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Hydrolase-Catalyzed Promiscuous Reactions and Applications in Organic Synthesis

Yun Wang and Na Wang

Abstract

The potential of biocatalysis becomes increasingly recognized as an efficient and green tool for modern organic synthesis. Biocatalytic promiscuity, a new frontier extended the use of enzymes in organic synthesis, has attracted much attention and expanded rapidly in the past decade. It focuses on the enzyme catalytic activities with unnatural substrates and alternative chemical transformations. Exploiting enzyme catalytic unconventional reactions might lead to improvements in existing catalysts and provide novel synthesis pathways that are currently not available. Among these enzymes, hydrolase (such as lipase, protease, acylase) undoubtedly has received special attention since they display remarkable activities for some unexpected reactions such as aldol reaction and other novel carbon-carbon and carbon-heteroatom bond-forming reactions. This chapter introduces the recent progress in hydrolase catalytic unconventional reactions and application in organic synthesis. Some important examples of hydrolase catalytic unconventional reactions in addition reactions are reviewed, highlighting the catalytic promiscuity of hydrolases focuses on aldol reaction, Michael addition, and multicomponent reactions.

Keywords: enzyme, biocatalysis, promiscuity, hydrolases, lipase, aldol reactions, Michael addition, multicomponent reactions

1. Introduction

Biocatalysis is the application of enzymes for chemical transformations of organic compounds. Enzymes as biocatalysts have many advantages [1–3]: (1) enzymes are very efficient catalysts. Typically the rates of enzyme-mediated processes are accelerated, compared to those of the corresponding nonenzymatic reaction, by a factor of 10^8 – 10^{10} . The acceleration may even exceed a value of 10^{12} , which is far above the values that chemical catalysts are capable of achieving; (2) enzymes are environmentally acceptable. Unlike heavy metals, for instance, biocatalysts are completely degraded in the environment; (3) enzymes act under mild conditions. Enzymes act in a temperature range of 20–40°C, under neutral aqueous, and in the absence of substrate functional group protection. This minimizes problems of undesired side reactions such as decomposition, isomerization, racemization, and rearrangement, which often plague traditional methodology; (4) enzymes display high chemoselectivity, regioselectivity, and enantioselectivity.

As a result, reactions that generally tend to be “cleaner” and laborious can largely be omitted; and (5) enzymes can catalyze a broad spectrum of reactions. There is an enzyme-catalyzed process equivalent to almost every type of organic reaction, such as oxidation, hydrolysis, addition, halogenation, alkylation, and isomerization. In addition, many enzymes accept unnatural substrates, and genetic engineering can further alter their stability, broaden their substrate specificity, and increase their specific activity. Thus, the application of enzymes in synthesis thus represents a remarkable opportunity for the development of industrial chemical and pharmaceutical processes [4–7].

Although it is well known that a given enzyme is able to catalyze a specific reaction efficiently, some unexpected experimental results have indicated that many enzymes have catalytic promiscuity [8–12]. Enzyme promiscuity is classified into three categories: (a) condition promiscuity, which is an enzyme’s ability to work under unexpected condition; (b) substrate promiscuity, which is an enzyme’s ability to work with unexpected substrates; and (c) catalytic promiscuity, which is an enzyme’s ability to catalyze unexpected reactions. Among them, catalytic promiscuity has gained much attention as it opens a wide scope for the industrial application of enzymes.

During the past decade, biocatalytic promiscuity, as a new frontier extending the use of enzymes in organic synthesis, has received considerable attention and expanded rapidly. A classic example of promiscuous enzymatic behavior is pyruvate decarboxylase, which not only decarboxylates pyruvate but also links acetaldehyde and benzaldehyde to form R-phenylacetylcarbinol. The use of pyruvate decarboxylase to form carbon–carbon bonds, which does not occur in the natural reaction, was first studied in 1921 and was applied in industry today [13]. As one of the most rapidly growing areas in enzymology, multifunctional biocatalytic reactions not only highlights the existing catalysts but may provide novel and practical synthetic pathways which are not currently available. Miao et al. reviewed enzyme promiscuity for carbon-carbon bond-forming reactions like aldol couplings, Michael(-type) additions, Mannich reactions, Henry reactions, and Knoevenagel condensations [14]. Gotor-Fernández et al. also highlighted the hydrolase-catalyzed reactions for nonconventional transformations in the same year [15].

