Recent advances in biocatalytic promiscuity. Hydrolase-catalyzed reactions for nonconventional transformations.

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Abstract: Enzymes has emerged in recent decades as ideal catalysts for synthetic transformations in mild reaction conditions. Their capacity to accelerate a myriad of biotransformations with high levels of selectivity and broad substrate specificity including excellent atom economy has led to a current full recognition. The six classes of enzymes (oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases) possess outstanding abilities to perform specific modifications in target molecules. Nevertheless, in the last fifteen years, novel examples have appeared related to nonconventional processes catalyzed by various classes of biocatalysts. Amongst these, hydrolases have received special attention since they display remarkable activities in initially unexpected reactions such as carbon-carbon and carbon-heteroatom bond formation reactions, oxidative processes and novel hydrolytic transformations. In this review, the main findings in this area will be disclosed, highlighting the catalytic properties of hydrolases not only to catalyze single processes but also multicomponent and tandem nonconventional reactions.

1. Introduction

Hydrolases are a class of enzymes that catalyze a significant number of hydrolytic or the reversible bond formation reactions depending on the specificity of each enzyme subclass.[1] The lack of cofactor requirements and the ability to work not only in aqueous systems but also in organic and neoteric solvents has made possible their incorporation in synthetic routes, attracting the attention of a wide number of organic and inorganic chemists due to their easy handling. All this has made possible the development of efficient methods for the production of interesting organic molecules such as alcohols, amines, esters, amides, epoxides and nitriles, among others. The application of hydrolases has been adapted to an industrial perspective, receiving special attention in the synthesis of agrochemicals, pharmaceuticals and other high added value compounds.[2] Beyond the hydrolase-catalyzed traditional transformations (hydrolysis, esterification, amidation, acylation…), the discovery of novel and nonconventional transformations involving the use of enzymes has opened new challenges, which have been grouped inside the term enzymatic promiscuity that covers the ability of a single enzymatic active site to catalyze different chemical transformations, and is linked to broader definitions such as catalytic promiscuity.[3] At this point is important to distinguish between the activity of a protein active site working in an orchestral manner to catalyze a desired transformation, from the action of a single or various amino acid residues outside of the active center able in some cases to accelerate a reaction, fact that is commonly known as unspecific catalysis.

Importantly, wild-type enzymes have exhibited significant levels of activity in selected transformations. Since the full development of directed evolution and protein engineering techniques has been achieved in recent years, the use of evolved enzymes has allowed the improvement of enzyme performances in challenging transformations.[4] Our aim in this review is to summarize remarkable contributions in the last five years regarding the use of hydrolases for nonconventional processes in order to update current existing revisions in the field.[5] Trying to provide a better understanding to the reader, we have classified the selected transformations depending on bond cleavage or formation reactions, covering the application in carbon-carbon bond formation reactions, carbon-heteroatom, unexpected hydrolytic reactions, oxidative processes and multicomponent reactions.

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Vicente Gotor-Fernández (1974) studied organic chemistry at the University of Oviedo, where he received his PhD in 2001 working in the development of chemoenzymatic routes for the production of vitamin D_3 derivatives. In June 2002 he moved to Edinburgh for a post-doctoral stage in the group of Professor Nicholas J. Turner, studying deracemization processes by combination of selective amine oxidase obtained by directed evolution methods and chemical reducing agents. Two years later he moved back to Oviedo where he was appointed first as a senior scientist, obtaining a permanent position as Associate Professor in June 2012. His main research interest involves the development of novel synthetic methodologies using chemical and enzymatic methods.

2. Carbon-carbon bond formation reactions

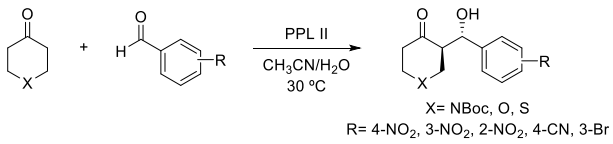
Carbon-carbon formation reactions are one of the fundamental transformations in organic chemistry. The use of biocatalysts for these transformations offers clear advantages in comparison with nonenzymatic processes providing access to complex molecules with a high degree of chemo-, regio- but distinctively stereoselectivity through the formation of multiple stereocenters with the desired stereochemistry under physiological conditions (aqueous systems, neutral pH and room temperature).[6] Enzymatic carbon-carbon bond formation reactions involve the use of various subdivisions such as aldolases, hydroxynitrile lyases and thiamin diphosphate-dependent enzymes, all of them inside the lyases class (EC.4).[7] Recently, new enzymes have also expanded the synthetic possibilities of enzymatic methods by using cyclases, Dields-Alderases or Pictet-Spenglerases enzymes,[8] also the benefits of directed evolution playing an important role for the discovery of more efficient enzymes.[9] Next, the main recent achievements in hydrolase-catalyzed nonconventional aldol, Henry, Knoevenagel and Michael reactions will be disclosed giving also an insight in the possibility to develop multistep transformations with synthetic purposes.

2.1. Aldol reaction

Since aldol reaction was discovered in 1872, this synthetic approach has emerged as one of the most powerful methods for C-C bond formation. Multiple conventional chemical processes have been reported for asymmetric aldol reaction to form new active β-hydroxycarbonyl compounds. Organocatalytic reactions have been conveniently employed for the formation of complex molecules with high yields and selectivities, while the development of alternative sustainable methods is an important feature to be taken into consideration. In this respect, some aldolases have been elegantly applied as environmentally benign choices to catalyze enantioselective aldol reactions. However, the limited number of substrates as well as the high cost of these biocatalysts has led to consider other more stable enzymes.

In contrast to moderate activities and selectivities found in earlier studies involving hydrolases, several efficient promiscuous asymmetric aldol reactions have been reported in recent years. In these studies, the couplings between 4-nitrobenzaldehyde or 4-cyanobenzaldehyde with acetone or cyclohexanone have been normally selected as model reactions in order to find suitable optimized conditions, extending later the studies to a broad family of substrates. Importantly, the action of the catalytic active site has been studied by means of control experiments including the inhibition or denaturation of the enzymes, and thus examples derived from unspecific enzymatic catalysis or the presence of impurities in the enzymatic preparations have been avoided.[10]

Among hydrolases, lipases are the most commonly used biocatalysts in organic synthesis because of their capability to act both in organic and aqueous medium. *Candida antarctica* lipase type B (CAL-B) has been the most recurrent hydrolase used for the development of carbon-carbon formation reaction. Nevertheless, other lipases have also acted with good levels of activity. Recently, Guan and He described the first direct asymmetric aldol reaction of heterocyclic ketones with aldehydes catalyzed by lipase from porcine pancreas type II (PPL II).[11] *N*-Boc protected piperidin-4-one and 4-nitrobenzaldehyde were chosen as model substrates for a preliminary enzymatic screening, and the influence of various reaction parameters (reaction medium, water content, molar ratio of ketone to aldehyde and enzyme concentration) was later studied. Under optimized reaction conditions various aromatic aldehydes and heterocyclic ketones were reacted in equimolecular amounts as depicted in Scheme 1, achieving after prolonged reaction times moderate enantioselectivities (up to 87% *ee*) into the corresponding *anti*-diastereoisomers (diastereoselectivities up to 83:17), which were isolated after silica gel chromatography (31-56% isolated yield).

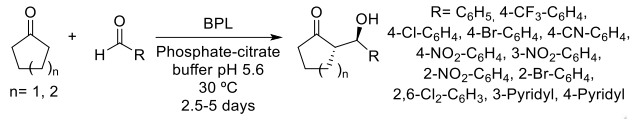


Scheme 1. PPL-catalyzed asymmetric aldol reaction between substituted aromatic aldehydes and heterocyclic ketones.

More recently, the same authors expanded the application of PPL II to direct asymmetric aldol reactions of cyclic ketones with aromatic and heteroaromatic aldehydes,[12] achieving in some cases better results in terms of activity and diastero- and enantioselectivity (10-98% yields; 48/52 to 87/13 *dr*, *anti*/*syn*; 53-94% *ee*). Among other features, the benefits of the steric hindrance in the *ee* of the products and the positive effect in the yields using aldehydes with electron-withdrawing substituents were investigated, finding the best results in a large excess of the starting ketones (20 equiv).

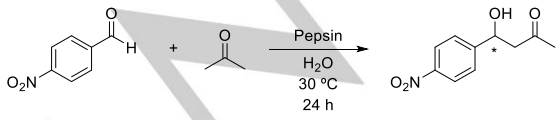
The PPL was also employed by Yu and co-workers in the cross-aldol reaction between aromatic aldehydes and cyclic aliphatic

ketones, the later themselves acting as the best solvents.[13] The large influence of water content in the catalytic activity and selectivity was demonstrated, as it played a major role as a "molecular lubricant". The best catalytic properties were found at 5% water (v/v). In addition, the authors observed a high degree of homology between PPL and lipase from bovine pancreas (BPL), their similar spatial conformation made them displaying comparable activity values. Thus, the same authors reported the first BPL-catalyzed aldol reaction between cyclic aliphatic ketones (20 equiv) and (hetero)aromatic aldehydes in a phosphate-citrate buffer pH 5.6 at 30 ºC, in which the formation of the *anti*-diastereoisomers was slightly favored although with low to moderate enantiomeric excess (Scheme 2).[14] More recently, Gupta and co-workers have reported the lipase-catalyzed reaction between 4-nitrobenzaldehyde and 2-cyclohexen-1-one in mixtures of DMSO and water to give mixtures of the aldol adduct and the Morita-Baylis-Hillman product.[15] Best results in terms of global conversion were found for *Pseudomonas cepacia* lipase while the highest stereoselectivities were attained with *Mucor javanicus* lipase.



