use of alanine dehydrogenase (Ala-DH), glucose dehydrogenase (GDH), lactate dehydrogenase (LDH), or others accompanying the main transamination reaction.[149]

The development of evolved transaminases has allowed the design of efficient transformations, greatly improving their substrate specificity, as well as the ability to accept highly hindered substrates such as esteroids[152] or complex organic molecules.[153,154] In this context, it is worth mentioning the work of

Savile *et al.* in the synthesis of the anti-diabetic (*R*)-Sitagliptin. Savile's research has been awarded a number of prestigious prizes after evolving an enzyme from non initial activity.[155]

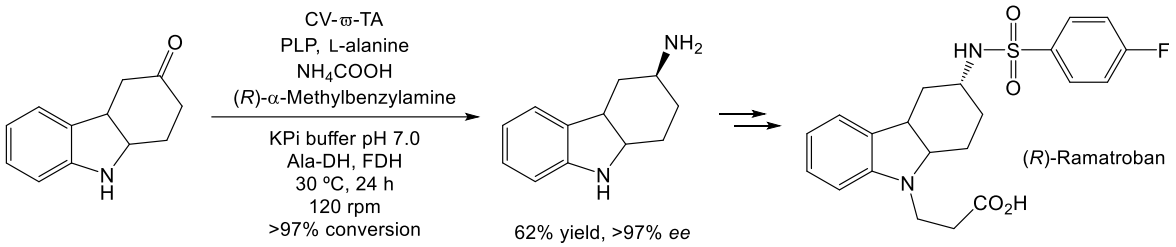
Remarkably, transaminases are also involved in cascade reactions via a perfect combination with other enzyme sources such as alcohol dehydrogenases,[156] synthases[157] and pyruvate decarboxylases[158] which have served for the production of interesting Vanillin and Ephedrine derivatives. Here, we have focused on the application of native enzymes, and so evolved transaminases and those of unknown origin, mainly those commercialized by biotechnological companies and named with their own acronyms, have not been included. Examples of the production of pharmacologically relevant compounds by using asymmetric transaminase-catalyzed reactions are described here.

Silodosin is a single enantiomer prodrug with (*R*)-configuration used in the treatment of urinary diseases. A key Silodosin intermediate has been obtained recently by the oxidative resolution of the racemic amine or transamination of the corresponding ketone, good levels of activity and stereodiscrimination being found with different lyophilized *E.coli* cells containing overexpressed ω-TAs.[159] In both cases 10% of DMSO as cosolvent was required for the solubilization of the starting materials. For the kinetic resolution of the amine, (*R*)-*Arthrobacter* sp. and *Bacillus megaterium* displayed the best results, acting with opposite selectivities (Scheme 30). Since the (*R*)-amine enantiomer was obtained in good yield, the ω-TA from (*R*)-*Arthrobacter* sp. provided the best access to the desired intermediate in quantitative conversion and perfect optical purity by a transamination approach (Scheme 30).



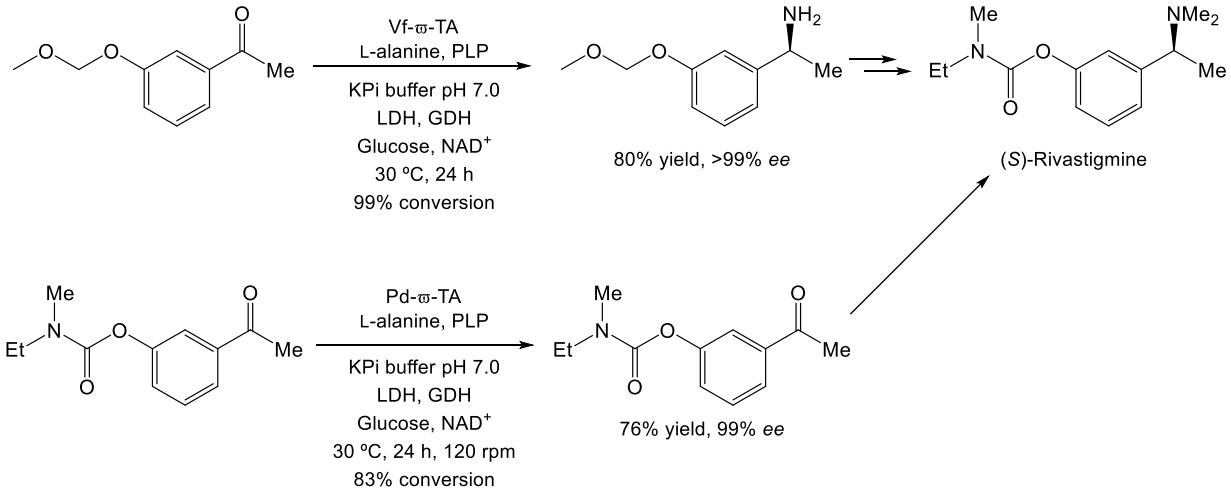
Scheme 30. Chemoenzymatic synthesis of Silodosin by oxidative resolution or transamination using different transaminases.