Hydrolases (such as lipase, protease, acylase) have received extensive attention as biocatalysts for a long time due to their many attractive properties like stability in

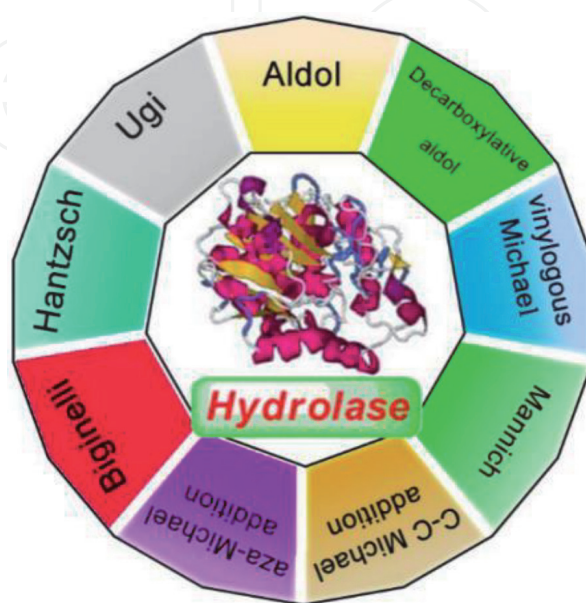


Figure 1.
Hydrolase-catalyzed promiscuous reactions.

organic solvents, not requiring cofactors, broad substrate tolerance, commercial availability, and high chemo-, regio-, and stereoselectivity. Hydrolases have demonstrated a great versatility in hydrolysis, transesterification, aminolysis reactions, etc. Some hydrolase-catalyzed promiscuous reactions have been done in the last decades (**Figure 1**). These research and other relevant reports encouraged us to believe that the catalytic activities for unconventional reactions rather than the well-known hydrolytic function may also have a natural role in hydrolase evolution.

The aim of the present chapter is to give a brief overview of the hydrolase-catalyzed C—C and C—N reactions and present some of the most recent applications in different fields for recent decade. The main work in our group will be disclosed, highlighting the catalytic properties of hydrolases to catalyze not only single processes but also multicomponent and tandem reactions. Consequently, the promiscuous hydrolase-catalyzed reactions are outlined with focus on Michael addition, aldol reaction, Mannich reaction, Biginelli reaction, etc.

2. Michael addition

Michael addition is a 1,4-addition of a nucleophile to α,β -unsaturated compounds, and it is one of the most fundamental and important reactions for the formation of carbon-carbon bonds and carbon-heteroatom bonds in organic synthesis. Michael addition reactions are traditionally catalyzed under strong basic or acidic conditions, which can cause unwanted side reactions such as further condensation or polymerization of α,β -unsaturated compounds. Thus, biocatalysis can afford a green and facile method for organic synthesis. Among different biocatalysts, hydrolases such as protease and lipase have been widely used as a green and efficient catalyst for Michael addition.

2.1 Carbon-heteroatom bond formation Michael addition

Michael addition is the early promiscuous reaction catalyzed by hydrolase. In 1986, Kitazume et al. reported the hydrolytic enzyme-catalyzed stereospecific Michael addition reactions in buffer solution (pH = 8.0) at 40–41°C (**Figure 2**) [16]. This discovery overthrows the long erroneous concept of enzymology that “biocatalysis must be carried out in aqueous solution,” making many organic reactions that cannot be carried out in water be completed in organic solvents and greatly expanding the application scope of enzymes as catalysts. Moreover, enzymes are frequently more stable in organic solvents than in water. Thus, some research groups began to focus on enzyme-catalyzed Michael addition reactions in organic solvents.

Lin and Gotor et al. firstly reported the hydrolase-catalyzed Michael addition of imidazole with acrylates catalyzed by alkaline protease from *Bacillus subtilis* in organic solvent in 2004 [17, 18]. Subsequently, other hydrolase-catalyzed Michael addition reactions were reported. In 2010, Bhanage et al. developed an efficient protocol for the regioselective aza-Michael addition of amines with acrylates using

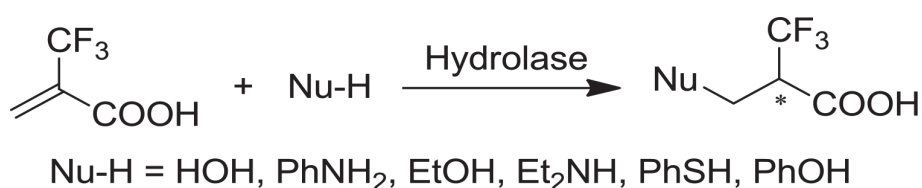


Figure 2.
The first hydrolase-catalyzed Michael addition in buffer.