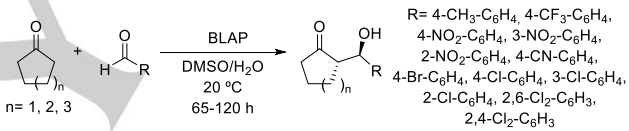
Scheme 2. BPL-catalyzed asymmetric aldol reaction between cyclic aliphatic ketones and (hetero)aromatic aldehydes in an acidic buffer.

In addition to lipases, other hydrolases has also acted with good levels of activity in aldol reactions as promiscuous biocatalysts, proteases being found as suitable catalyst for this type of transformation. In 2010, Yu and co-workers described the superior enantioselectivity of pepsin, a gastric aspartic proteinase, compared to PPL in the crossed aldol reaction between acetone and 4-nitrobenzaldehyde under "wet" reaction conditions (Scheme 3).[16] Although native environment of pepsin is comparable to acidic buffer of pH 2.2, the authors detected that pepsin is properly folded and stable under the optimal neutral conditions of water/acetone reaction medium (10% v/v). Unfortunately, a significant loss of activity was observed after the first recycling use of the enzyme (23-54% for the first three cycles), while selectivity was satisfactorily maintained. Low to moderate yields (5-69%) were achieved for selected substrates, the higher reactivity being found for aldehydes with electron-withdrawing functional groups (NO_2) and ketones with minimal steric bulk (acetone).



Scheme 3. Pepsin-catalyzed aldol reaction between 4-nitrobenzaldehyde and acetone in aqueous medium.

Guan and co-workers also reported several examples of protease-catalyzed aldol additions. For instance, *Bacillus licheniformis* (BLAP) resulted to be an efficient catalyst of the reaction between 4-cyanobenzaldehyde and cyclohexanone.[17] The highest yield (59%) in compromise with the best enantioselectivity (51% *ee*) was found at 20 ˚C using hydrated DMSO (0.15% water) as solvent. The enzyme showed a wide range of substrate specificity towards aromatic aldehydes and cyclic ketones, yielding in most cases the favored *anti*-diastereoisomers in 28-92% isolated yield and moderate to high selectivities (Scheme 4). Similarly, the same authors reported the use of chymopapain, a cystein proteinase isolated from the latex of the unripe fruits of *Carica papaya*, in the same model reaction between 4-cyanobenzaldehyde and cyclohexanone.[18] Acceptable stereoselectivity was observed after a detailed study of different reaction parameters such as solvent, temperature, pH and reaction time, although a clear deactivation and destabilization of the enzyme was suggested as a reason for limitations in the final product yield. In a 0.12% water content solution in acetonitrile (MeCN) at pH 4.91 a maximum yield of 45% was obtained in the aldol product with 72:28 *dr* and 78% *ee*, to later extend the same conditions to related aromatic aldehydes and aliphatic cyclic ketones.



Scheme 4. Alkaline protease from *Bacillus licheniformis*-catalyzed aldol reaction between aromatic aldehydes and aliphatic cyclic ketones in wet DMSO.

The authors proposed the reaction mechanism involving the coordinating action of the chymopapain catalytic triad formed by single cysteine (Cys), histidine (His) and asparagine (Asn) residues. Firstly, the carbonyl of the ketone is coordinated in the Asn-His dyad and the oxyanion hole in the enzyme active site. Then, a proton is transferred from the ketone to the His and an enolate is formed. Finally, an aldehyde molecule accepts the proton from the imidazolium cation interacting with the ketone to form a new carbon-carbon bond. Once the aldol product is formed, it is released from the oxyanion hole and separates from the chymopapain active site.

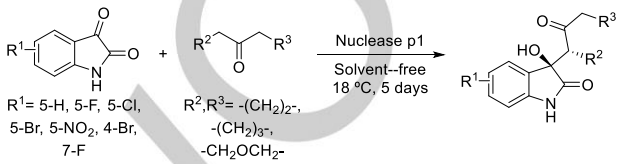
The acidic protease from *Aspergillus usamii* No. 537 (AUAP) has been also found to be responsible of the acceleration of asymmetric aldol reactions.[19] The reaction between cyclohexanone and 4-nitrobenzaldehyde in a mixture of (90:10) MeCN/H_2O at 25 ˚C led to the aldol product in good yield (63%) and good stereodiscrimination towards the *anti*-diastereoisomer (82% *ee* and 83/17 *dr*). Enantioselectivities up to 88% *ee* and diastereoselectivities up to 92/8 (*anti*/*syn*) were achieved after extending the optimized conditions towards a wide range of substrates. Similarly, the applicability of trypsin from porcine pancreas in asymmetric aldol reactions was successfully reported by the same research group.[20] The reaction took place

under solvent free conditions using 10 equivalents of ketone and a minimum amount of water to prevent the enzyme deactivation. Consonantly with the one reported for the chymopapain-catalyzed aldol reaction,[18] a mechanism was proposed identifying the orchestral action of the catalytic triad composed in this case by three residues: Histidine (His-57), Aspartic acid (Asp-102) and Serine (Ser-195). The commercially available proteinase from *Aspergillus melleus*, type XXIII (AMP) has also efficiently catalyzed aldol reactions leading to similar activity values than the previously reported proteases.[21] A comparison between AMP, AUAP, BLAP and chymopapain is reported, finding generally the best values for AMP.

Some trends can be concluded from these studies. Aromatic aldehydes with electron-withdrawing substituents react faster, and those with hinder substituents lead to the highest enantio- and diastereoselectivities. On the other hand, cyclochexanone seems to be the best aldol donor in comparison to acetone, cyclopentanone and cycloheptanone. The *anti*-diastereoisomers are the major products in almost all cases, while when the *syn*-isomers are preferred they are formed with usually low selectivities.

Ficin from fig tree latex is a plant cysteine proteinase, which has been found to display catalytic promiscuity in the direct asymmetric aldol reaction of heterocyclic ketones with aldehydes in organic medium containing a minimum amount of water (0.15%).[22] After optimization of the reaction parameters such as temperature (30 ºC), molar ratio of substrates (2:1 aldehyde to ketone) and enzyme concentration (75 mg/mL), yields between 21-44% and up to 81% *ee* were achieved for the reaction of a wide range of nitrogen, oxygen and sulfur containing heterocyclic ketones and substituted benzaldehydes. In contrast to *Carica papaya* experiments,[18] none improvement was observed in either yields or selectivities when water was replaced by a buffer solution.

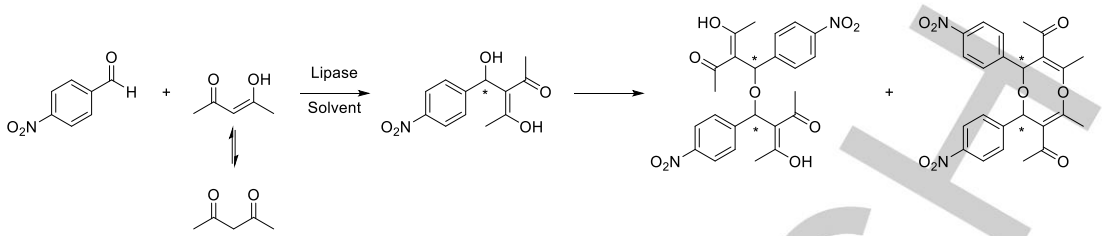
Leaving aside the proteases, a novel thermophilic esterase (APE1547) from the archaeon *Aeroynum pernix* K1 has been reported to catalyze the asymmetric aldol addition of 2-butanone and 4-nitrobenzaldehyde in organic solvents.[23] The effects of organic solvent, temperature, water content and substrate concentration were investigated, leading to the corresponding (*S*)-product in approximately 69% yield and 71% *ee* after 120 h at 65 ºC. On the other hand, nuclease p1 from *Penicillium citrinum*, a zinc-dependent endonuclease, has been reported in different asymmetric aldol reactions.[24] The aldol addition of 4-cyanobenzaldehyde and cyclohexanone was optimized by Guan and co-workers, extending later this methodology to a vast number of substituted benzaldehydes and five-, six- and seven-membered cyclic ketones, which served as aldol donor as well as solvents.[24a] In general moderate yields but good to excellent selectivities were found, the best enantioselectivities being found at 15 ºC, while higher temperatures were required (45 ºC) to exhibit the best nuclease catalytic activity. At the same time, Lin and co-workers observed that this nuclease was able to catalyze the aldol reaction between isatin derivatives and cyclic ketones to synthesize 3-alkyl-3-hydroxyindolin-2-ones in the presence of a minimum amount of deionized water (Scheme 5).[24b] High isolated yields (70-95%) and moderate to good stereoselectivities (57:43 to >99:1 *dr* and 27-82% *ee*) were found after 5 days at 18 ºC. In this intermolecular ketone-ketone asymmetric aldol addition, the authors detected a little influence related to the size and electronic nature of the substituents on the isatin phenyl ring, although large effects in activity and selectivity were found depending on the cyclic ketone size.



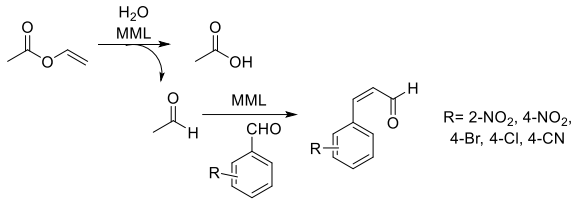
Scheme 5. Nuclease p1 from *Penicillium citrinum*-catalyzed asymmetric aldol reaction between isatin derivatives and cyclic ketones.