The asymmetric synthesis of Ramatroban, an anti-allergic drug also used for the treatment of coronary diseases, has been achieved by transamination of a ketone precursor.[160] The 2,3,4,9-tetrahydro-1*H*-carbazol-3-amine isomers were successfully obtained using the transaminase from *Chromobacterium violaceum* (Cv-ω-TA) in lyophilized form for the (*S*)-enantiomer (Scheme 31), while for the (*R*)-enantiomer a semi-purified mutant of (*R*)-*Arthrobacter* led to the best results. This synthetic route seems to be more efficient in terms of E-factor, isolated yield, reaction time and the number of steps than the previous chemoenzymatic approach described using lipases. Inside the indazoyl class, a potent calcitonin gene-related peptide (CGRP) receptor antagonist has been synthesized finding *Bacillus thuringiensis* as an effective enzyme for the production of a key intermediate.[161] Better results in terms of productivity and reaction time were achieved when the commercial available enzyme was purified, cloned and expressed in *E. coli*.



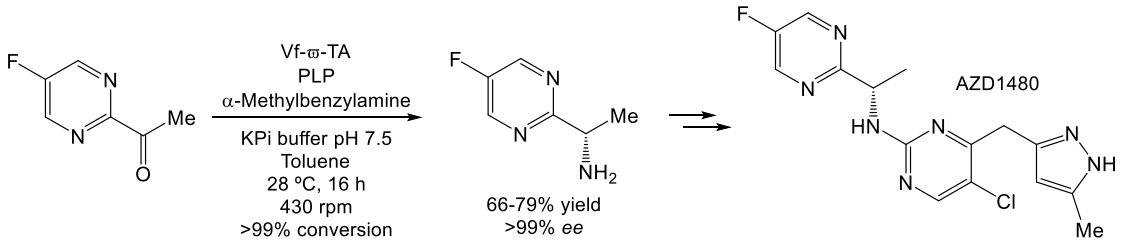
Scheme 31. Chemoenzymatic synthesis of (*R*)-Ramatroban using transaminases.

An example of chemoenzymatic synthesis of anti-cholinergic agent (*S*)-Rivastigmine has been already described in this review using a bioreduction approach.[57] The synthesis of this active pharmaceutical ingredient (API) has been elegantly achieved by Kroutil and co-workers, ω-transaminases from *Vibrio fluvialis* (Vf-ω-TA)[162] and *Parococcus denitrificants* (Pd-ω-TA)[163] proving to be very efficient enzymes in the transamination of the corresponding ketone precursor using L-alanine as amine donor. The transamination of an *O*-protected ketone occurred in complete conversion and excellent selectivity using Vf-ω-TA, while Pd-ω-TA allowed the transamination of the corresponding ketone including the required carbamate functionality in *metha*-position (Scheme 32).