Candida antarctica lipase B (CALB) as a biocatalyst at 60°C (**Figure 3**) [19]. The universality of the reaction, including the reactions of various primary and secondary amines with different acrylates, was also studied. Higher yields were obtained.

Gotor et al. have explored new synthetic possibilities of commercially available protease from *Bacillus licheniformis* immobilized as cross-linked enzyme aggregates (Alcalase-CLEA) in 2011, since the CLEA immobilization improves the stability of the protein toward denaturalization by heating, organic solvents, and autoprolysis [20]. Alcalase-CLEA has achieved the best results in the aza-Michael addition of secondary amines to acrylonitrile (**Figure 4**). In all cases the formations of the corresponding Michael adduct were faster than in the absence of biocatalyst, but also in comparison with the inhibited enzyme, the reaction rates being highly dependent of the amine structure.

In 2012, Baldessari et al. firstly reported the synthesis of N-substituted β -amino esters by application of *Rhizomucor miehei* lipase in aza-Michael addition of mono- and bifunctional amines to α,β -unsaturated esters [21]. The authors selected ethyl acrylate and propyl acrylate as the Michael acceptors and different alkylamines, alkanolamines, and diamines as the Michael donors (**Figure 5**). The reactions were carried out in low-polarity solvents (hexane, toluene, and diisopropyl ether (DIPE)) at 30°C for 16 h with yields from 12 to 100%. Subsequently, the authors investigated the effect of the reaction conditions on the Michael addition systematically, such as commercially available enzyme sources, organic solvents, and the structure of the Michael acceptor and donor. The results showed that the alkanolamines in n-hexane were not selective and double Michael adducts could be obtained. Substrate concentration also plays an important role in enhancing the catalytic effect of enzymes on spontaneous reactions. High substrate concentration limits the efficiency of biocatalysts.

In 2013, Demeunynck et al. have optimized the lipase-biocatalyzed addition of benzylamine to ethyl propiolate. Immobilized *Candida antarctica* lipase B was

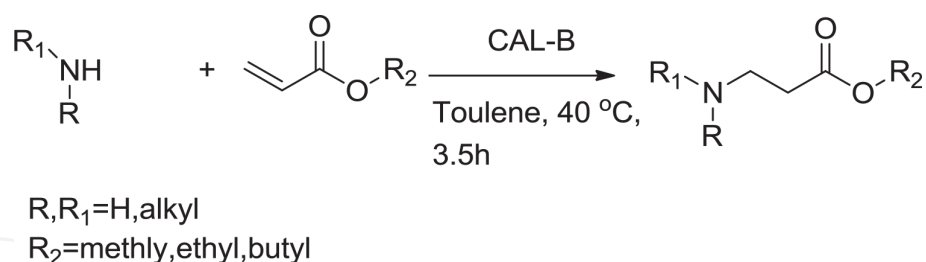


Figure 3.
CALB-catalyzed aza-Michael addition of amine to acrylate.

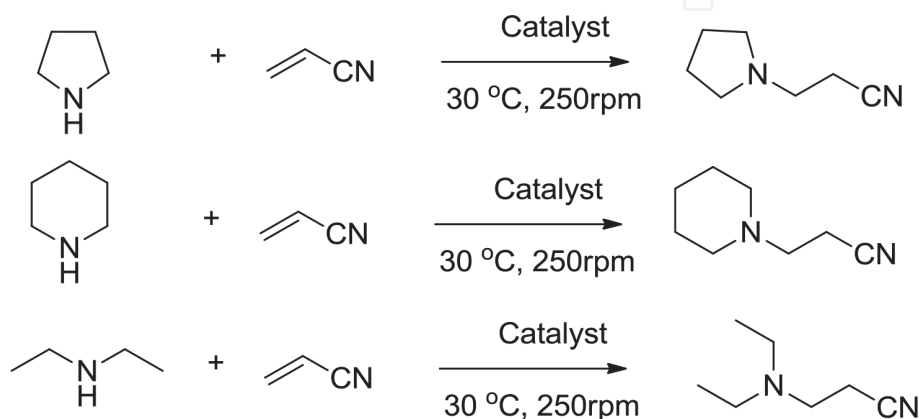
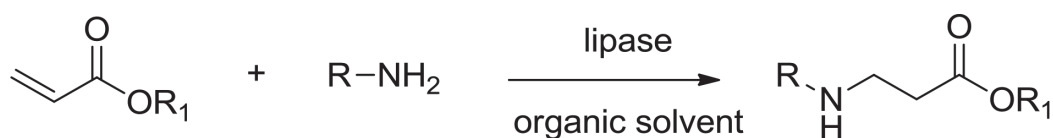


Figure 4.
Hydrolase-catalyzed Michael-type additions between secondary amines and acrylonitrile.