2.2. Tandem aldol reactions

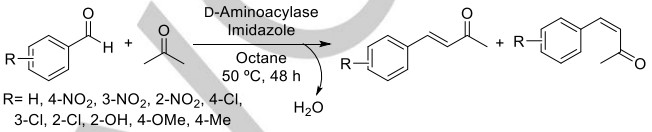
Examples of nondirect hydrolase-catalyzed aldol reactions have been also found in the literature. A series of authors has reported the advantages to synthesize acetaldehyde but also to develop multiple transformations in one-pot. For instance, Yu and co-workers reported the combined conventional and promiscuous catalytic activity of *Mucor miehei* (MML) to prepare α,β-unsaturated aldehydes in a one-pot strategy (Scheme 6).[25] The lipase promoted the in situ generation of acetaldehyde through vinyl acetate hydrolysis in a first step, and the subsequent aldol reaction with a series of (hetero)aromatic aldehydes. Thus, problems associated to the volatilization loss, oxidation and polymerization of acetaldehyde are suppresses, thereby avoiding the negative effect on the enzyme of high amounts of acetaldehyde in the reaction medium. MML showed the best results from a panel of lipases such as CAL-B, PPL, *Candida rugosa* lipase (CRL) and lipase from *Mucor javanicus* (MJL), and proteases (pepsin and trypsin), observing no appreciable reactivity in control experiments with a carrier protein such as bovine serum albumin (BSA) and denatured MML. Optimized reactions in terms of solvent, enzyme loading, temperature and reaction time led to moderate to good isolated yields (38-78%) after column chromatography purification on silica gel, using vinyl acetate as reactant and solvent in the presence of 50% water (v/v) at 60 ºC for 5 days. Generally benzaldehydes with electron withdrawing substituents were better acceptors and gave better yields. This article clearly differs from others reporting the combination of a lipase such as CAL-B and an organocatalyst or a benzaldehyde lyase for cross-aldol reactions, the carbon-carbon bond formation being exclusively catalyzed for the nonenzymatic catalyst.[26]



Scheme 7. Lipase-catalyzed formation of acyclic and cyclic 2:2 adducts through carbon-carbon bond formation reactions.



Scheme 6. MML-catalyzed aldol condensation using an in situ generated acetaldehyde in a route to α,β-unsaturated aldehydes.

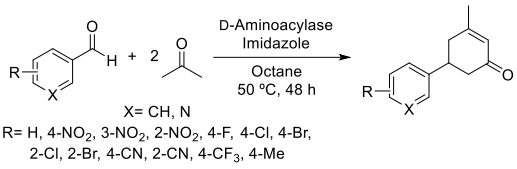


Scheme 8. Tandem aldol condensation/dehydration between aromatic aldehydes and acetone using D-aminoacylase in octane.

Majumder and Gupta explored the aldol condensation reaction between 4-nitrobenzaldehyde and acetyl acetone in phosphate buffer (50 mM, pH 7.0), using several commercially available lipases as biocatalysts (CAL-B, MJL, MML and *Pseudomonas cepacia* lipase). In addition to the aldol product, two unexpected ones were obtained (Scheme 7).[27] After lipase-catalyzed condensation, the dimerization of the aldol led to the 2:2 adduct product, and also the formation of an unsaturated cyclic ether was observed as third reaction product through subsequent removal of the two enolic protons of the 2:2 aldol adduct during the ring closure. A clear regioselectivity was attained, since the dimerization took place only via C-3 atom of the acetylacetone. The choice of type and source of lipase and solvent dramatically affected the ratio of final products, stopping in the 2:2 adduct as the major product for those reactions carried out in aqueous medium. These findings revealed that combination of the natural function of biocatalysts with the promiscuous one allowed the use of one single-enzyme to catalyze tandem reactions.

Other challenging tandem reactions have been reported involving a hydrolase-catalyzed aldol reaction. For instance, Lin and co-workers reported the preparation of unsaturated carbonyl compounds starting from aldehydes and ketones in a tandem aldol condensation/dehydration process catalyzed by D-aminoacylase and imidazole.[28] Control experiments made clear that the enzyme active site and also imidazole were essential to promote the tandem process, but inefficient to individually catalyze the reaction. Furthermore, evaluation of other *N*-heterocyclic compounds showed that imidazole was the best option as it exhibited the greatest enhancement of the D-aminoacylase activity. Thus, the reaction between a series of benzaldehydes and acetone in octane favored the formation of the *E*-isomers in generally good yields after 48 h and 50 ºC (Scheme 8).

The same catalytic system has been efficiently described for the one-pot tandem aldol condensation/Robinson annulation between aldehydes and acetone for the synthesis of substituted cyclohex-2-enones in moderate to good yields (21-74%), finding octane as the best solvent (Scheme 9).[29] The tentative mechanism for this zinc-dependent acylase involves the coordination action of the zinc ion and the Asp366 residue for the formation of a Knoevenagel condensation intermediate, which later evolved to the final product by the elimination of two molecules of water.

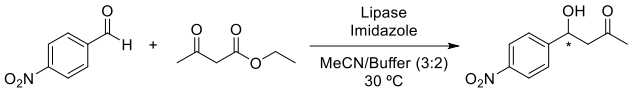


Scheme 9. D-Aminoacylase catalyzed Aldol condensation/Robinson annulation cascade reaction between (hetero)aromatic aldehydes and acetone.

The decarboxylative aldol reaction is an important carbon-carbon bond forming reaction, which can be catalyzed by hydrolases. The effects of the choice of lipase, enzyme immobilization, reaction medium, presence of additives and temperature have been examined by Gupta and co-workers for the reaction between 4-nitrobenzaldehyde and ethyl acetoacetate (Scheme 10).[30] Best results were obtained using a mixture of MeCN/buffer (3:2 v/v) for both MJL and CAL-B as biocatalysts, finding that the free and immobilized CAL-B displayed opposite and low stereoselectivities (up to 14% *ee*). Interestingly the use of 30% imidazole as additive allowed a notably increase in the reaction rate, although a significant loss

of the optical purity of the product was also observed. The main limitation of the method resides in the negligible conversions attained with less active acceptor aldehyde molecules such as benzaldehydes with different substiutions (H, Cl, methyl or methoxy).

The decarboxylative aldol reaction was also carried out in nearly anhydrous MeCN, in order to far investigate the controversial role of CAL-B in this catalytic reaction.[31] Interestingly, several CAL-B formulations showed significant levels of aldol reaction, which did not occur with other lipases. Along with the minimum amount of water in the reaction medium, this indicates that CAL-B catalyzes the final product formation.



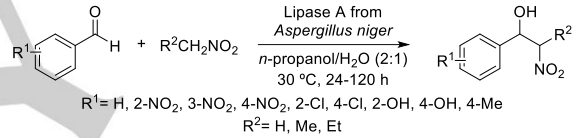
Scheme 10. Decarboxylative aldol reaction between 4-nitrobenzaldehyde and ethyl acetoacetate catalyzed by CAL-B or MJL in the presence of imidazole.

Taking advantage of the previous results,[30] the two-step sequential process for the synthesis of optically active hydroxyesters has been possible through a lipase-catalyzed decarboxylative aldol followed by a kinetic resolution (KR) using an acyl donor (Scheme 11).[32] Thus, an adequate combination of conventional and promiscuous catalysis provides a useful access to enantiopure compounds. CAL-B catalyzed the reaction between a series of nitro-substituted benzaldehydes and ethyl acetoacetate, obtaining β-hydroxyesters, which were subsequently resolved using CRL as biocatalyst in the presence of vinyl acetate at 37 ºC. Unfortunately, the two-step combination was no possible since CAL-B inhibited the KR process, so resolution was accomplished after purification of the corresponding decarboxylative aldol adduct by column chromatography. This approach allowed the isolation of the final esters with good to excellent enantioselectivities (84-98% *ee*), although yields were poor for some substrates due to the steric hindrance.

2.3. Henry reaction

The nitroaldol or Henry reaction is one of the most classical and useful carbon-carbon bond forming reactions in organic chemistry.[33] This atom-economical strategy provides access to valuable racemic and optically active β-nitro alcohols, which are key building blocks in the synthesis of bioactive pharmaceutical ingredients. Efficient nonconventional biocatalytic approaches have been reported using mainly hydroxynitrile lyases and hydrolases for the reaction between aldehydes and 1-nitroalkanes.[34]

For instance, the Henry reaction between aromatic aldehydes and a large excess of nitroalkanes (26 equiv) can be carried out using Lipase A from *Aspergillus niger* in an organic/water medium (Scheme 12).[35] The corresponding β-nitro alcohols were obtained in yields up to 94% at 30 ºC, finding low conversions for the denatured enzyme and the BSA-catalyzed reactions. Gotor and co-workers reported the activity of porcine pancreas lipase (PPL) in a biphasic system composed by nitromethane and water (4:1 w/w) in the reaction with 4-nitrobenzaldehyde at 30 ºC, obtaining a 65% conversion after 48 h at 250 rpm.[36] Importantly, the denatured PPL was also active (31% conversion), suggesting an unspecific catalytic action of the enzyme. This was corroborated by the fact that the BSA-catalyzed reaction reached a remarkable 91% conversion in the same reaction conditions.



Scheme 12. Nitroaldol reaction between aromatic aldehydes and nitroalkanes catalyzed by Lipase A from *Aspergillus niger*.