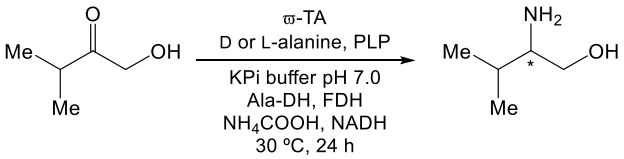
Scheme 32. Chemoenzymatic synthesis of (*S*)-Rivastigmine using transaminases.

After an exhaustive screening of 60 enzymes, the (*S*)-1-(5-fluoropyrimidin-2-yl)-ethylamine, an important intermediate in the synthesis of JAK2 kinase inhibitor AZD1480, has been obtained using Vf-ω-TA as biocatalyst and α-methylbenzylamine as amine donor in the transamination of the corresponding ketone (Scheme 33).[164,165] Optimization of the reaction conditions led to the successful scale-up of the multi-gram Vf-ω-TA in whole cell form or using the commercially available alternative.



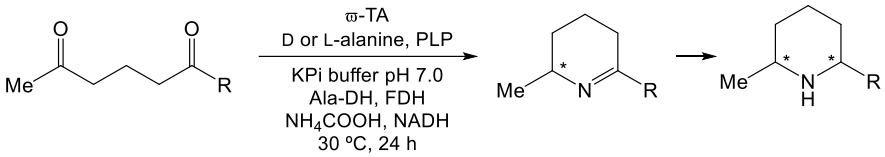
Scheme 33. Multi-gram Vf-ω-TA-catalyzed transamination reaction for the synthesis of an AZD1480 precursor.

Valinol is a highly versatile 1,2-amino alcohol with applications as a chiral shift agent, a building block for pharmaceuticals and in organocatalytic reactions. Its synthesis has been achieved by transamination of 1-hydroxy-3-methylbutan-2-one in water-saturated organic solvents, or in pure aqueous medium.[166] The best results were found with lyophilized *E.coli* cells containing overexpressed transaminases from (*R*)-*Arthrobacter* sp., *Aspergillus terreus*, *Gibberella zeae* and *Neosartoria fischeri* for the production in high to complete conversions of the (*S*)-enantiomer, while *Bacillus megaterium* seemed to be the most efficient enzyme for the production of the antipode in 98% yield (Scheme 34).



Scheme 34. Enzyme-catalyzed transamination of 1-hydroxy-3-methylbutan-2-one for the production of Valinol enantiomers.

Finally, in this section it is worth mentioning the ability of transaminases for the regio- and stereoselective monoamination of diketones. This has allowed the production of all of the possible diastereoisomers of 2,6-substituted piperidines, versatile building blocks in the synthesis of alkaloids.[167-169] The adequate selection of an active and selective transaminase allowed the monoamination of one of the keto groups, which immediately cyclized in the reaction medium to afford the corresponding imines (Scheme 35). Their reduction using palladium-catalytic hydrogenation led to interesting structures such as the natural alkaloid (+)-dihydropynidine, its enantiomer and both diastereoisomers (R= propyl), or isosolenopsin stereoisomers (R= *n*-C_9H_{19}).



Scheme 35. Stereoselective transaminase-catalyzed monoamination of 1,5-diketones for the production 2,6-substituted piperidine diastereoisomers.

5. Conclusions.

Nowadays, the pharmaceutical industry faces significant challenges and must take advantage of the enzyme toolbox for the development of efficient transformations.[170-173] The application of enzymes has taken on an extraordinary importance over the last decades in the development of efficient and sustainable transformations with a high level of selectivity,[174-177] a one-pot combination with chemical catalysis also being possible for the production of a desired target in high purity, yield and stereoselectivity.[178] Native hydrolases, oxidoreductases, transferases and lyases have demonstrated their versatility in synthesis over the years, although there is still a big gap for ligases and isomerases.[179] However, novel enzymes such as imine reductases[180,181] are emerging at an exponential rate, and are attracting considerable attention nowadays,[182-184] thanks in particular to the

rational design of proteins by directed evolution techniques.[185-188] Because of all this, it is clear that biocatalysis has still a long way to go and will continue helping organic chemists improve existing synthetic strategies and develop valuable, novel products both for drug design and, importantly, for other sectors of industry.

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