$\text{R}_1 = \text{CH}_2\text{CH}_3, (\text{CH}_2)_3\text{CH}_3$

$\text{R} = \text{CH}_3(\text{CH}_2)_2-, (\text{CH}_3)_2\text{CH}-, \text{Ph}(\text{CH}_2)_2-, \text{HO}(\text{CH}_2)_2-, \text{HO}(\text{CH}_2)_3-, \text{HO}(\text{CH}_2)_4-, \text{H}_2\text{N}(\text{CH}_2)_3, \text{H}_2\text{N}(\text{CH}_2)_6, \text{H}_2\text{N}(\text{CH}_2)_8, \text{H}_2\text{N}(\text{CH}_2)_{12}$

Figure 5.

Lipase-catalyzed aza-Michael addition of amines to α,β -unsaturated esters.

beneficial to the chemoselective 1,2-addition, using TBME, dioxane, or toluene under overnight (15 h) gentle magnetic stirring at 50°C (**Figure 6**) [22]. Under these conditions, the yield of acrylamide was good at the Gram scale. S-trans-z and e-diphenylamine were formed as by-products. The reactions worked well with other primary amines but not with secondary amines that only gave the corresponding aminoacrylates. The chemoselectivity of CALB with N- and S-nucleophiles was also checked. The transesterification also worked in good yields in TBME, toluene, or dioxane. The best yields (near quantitative) were observed when the reactions were carried out in open vessels under gentle magnetic stirring at 50°C for 6 h.

In the same year, Franssen and co-workers demonstrated lipases from *Pseudomonas stutzeri* (PSL) and *Chromobacterium viscosum* (CVL) are excellent catalysts for the aza-Michael addition of amines to substituted or unsubstituted acrylates with high product selectivity and good yields (**Figure 7**) [23]. Comparative studies of other lipases, including Novoxin 435, have proven ineffective. The selective Michael addition of diamines to these substituted acrylates was also realized. In this paper, the catalytic effects of various lipases on aza-Michael addition reaction, especially on the lipase catalysis of *Pseudomonas aeruginosa* OM2 and PSL, are introduced. The 1,4-adducts of acrylate and benzylamine have high yield and selectivity.

Chemoselective synthesis of N-protected β -amino esters involving lipase-catalyzed aza-Michael additions is mainly hampered by the two electrophilic sites present on these compounds. In order to control the chemoselectivity, a solvent engineering strategy based on the thermodynamic behavior of products in media of different polarity was designed by Castillo et al. (**Figure 8**) [24]. This strategy can

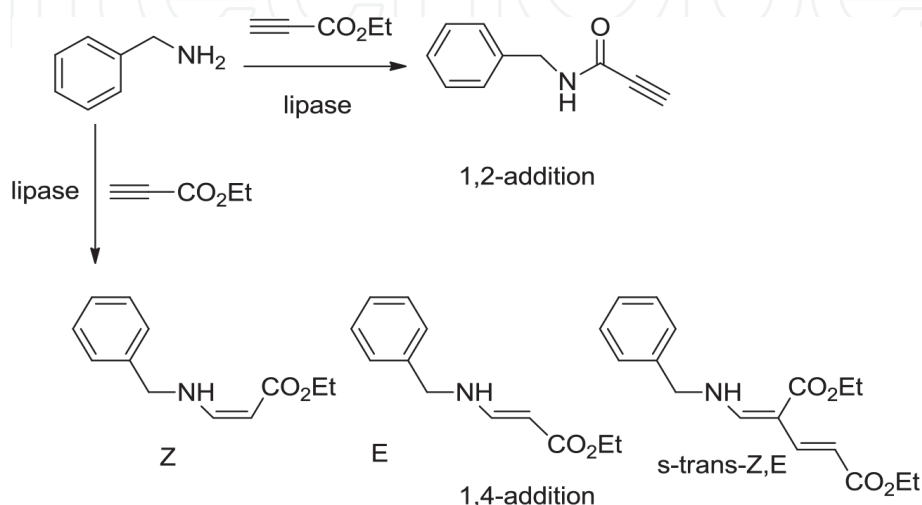


Figure 6.