Similarly, two other well-known lipases such as *Pseudomonas cepacia* lipase (PSL) and CAL-B were found to catalyse the Henry reaction although the mechanism was not associated with the corresponding active site action.[37] Nevertheless, spectroscopical experiments showed that the immobilization protocols have a significant importance in the enzymatic promiscuous action as they contribute to a variation in the secondary structure of the enzyme, which leads to improved conversion values.

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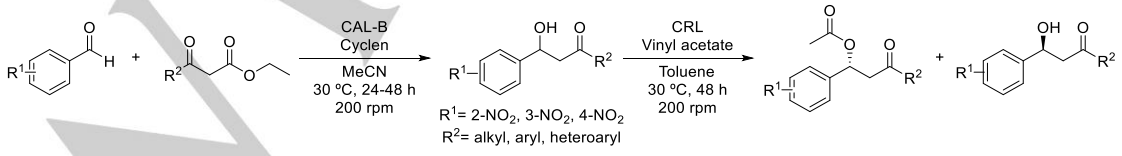
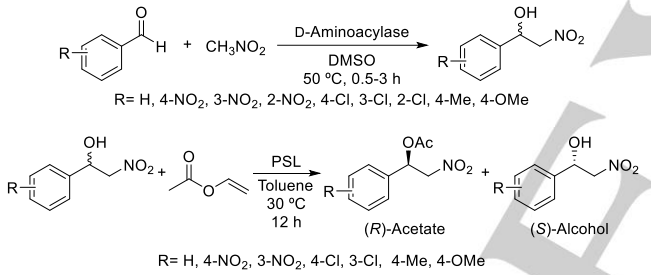


Scheme 11. Two-step sequential biocatalytic process for the synthesis of optically active β-hydroxyesters and derivatives through a decarboxylative aldol addition and a KR process.

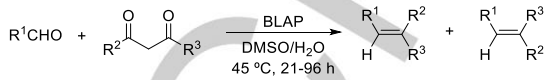
In addition, other hydrolases such as glucoamylase from *Aspergillus niger* (AnGA)[38] and a D-aminoacylase[39] have also been able to catalyze the Henry reaction. A large and scalable AnGA-catalyzed Henry reaction has been described between (hetero)aromatic aldehydes and nitroalkanes (5 equiv) such as nitromethane, nitroethane and nitropropane in a mixture of ethanol and water (85:15 v/v) at an optimum temperature of 30 ºC.[38] The specific catalytic activity of AnGA on the Henry reaction was nicely demonstrated by a rational design of experiments including the presence of possible inhibitors such as metal ions (Ag^+ and Cu^{2+}) or miglitol, but also denaturation reagents such as urea or guanidine. On the other hand, the Henry can also be catalyzed in a unique organic solvent, finding DMSO as the best solvent at 50 ºC when using the D-aminoacylase from *Escherichia coli* as promiscuous biocatalyst.[39] In both cases negligible stereodiscriminations were achieved although the specific catalytic activities of both hydrolases were demonstrated. Interestingly, the synthesis of optically active β-nitro alcohols was achieved by a two-step strategy combining the D-aminoacylase-catalyzed nitroaldol reaction with the PSL-catalyzed resolution of the so-obtained racemic β-nitro alcohols (Scheme 13).[40] Both alcohols and acetates were isolated in good yields and high enantiomeric excess values (>84% ee_S; >96% ee_P; E>150), the process resulting only limited for the *ortho*-substituted aldehydes, which were found inert in the KR step.



Scheme 13. Two-step method to obtain β-nitro alcohols and derived acetates of both configurations based on an D-aminoacylase catalyzed reaction and PSL-mediated kinetic resolution using vinyl acetate as acyl donor.

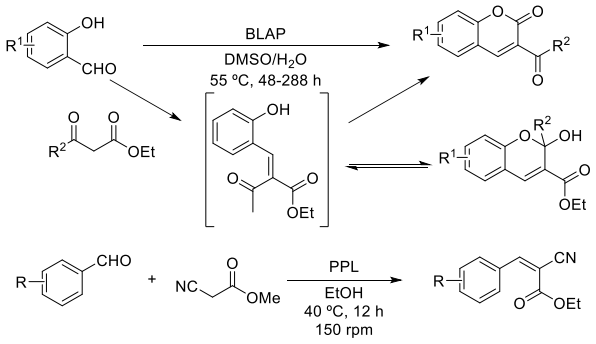
2.4. Knoevenagel reaction

The Knoevenagel reaction consists in the nucleophilic addition of an active hydrogen compound to an aldehyde or ketone, often followed by dehydration forming a carbon-carbon double bond. Discussion about the mechanistic action has been debated for the lipase-catalyzed Knoevenagel reaction based on the role of the enzyme and also specific amino acids.[31b,41] Applications to the synthesis of a series of compounds have been successfully developed in recent years. For instance, the *Aspergillus oryzae* lipase have served to promote the synthesis of novel chromophores by reaction between 4,4'-hexylimino-bisbenzaldehydes with different active methylene compounds.[42] This study describes the advantages in terms of yield, time, temperature and recyclability for independent catalytic systems as a lipase or piperidine in ethanol, or the reaction in deep eutectic solvent (DES) without an external catalyst, finding generally the best conditions in the latest system using a choline chloride:urea mixture (1:2 mol %). In addition, the alkaline protease from *Bacillus licheniformis* (BLAP) catalyzed the Knoevenagel condensation between (hetero)aromatic or α,β-unsaturated with a little excess of acetylacetone or ethyl acetoacetate in organic solvent and the presence of water (Scheme 14), yielding trisubstituted alkenes and α,β,γ,δ-unsaturated carbonyl compounds in 24-82% yield and low to complete *E*/*Z* selectivity using DMSO in the presence of 5% water (v/v).[43]



Scheme 14. BLAP-catalzyed Knoevenagel condensation between aldehydes and 1,3-dicarbonyl compounds

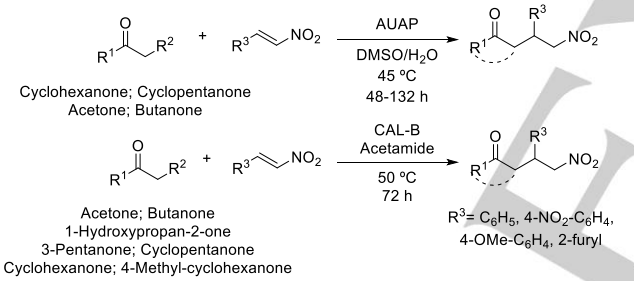
A series of tandem processes has been reported involving an initial Knoevenagel reaction, which have led to the preparation of interesting organic compounds. 2*H*-1-Benzopyran-2-ones have been obtained through a domino Knoevenagel reaction-intramolecular transesterification catalyzed by BLAP in organic solvent.[44] A significant influence in the chemoselectivity of the reaction has been observed in terms of solvent, water content and temperature, parameters that affect the ratio of the desired benzopyran-2-one derivative or the by-product obtained by domino Knoevenagel-intramolecular hemiketalization (Scheme 15). Best results including yields up to 58% were obtained at 55 ºC using a (1:3) molar ratio of salicyladehyde:ethyl acetoacetate in DMSO with 10% water content, finding the preferential formation of the byproduct when the water content reached 30%. Remarkably, the process was extended with yields between 10-75% to a family of aromatic aldehydes and ethyl 1,3-ketoesters, demonstrating the specific activity of the lipase for the Knoevenagel reaction while the intramolecular transesterification was only promoted by the enzyme in a little extent. PPL, among others hydrolytic enzymes such as amylase from hog pancreas, lipase A from *Aspergillus niger* or MJL, has efficiently catalyzed the Knoevenagel reaction between benzaldehyde and methyl cyanoacetate in stoichiometric amounts, occurring the catalyzed transesterification reaction of the resulting intermediate in an alcoholic solvent such as ethanol (Scheme 15).[45]



Scheme 15. Domino Knoevenagel-transesterification reactions catalyzed by different hydrolases.

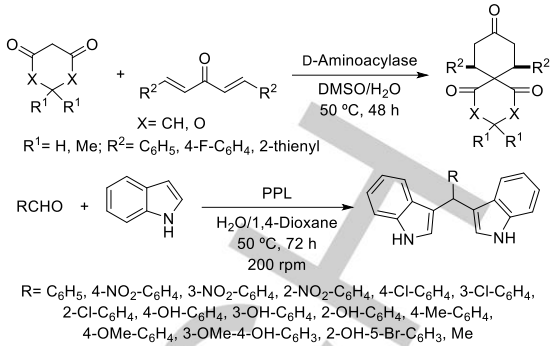
2.5. Michael reaction

The Michael addition consists in the nucleophilic conjugate addition of a carbanion or another nucleophile to an α,β-unsaturated compound. Their possibilities have allowed the synthesis of a wide variety of compounds in an enantioselective fashion.[46] Regarding the use of hydrolases, acidic proteinase from *Aspergillus usamii* (AUAP) has been identified as the best biocatalyst from a panel of 10 enzymes in the reaction between cyclohexanone and *trans*-β-nitrostyrene.[47] The reaction was extended to other aliphatic ketones and nitroolefins in DMSO and in the presence of water (2:0.3 v/v) at 45 ºC using a large excess of the ketone (20 equiv) and generally prolonged reaction times were required. Michael adducts were obtained in 47-84% isolated yields with moderate to excellent diasteroselectivity towards the *syn*-isomers as a result of thermodynamic control although with negligible enantioselectivity (Scheme 16). Recently, Lin and co-workers reported the benefits of using acetamide as co-catalyst in the CAL-B catalyzed Michael addition of less activated ketones and aromatic nitroolefins, which is particularly interesting because neither CAL-B nor acetamide can independently catalyze the reaction in an appreciable extension.[48] A variety of nitroolefins and ketones were tested, leading to the desired Michael adducts in 25-72% yield after 72 h at 50 ºC (Scheme 16). Formamide displayed also a good promotion effect in the two-catalyst system action.