Reactivity of benzylamine with ethyl propiolate.

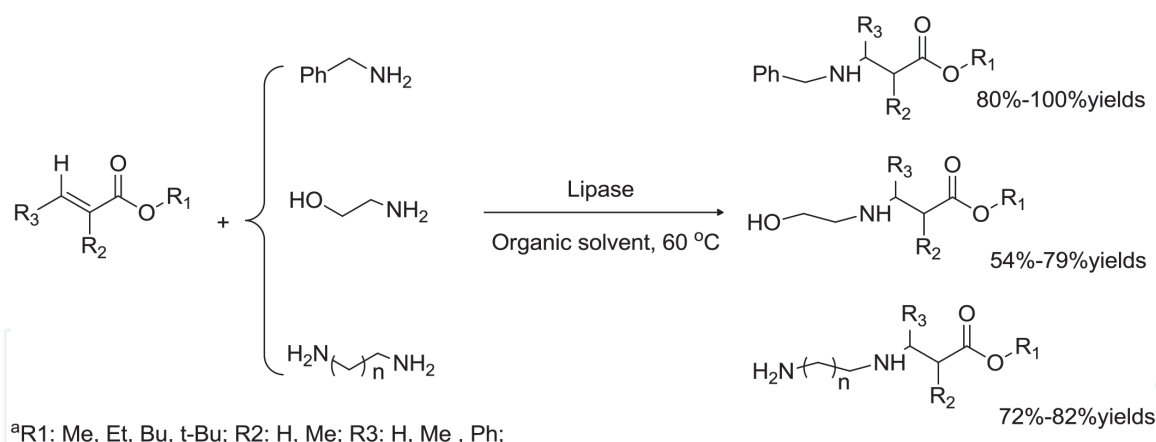


Figure 7.
Lipase-catalyzed aza-Michael addition of amines with different substituted acrylates.

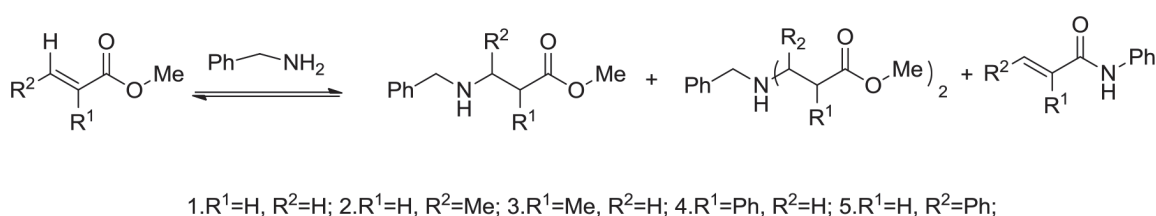


Figure 8.
Chemoselectivity of the addition of benzylamine to α,β -unsaturated esters.

obtain highly selective aza-Michael adducts from benzylamine and different acrylates. Ammonia hydrolysis is avoided in almost all reactions with n-hexane (a nonpolar solvent) as solvent, while the corresponding Michael adduct is synthesized in 53–78% yield. On the contrary, if the reaction is carried out in polar solvents (e.g., 2-methyl-2-butanol (2M2B)), the product of ammonia hydrolysis will be advantageous. Thermodynamic analysis of these processes using the actual solvation conductor-like screening model (COSMO-RS) helps to understand some key factors affecting chemical selectivity and confirms that reliable estimates of the thermodynamic interactions between solutes and solvents allow adequate selection of reaction media that may lead to chemical selectivity.