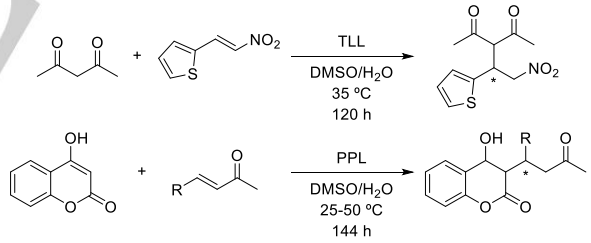
Scheme 16. Biocatalyzed Michael additions of ketones to nitrostyrenes.

In some cases, the formation of di-Michael products is observed in detriment of the single Michael adduct, as described by Lin and co-workers in the D-amino acylase-catalyzed reaction between 1,3-diones and penta-1,4-dien-3-ones (4 equiv) for the synthesis of (hetero)spiro[5.5]undecane derivatives (Scheme 17).[49] The racemic *cis*-products were obtained in moderate yields (30-61%) through the double Michael addition at 50 ºC using a mixture of DMSO and water as solvent (9:1 v/v). Recently, a cascade reaction between aldehydes (1.9 equiv) and indole (1 equiv) in a water/dioxane mixture (4:1 v/v) has been reported, PPL displaying a high activity at 50 ºC for the formation of a series of bis(indolyl)alkanes in moderate to excellent conversions (38-98%) after 48 h (Scheme 17).[50]



Scheme 17. Formation of di-Michael products in hydrolase-catalyzed reactions.

Only a few cases report examples of asymmetric Michael additions, which generally occurred with poor selectivities. For instance, the lipase from *Thermomyces lanuginosus* (TLL) has catalyzed the addition between a wide range of donor and acceptors including ketones, nitrostyrenes and 1,3-dicarbonyl compounds, affording the final products with yields up to 90% while the selectivities were generally low.[51] Just in the reaction between acetylacetone and thienyl nitroolefin (3 equiv) an 83% *ee* was attained (Scheme 18), observing a dramatic loss of the yield and stereoselectivity when the enzyme was recycled. Besides, the asymmetric Michael addition of 4-hydroxycoumarin to α,β-unsaturated ketones was reported using PPL, although after an optimization of the reaction conditions poor enantioselectivities were also observed (up to 28% *ee*) towards the formation of warfarins (Scheme 18), which are effective anticoagulants.[52]

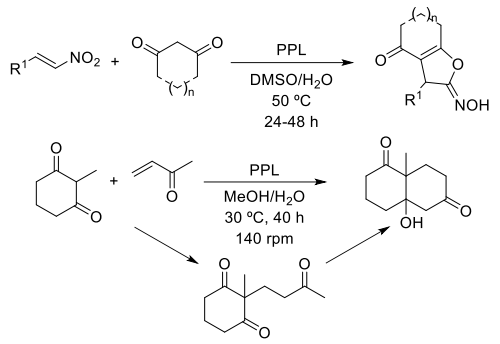


Scheme 18. Hydrolase-catalyzed catalyzed asymmetric Michael additions.

2.6. Tandem Michael reactions

Finally, some examples of tandem carbon-carbon bond forming reactions are here reported, all of them based on an initial Michael reaction followed by an intramolecular cyclization, favouring the formation of a more stable compound. Thus, the selective syntheses of 5-hydroxyimino-4,5-dihydrofurans and 2,3,6,7-tetrahydrobenzofurans have been described via tandem coupling between β-nitrostyrenes and 1,3-dicarbonyl compounds using PPL as biocatalyst (Scheme 19).[53] This one-pot strategy led in most of the cases to the *Z*-isomers by reaction of the corresponding nitrostyrene with 2 equivalents of the dione (2,4-pentanedione or 1,3-cyclohexanedione) in a mixture of DMSO/H_2O (3:2 v/v) for 24 or 48 h at 50 ºC. Recently, the one-pot synthesis of the Wieland-Miescher ketones has been

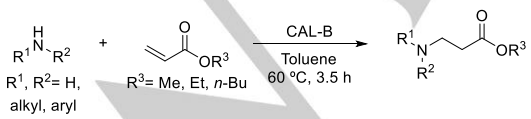
reported through a global Robinson annulation that occurred via Michael addition of 2-methyl-cyclohexane-1,3-dione to methyl vinyl ketone and subsequent intramolecular aldol condensation (Scheme 19).[54]



Scheme 19. PPL-catalyzed tandem reaction involving a Michael reaction.

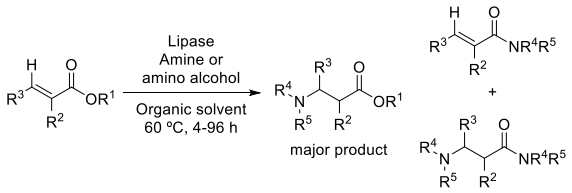
3. Carbon-heteroatom bond formation reactions

Michael-type additions are fundamental transformations in organic synthesis leading to the formation of carbon-heteroatom transformations with a perfect atom economy. In this context, biocatalytic promiscuous Michael-type transformations have been disclosed, as they offer greener alternatives to C-N and C-S bond formation reactions in comparison with traditional chemical methods. Firstly, the role of hydrolases in aza-Michael reactions will be discussed. The reaction between methyl acrylate and secondary amines such as diethylamine, pyrrolidine and piperidine has been efficiently accelerated by BLAP immobilized as cross-linked aggregates.[55] Similarly, CAL-B resulted as an effective enzyme for the regioselective aza-Michael reaction of primary and secondary amines with alkyl acrylates, forming the corresponding β-amino esters in 31-94% yield after a short reaction time at 60 ºC in toluene (Scheme 20).[56] Recently, Castillo and co-workers have developed a solvent engineering strategy to control the chemoselectivity of the CAL-B catalyzed aza-Michael addition between benzylamine and different α,β-unsaturated alkyl and phenylacrylate esters to explain the formation of aza-Michael adducts or aminolysis product taking into account thermodynamic interactions.[57] The formation of aza-Michael products was favored using nonpolar solvents while high chemoselectivity but lower yields were observed for the aminolysis products in polar solvents.



Scheme 20. CAL-B catalyzed the aza-Michael reaction between amines and alkyl acrylates.

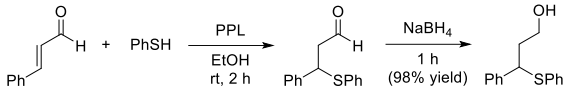
Rhizomucor miehei lipase (RML) has also efficiently promoted the aza-Michael reaction between alkyl acrylates and alkylamines, alkanolamines and diamines, leading to the selective preparation of 22 *N*-substituted β-amino esters in 45-100% yield.[58] A few organic solvents were tested such as hexane, toluene or diisopropyl ether, parameter that resulted highly depended on the nucleophile nature. Interestingly, when the RML-catalyzed Michael addition was carried out with etanolamine in hexane, the double Michael adduct was observed as unique product. This difference could be attributed to the low solubility of the amino alcohol in hexane creating a large excess of acrylate in the reaction medium, and thus driving to the formation of the unexpected di-aza-Michael product. On the other hand, when diamines were used as nucleophiles (1,3-propanediamine and 1,12-dodecyldiamine), the lipase catalyzed exclusively the addition through one of the amino groups. *Chromabacterium viscosum* lipase and *Pseudomonas stutzeri* lipase have served among other lipases to perform the reaction between primary, secondary amines or linear amino alcohols and a series of acrylates such as methyl acrylate, ethyl acrylate, butyl acrylate, methyl crotonate or methyl methacrylate (Scheme 21).[59] A clear preference was observed for the chemoselective formation of the 1,4-addition aza-Michael products, although in some cases significant amounts of the aminolysis reaction and the 1,4-addition followed by successive aminolysis were observed (up to 29%). An exhaustive study for the modulation of the enzyme chemoselectivity was achieved by a correct choice of enzyme source, substrate concentration and solvent. In a similar manner, the competition between aza-Michael addition and aminolysis reaction was observed in the reaction between aliphatic amines and ethyl propiolate.[60] The ratio of products obtained in the biotransformations catalyzed by CRL, CAL-B, PPL, PSL and RML were highly dependent of the solvent employed, observing changes in the preferred product just using different types of solvents such as 1,4-dioxane, isooctane, MeCN, toluene or MTBE among others. CAL-B was found as an excellent biocatalyst for the formation of *N*-substituted propiolamides, observing a significant influence also with the stirring mode as a gentle magnetic stirring led to complete conversions while the use of an orbital shaker led to slower reaction kinetics. The scope of the process was also extended to other nucleophiles such as benzyl alcohol and benzyl mercaptan both favoring the 1,2-addition for the formation of the corresponding ester and thioester, respectively.



Scheme 21. Competition between 1,4-aza-Michael addition, aminolysis reaction and 1,4-aza-Michael addition-aminolysis sequence in the lipase-catalyzed reaction between amine derivatives and different acrylates.