Reaction media has an important effect on the yield and chemo- and enantioselectivity of biocatalytic reaction. Solvent engineering is an effective tool to direct chemo- and enantioselectivity of the aza-Michael addition and the subsequent kinetic resolution of the Michael adduct [25]. In the reaction of benzylamine and methyl crotonate catalyzed by CALB, three possible adducts can be isolated: aminolysis product, aza-Michael addition product, and double addition product (**Figure 9**). The authors selected n-hexane and 2-methyl-2-butanol (two solvents of opposite polarity) as solvents in the experiments. The Michael adduct is favored in more hydrophobic media, while the amide product in more polar solvents, and the best values of ee for aza-Michael adduct were obtained in almost 100% 2M2B. The experiment results clarify the origin of the enantiomeric excess of the aza-Michael

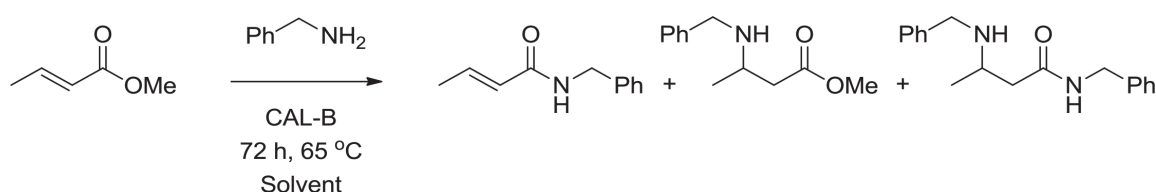


Figure 9.
Lipase-catalyzed addition and aminolysis reaction of benzylamine to methyl crotonate.

addition product, obtained in 2M2B, by a resolution process with CALB on the enantiomers of aza-Michael addition product.

Our group demonstrated that 3-substituted 2H-chromene derivatives were synthesized via biocatalytic domino oxa-Michael/aldol condensations (**Figure 10**) [26]. α -Amylase from *Bacillus subtilis* shows excellent catalytic activity and exerts good adaptability to different substrates in the reaction. The reaction conditions including organic solvents, water content, temperature, molar ratio of substrates, and enzyme loading were optimized.

It is generally believed that the hydrolysis site of hydrolase is also the active site of its miscible catalysis. On this basis, the possible mechanism of domino reaction catalyzed by hydrolase was proposed. First, salicylaldehyde was activated by amino residues and oxygen anions of amylase. Then methyl vinyl ketone is attacked by activated salicylaldehyde, and a new C—O bond is formed by oxa-Michael addition reaction. Next, an intramolecular aldol reaction begins to form carbon–carbon bonds. Finally, the adduct was dehydrated, and the required product was released (**Figure 11**).

Very recently, our group conducted an aza-Michael addition of aniline compounds and acrylate derivatives catalyzed by CALB and several mutants in order to investigate reaction mechanistic (**Figure 12**) [27]. The influence factors of the reaction were discussed systematically, including solvent, enzyme loading, temperature, and time of reaction. On this basis, dozens of substrates with different structures were conducted to occur aza-Michael addition on the optimized conditions. The results demonstrated that the structures of substrates had a great influence on the activity.

Four different reaction intermediates (Intermediate 1, 2, 3, and 4) were matched with the catalytic activity site of CALB to perform molecular docking simulation (**Figure 13A**). We can see that the binding mode of all the four intermediates with the active site is basically the same. The binding modes of four intermediates with CALB catalytic active sites were analyzed, in order to further study the binding modes of aza-Michael addition intermediates and CALB and the driving forces of their mutual recognition. As shown in **Figure 13b**, it can be seen from the figure