The biocatalytic thia-Michael is an attractive strategy to develop C-S bond formation reactions, which was reported for the first time by Domingues and co-workers in the reaction between cinnamaldehyde (8.25 mmol) and thiophenol (8.18 mmol).[61] Several hydrolases such as PPL, Lipozyme®, chymosin and papain has demonstrated different levels of activities, finding application in multigram-scale for PPL (Scheme 22). Kiełbasiński

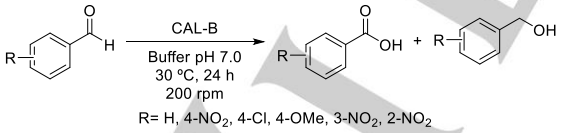
and coworkers have reported the use of a number of lipases as the own PPL, MJL, CAL-B or PSL among others in the addition of benzenethiol to racemic phenyl vinyl sulfoxide or 2-phosphono-2,3-didehydrothiolane S-oxide (2 equiv) in organic solvents at room temperature.[62] In general, low to moderate conversions were attained, with optical purities up to 25% *ee* after prolonged reaction times.



Scheme 22. PPL-catalyzed C-S bond formation reaction between cinnamaldehyde and thiophenol.

4. Other applications of hydrolases in single nonconventional processes

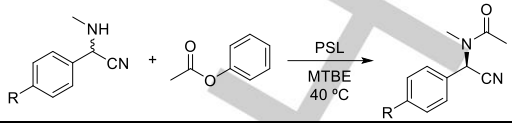
Apart from bond forming reactions, hydrolases have received consideration in recent years due to their role in oxidation, racemization and unconventional hydrolytic processes. Recent examples will be here discussed focusing in the importance for their future synthetic applications. The unusual participation of lipases for global oxidative processes has attracted great attention in recent years since they lack of cofactor dependency, acting under mild reaction condition.[63] Although lipases generally do not catalyze the oxidation on their own, lipase-mediated chemoenzymatic processes represent an alternative to traditional methods for Baeyer-Villiger or epoxidation reactions. However, in some cases, lipases have shown potential for a direct role such as in the Canizzaro type-reaction of substituted benzaldehydes in aqueous medium in which mixtures of the corresponding carboxylic acids and primary alcohols were afforded without the addition of external redox reagents (Scheme 23).[64] CAL-B displayed higher activities than other lipases and denatured CAL-B, obtaining mixtures of products with conversions between 78 and 98% in a phosphate buffer pH 7.0 at 30 ºC. Although it has not a direct synthetic applicability due to the formation of mixtures and the low substrate concentration (1 mM), the discovery open new possibilities for lipase-catalyzed transformations.



Scheme 23. CAL-B catalyzed Cannizaro-type reaction of benzaldehydes in aqueous medium.

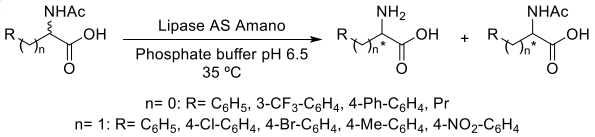
The development of dynamic kinetic resolutions (DKRs) is a challenging task as overcomes the inherent limitation of classical kinetic resolution in terms of maximum theoretical 50% yield. Interestingly, Ramström and co-workers reported an experimental and computational study regarding the racemase activity displayed by PSL[65] in the resolution of *N*-methyl-α-aminonitriles through acetylation of the amino group using 3 equivalents of phenyl acetate as acyl donor (Table 1).[66] The corresponding acetamides were obtained in good optical purity with conversions up to 94%.

Table 1. DKR of *N*-methyl-α-aminonitriles using PSL and phenyl acetate as acyl donor.



R	t (days)	Conversion (%)	Isolated yield (%)	*ee* (%)
H	8	94	90	85
OMe	7	92	89	86
F	13	92	87	88

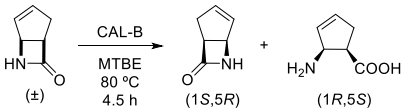
Hydrolases conventionally catalyze the hydrolysis of specific bond, which is their natural action. However, recent examples of nonconventional amidase and lactamase activities have been found mainly in lipases, and thus their possibilities to carry out stereoselective transformations have been highlighted. For instance, lipase AS Amano has efficiently catalyzed the classical kinetic resolution of 2-acetamides-2-substituted acetic acids including 2-alkyl-, 2-aryl- and substituted 2-benzyl rests, through the hydrolysis of the acetamide functionality in a buffer pH 6.5 after 4 h (Scheme 24).[67] Although none information was reported regarding the remaining amide isolation, the corresponding (*S*)-amino acids were obtained with high selectivity (up to 91% *ee*) and conversions between 31-48%. The possible contamination of the lipase with an amidase was discarded by performing protein electrophoresis analyses.



Scheme 24. A lipase showing an amidase activity for the kinetic resolution of amino acid amides in aqueous medium.

The lactamase activity has been found in hydrolases such as lipases, lipolases and esterases, providing access to lactams through stereoselective transformations. Importantly, these biocatalytic steps have served for the synthesis of relevant bioactive molecules. For instance, Fülöp and co-workers took advantage of the kinetic resolution of (1*R**,2*S**)-9-azabicyclo[6.2.0]dec-4-en-10-one to perform its enantioselective ring opening reaction using a lipolase and diisopropyl ether at 70 ºC.[68] The resulting amino acid was used as intermediated in the synthesis of enantiopure 6-hydroxy- and 5,6-dihydroxy-2-aminocyclooctanecarboxylic acid derivatives.[69] Similarly, the resolution of 7-azabicyclo[4.2.0]oct-3-en-8-one with CAL-B in MTBE using water as hydrolytic agent in stoichiometric conditions led to a 50% conversion after 4.5 h at 80 ºC, obtaining the corresponding (–)-lactam and the (+)-amino acid both in enantiopure form (Scheme 25).[70] Ostaszewski and co-

workers reported the kinetic resolution of 2-acetyl-4-aryl-1,4-dihydro-2*H*-isoquinolin-3-ones using lipases such as PSL in organic medium (MTBE and Et_2O) or live acetone powders (LAPs) in aqueous medium, finding an excellent selectivity (*E*>500) with turkey LAP for the production of the desired enantiopure unprotected (*R*)-lactam.[71]



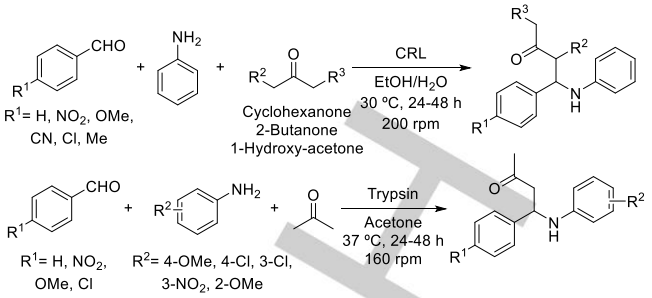
Scheme 25. CAL-B catalyzed the kinetic resolution of 7-azabibyclo[4.2.0]oct-3-en-8-one through a hydrolytic procedure.

The amino acid sequence of wild-type *Pseudomonas fluorescens* esterase I (PFEI) was modified using directed evolution methods, finding an improvement of 200-fold for the new variant Leu29Pro in the lactamase activity for the kinetic resolution of the Vince lactam (2-azabicyclo[2.2.1]hept-5-en-3-one).[72] Dramatic improvements were achieved in the reaction turnover without affecting the enantioselectivity by the introduction of a single point mutation. These changes were explained through docking and molecular dynamic simulations.

5. Hydrolases in multicomponent and tandem nonconventional reactions

Multi-step one pot processes are attractive tools for the production of organic compounds such as fine chemical or pharmaceutical precursors without the necessity of isolate and purify reaction intermediates, and providing outstanding economic and time benefits. In this context, hydrolases have allowed the development of multiple transformations in recent years, which mainly have served for an optimum manufacture of heterocyclic compounds with high complexity in high yields. Although in this revision we have previously described examples of tandem reactions, this section will focus in those occurring in a multicomponent fashion involving mainly three compounds for a one-pot transformation. The Mannich reaction is a typical example, which traditionally requires the action of chiral transition-metal complexes and organocatalysts for the production of β-amino carbonyl compounds in an asymmetric mode.[73]

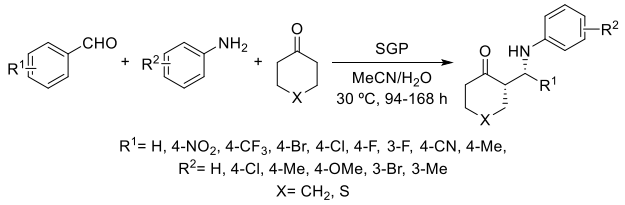
CRL has been identified as the most active lipase in the reaction between 4-nitrobenzaldehyde (1.0 mmol), aniline (1.1 mmol) and cyclohexanone (30 mmol) in a benign solvent system as ethanol with a 5% water content.[74] The extension to other aromatic aldehydes and aliphatic ketones under optimized conditions of this study led to the formation of 12 direct Mannich products in 20-94% isolated yield and moderate diasteroselectivity, after silica gel column chromatography (Scheme 26).



Scheme 26. CRL-catalyzed direct Mannich reaction between aromatic aldehydes, aniline and aliphatic ketones.

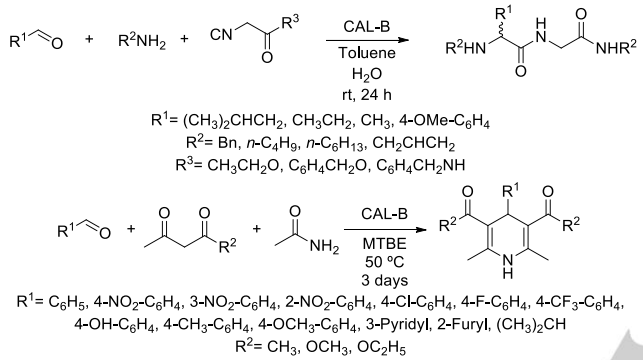
Similarly, trypsin from hog pancreas was found an active catalyst for the formation of Mannich adducts in 45-93% isolated yield for the reaction between aromatic aldehydes, substituted anilines and a large excess of acetone, which served as both solvent and reactant (Scheme 26).[75] The benefits of using acetamide as co-catalyst in the CAL-B catalyzed Mannich reaction between (*E*)-*N*-(4-nitro-benzylidene)aniline with cyclohexanone has been demonstrated by Wu, Lin and co-workers increasing the reaction yield from 25 to 38%.[48]

The first enzyme-catalyzed direct asymmetric Mannich reaction has been reported by He, Guan and co-workers using the protease type XIV from *Streptomyces griseus* (SGP), obtaining yields up to 92%, enantioselectivities up to 88% *ee* and diasteroselectivities *syn*:*anti* up to 92:8.[76] Optimization studies were performed considering the reaction between 4-nitrobenzaldehyde, aniline and a large excess of cyclohexanone in a mixture of MeCN and water (9:1 v/v) as the best solvent (Scheme 27). Surprisingly and although in control experiments the reaction in absence of enzyme and with albumins proceeded in some extent for the formation of the racemic Mannich product (21-28% yield), the SGP-catalyzed reaction allowed a 66% yield with 82% *ee* and 85:15 *dr*. Furthermore, the denatured GSP allowed the recovery of the Mannich product in a superior 87% isolated yield although with a significant loss of the optical purity (8% *ee*). All these results suggest that the enzyme is responsible of the catalytic action mainly directing the stereoselective pathway of the reaction. The scope of the reaction was demonstrated for a variety of ketones, aldehydes and anilines, observing a clear dependence in the reactivity and the selectivity due to the nature of the pattern substitution. Similarly, the same research group has recently reported the use of acylase I from *Aspergillus melleus* in the asymmetric Mannich reaction of the same substrates, enantioselectivities up to 89% *ee*, *syn*/*anti* diasteroselectivities up to 90:10 and yields up to 82% were achieved in a mixture of MeCN and phosphate buffer pH 8.1 (85:15 v/v) at 30 ºC.[77]



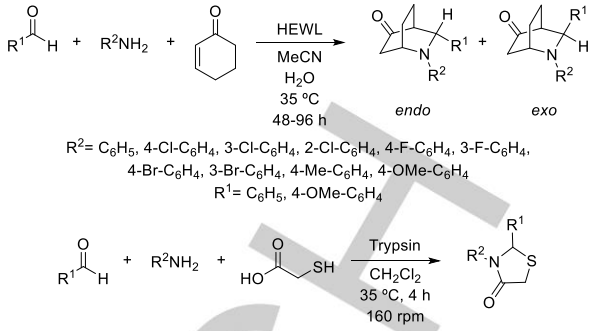
Scheme 27. SGP-catalyzed the direct asymmetric Mannich reaction between aromatic aldehydes, anilines and cyclic ketones.

Ostaszewski and co-workers reported the first example of a lipase-catalyzed Ugi reaction, in which CAL-B was found as a suitable enzyme in the formation of dipeptides in toluene.[78] The model reaction between iso-valeric aldehyde (1 equiv), benzylamine (2 equiv) and ethyl isocyanoacetate (1 equiv) proceeded with a 75% yield after 24 h (Scheme 28) and the potential of this reaction was extended to a representative number of amines using also benzyl isocyanoacetate. CAL-B has also efficiently catalyzed other three-component transformation such as the Hantzsch-type reaction (Scheme 28).[79] The reaction of an aldehyde, a large excess of 1,3-dicarbonyl compound and acetamide as a novel source of ammonia occurred in MTBE at 50 ºC, obtaining the corresponding 1,4-dihydropyridines in low to very high yield after 3 days (17-92%).



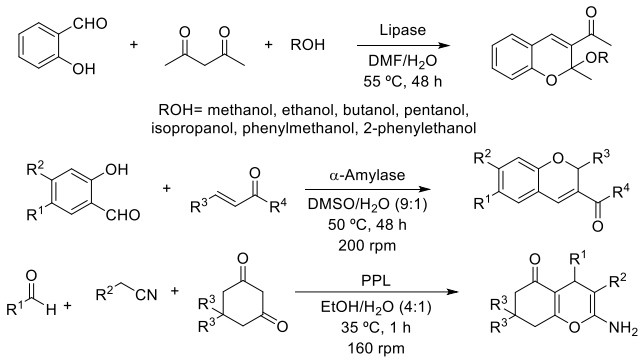
Scheme 28. CAL-B catalyzed three-component Ugi and Hantzsch reactions in organic solvent.

The production of heterocycles by promiscuous hydrolase-catalyzed reactions has also been achieved by a number of contributions. For instance, the direct three-component aza-Diels Alder reaction between an aromatic aldehyde (1 equiv), aromatic amine (3 equiv) and 2-cyclohexen-1-one (3 equiv) was catalyzed by hen egg white lysozyme (HEWL), hydrolase from the glycosylase family. The azabicycles were obtained in 69-97% isolated yields and an *endo*/*exo* ratio selectivity up to 90:10 after several days in a mixture of MeCN/water (9:1) at 35 ºC (Scheme 29).[80] 4-Thiazolodinones have been efficiently obtained through trypsin from porcine pancreas-catalyzed one-pot reaction between an amine, an aldehyde, and mercaptoacetic acid used in equimolar amounts (42-89% isolated yield, Scheme 29).[81] From a set of active lipases, diastase and amylases, this trypsin displayed the best catalytic activities, finding dichloromethane as the best organic solvent while no reaction was observed in water. A significant activity was also observed for the bovine serum albumin (BSA) suggesting the possibility of partial unspecific catalysis.



Scheme 29. Aza-Diels Alder reaction catalyzed by hen egg white lysozyme (top), and trypsin-catalyzed multicomponent reaction for the synthesis of 4-thiazolodinones.

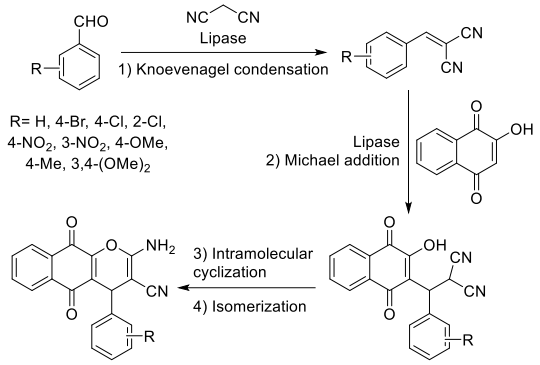
Alkoxy and aryloxy 2*H*-chromenes were obtained by a lipase-catalyzed three-component one-pot reaction between salicylaldehyde (1 equiv), acetoacetone (1 equiv) and a variety of alcohols (10 equiv) in DMF after 48 h at 55 ºC (45-89% yield, Scheme 30).[82] PPL displayed the highest activity although good results were also achieved with CAL-B, PSL and CRL. On the other hand, a biocatalytic domino oxa-Michael-aldol condensation has been reported for the synthesis of 3-substituted 2*H*-chromenes using salicylaldehydes and α,β-unsaturated ketones or aldehydes (5 equiv). α-Amylase from *Bacillus subtilis* provided the best activities, yielding the targeted heterocycles in moderate yields (18-70%, Scheme 30).[83] An unprecedented lipase-catalyzed one-pot synthesis of tetrahydrochromenes was reported by combining aromatic or aliphatic aldehydes, malonitrile or cyanoacetic ester and 1,3-dicarbonyl compounds in equimolar amounts and the presence of water in organic solvent.[84] Best results were found with PPL and ethanol, leading to 31 tetrahydrochromene derivatives in high to excellent yields (76-97%) at 35 ºC for just 1 h (Scheme 30).



Scheme 30. Hydrolase-catalyzed synthesis of chromene derivatives through tandem and multicomponent reactions.

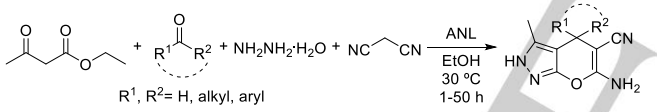
Recently, the synthesis of benzo[*g*]chromenes has been described though a three-component reaction, which includes the use of stoichiometric amounts of an aromatic aldehyde, malonitrile and 2-hydroxy-1,4-naphthoquinone.[85] After a reaction optimization study, ethanol, 55 ºC and *Candida species* lipase (CSL) were identified as the best conditions to yield the desired heterocycles in high yields (81-93%) after a

Knoevenagel condensation-Michael addition-intramolecular cyclization and isomerization sequence, as depicted in Scheme 31.



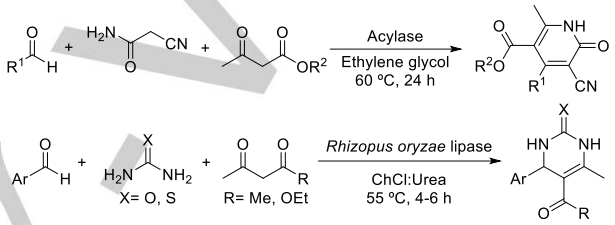
Scheme 31. CSL-catalyzed synthesis of synthesis of benzo[*g*]chromenes through a four-steps sequence.

The synthesis of dihydropyrano[2,3-*c*]pyrazoles has been achieved through a four-component and one-pot reaction catalyzed by lipase from *Aspergillus niger* (ANL).[86] By mixing ethyl acetoacetate, hydrazine hydrate, malonitrile with an aldehyde or a ketone in stoichiometric amounts at 30 ºC and ethanol as solvent (Scheme 32), the synthesis of the heterocycles was possible for 15 different aldehydes in short reaction times (1-3.5 h, 75-98% isolated yield) and slower for 7 ketones (36-50 h, 70-80% yield).



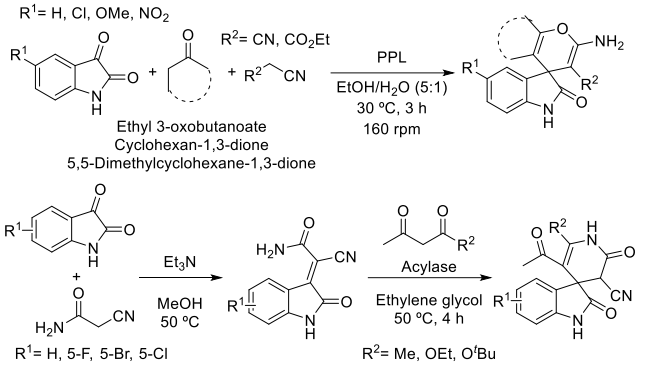
Scheme 32. ANL-catalyzed four-component reaction for the synthesis of dihydropyrano[2,3-*c*]pyrazoles.

The Amano acylase from *Aspergillus oryzae* has catalyzed the synthesis of a series of pyridin-2-ones in 37-93% yield, starting from an aldehyde (0.5 mmol), cyanoacetamide (0.75 mmol) and ethyl acetoacetate or cyclohexyl acetoacetate (0.25 mmol) in ethylene glycol at 60 ºC for 24 h (Scheme 33).[87] This challenging process has been possible through a domino Knoevenagel condensation-Michael addition-intramolecular cyclization-oxidation in one-pot. This works extends the applicability of the same acylase in the synthesis of highly substituted 3,4-dihydropyridin-2-ones via a three-component condensation of an aldehyde, cyanoacetamide and a 1,3-dicarbonyl compound including acetylacetone, methyl acetoacetate or acetoacetanilide.[88] The biocatalytic preparation of related nitrogen heterocycles such as novel dihydropyrimin-2(1*H*)-ones has been described by the proper combination of *Rhizopus oryzae* lipase in a deep eutectic solvent such as a mixture of choline chloride (ChCl) and urea (1:2 mol %).[89] The reaction between an aromatic aldehyde, ethyl acetoacetate or acetyl acetone and urea or thiourea was studied in terms of molar ratio, solvent, amount, type and reusability of catalyst, yielding dihydropyrimin-2(1*H*)-ones in 73-95% isolated yield after short reaction times at 55 ºC (Scheme 33). Similarly but in ethanol as solvent, this Biginelli reaction between an aromatic aldehyde, ethyl acetoacetate and urea or thiourea has been efficiently catalyzed by a series of hydrolases such as lipase M from *Mucor javanicus*, α-amylase from hog pancreas, pepsin from hog stomach and trypsin from porcine pancreas.[90] This trypsin has allowed the production of 18 dihydropyrimidiones in yields over 80%, and just a little loss of enzyme activity was observed after five cycles (89-95% yield). Trypsin has also efficiently catalyzed a tandem Biginelli reaction between substituted (thio)ureas and 1,3-dicarbonyl compounds using an in-situ generated acetaldehyde from vinyl acetate and isobutanol at 60 ºC for 5 days. The main advantage of this method is the reduction of problems associated with the volatilization loss, oxidation and polymerization of acetaldehyde, together with the avoidance of enzyme deactivation at large excess of the mentioned aldehyde.[91]



Scheme 33. Synthesis of pyridin-2-ones using Amano acylase from *Aspergillus oryzae* (top) and dihydropyrimidin-2-(1*H*)-ones using *Rhizopus oryzae* lipase (bottom).

Last but not least, the effectiveness of hydrolases in nonconventional multicomponent one-pot processes for the synthesis of spirocompounds is here described. For instance, 20 spirooxindole derivatives were prepared by a PPL-catalyzed sequence involving a Knoevenagel condensation, Michael addition and intramolecular cyclization in a mixture (5:1) of ethanol and water (Scheme 34).[92] The reaction between an isatin, malonitrile or cyanoacetic ester and a 1,3-dicarbonyl compound in stoichiometric amounts occurred at 30 ºC, yielding the spirooxindoles in very high yields (82-95%). Similarly, the acylase from *Aspergillus oryzae* has been identified as a powerful catalyst for the synthesis of spirooxindole derivatives in moderate yields (19-62%) and moderate diasteroselectivities (up to 80:20).[93] The reaction between a series of isatins (0.2 mmol), 1,3-dicarbonyl compounds (1.2 mmol) and cyanoacetamide (0.6 mmol) showed the best values in ethylene glycol at 50 ºC. Significantly, better results were obtained through a chemoenzymatic two-step route involving a trimethylamine-catalyzed Knoevenagel reaction followed by acylase-catalyzed reaction of the condensation products with 1,3-dicarbonyl compounds, improving the yields to 91-100% while the diasteroselectivities did not suffer significant changes (Scheme 34). On the other hand, CAL-B has been demonstrated to initiate the multicomponent reaction between an aldehyde, a nitrostyrene and acetamide to form a series of spirooxazine derivatives in low to good yield after 144 h at 50 ºC.[94]



Scheme 34. Synthesis of spirooxindoles involving a PPL-catalyzed Knoevenagel condensation-Michael addition and intramolecular cyclization sequence (top), and the chemoenzymatic approach using Et_3N and the acylase from *Aspergillus oryzae* (bottom).

6. Conclusions

The discovery of novel catalytic activities is a challenging task in organic chemistry, enzymes contributing to the design of efficient and environmentally friendly synthetic routes. The unnatural action of a wide range of existing hydrolases have been summarized and grouped in the category of biocatalytic promiscuity. Importantly, some enzyme catalytic mechanism have been discussed, and the action of the catalytic active sites demonstrated. In this context, it is important that authors will unequivocally address control experiments regarding the use of inhibited, denatured enzymes and of carrier proteins such as serum albumins[95] to assure the specific activity of the studied enzyme preparations. Then, the application of hydrolases in selected transformations will be justified, instead of using single amino acids with catalytic properties such as proline. In this context, it is noteworthy to rationalize the experiments as performed by Lin and co-workers in the pseudopromiscuous or apparent acylase-catalyzed Markovnikov addition between imidazoles, aldehydes and isopropenyl acetate in organic solvent.[96] The authors made efforts to develop control experiments and a mechanistic proposal to suggest that the acylase is just involved in a conventional acylation process although finally an unexpected carbon-nitrogen bond is formed through an acylation, hemiaminal intermediate formation and transesterification sequence, the formation of acetaldehyde being crucial for the evolution of the reaction and the enzyme activity.

The access to new enzyme variants through metagenomic libraries[97] and the development of alignment analyses and computational methods[98] can also lead to the discovery of novel catalytic activities expanding the potential of enzymes with synthetic purposes. Directed evolutions techniques offer significant advantages, providing a plethora of improved biocatalysts, which can also catalyze novel interesting transformations.[99] The combination of amino acid sequence modification with computational studies will open new possibilities allowing the rationalization of mechanistic actions for novel and intriguing biotransformations,[100] and hopefully also allowing the development of asymmetric transformations with until now remains in a premature stage for nonconventional hydrolase-catalyzed reactions.[101] In addition, enzyme immobilization techniques provides a useful access to enhanced enzyme activities by using different strategies and a variety of carriers, making possible the improvement or tuning of catalytic properties in the search of more efficient biocatalysts, which would facilitate the performance of novel enzyme-catalyzed reactions.[102]

Hydrolases possess remarkable advantages in comparison with other classes of enzymes such as an easy handling, commercial availability and lack of cofactor requirements, so their applications in nonconventional reactions have attracted great attention in the last two decades. Nevertheless, there are still some limitations as substrate specificity, stereodiscrimination and level of catalytic activity that must be overcome to bridge the gap with the values obtained by the natural enzymes in selected transformations. Hopefully the advances in enzyme modification techniques will provide even more efficient hydrolases in the next few years.

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Keywords: Biocatalytic Promiscuity • Enzymes • Hydrolases • Lipases • Organic Synthesis

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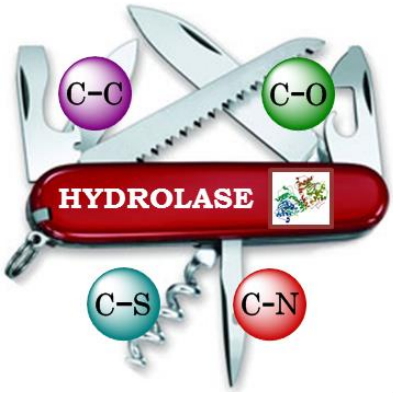
RECORD REVIEW

The discovery of novel catalytic activities is a challenging task in synthetic chemistry. Biocatalytic promiscuity has emerged in recent years providing a simple access to a vast number of organic compounds. An update of the action of hydrolases for the development of single and tandem carbon-carbon and carbon-heteroatom bond formation reactions, unexpected hydrolytic and global oxidative processes is here disclosed.



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