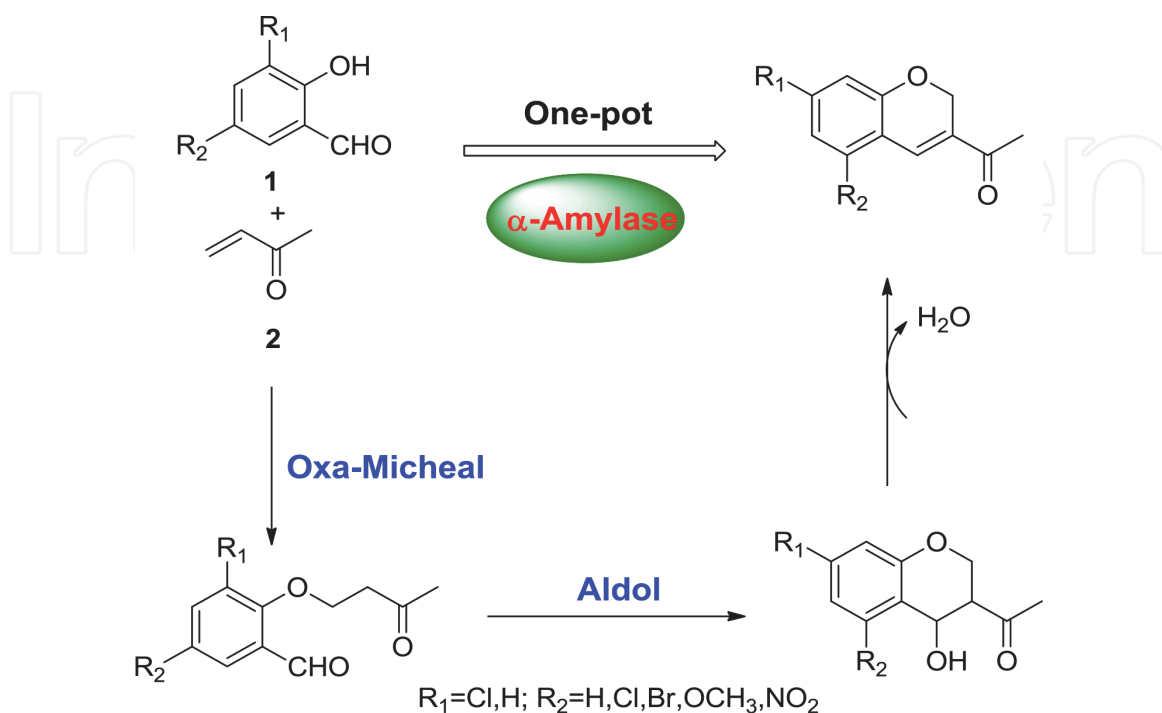


Figure 10.
 α -Amylase-catalyzed synthesis of 3-substituted 2H-chromene derivatives.

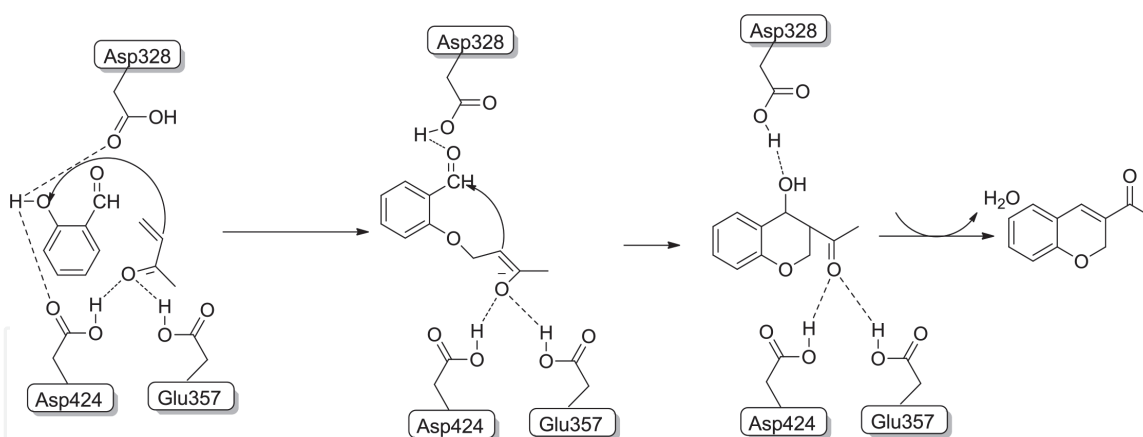


Figure 11.
The proposed mechanism for the α -amylase-catalyzed synthesis of 3-substituted 2H-chromene derivative.

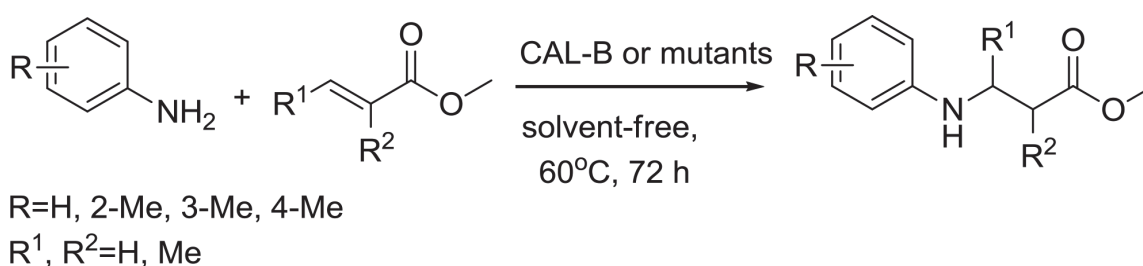


Figure 12.
CALB and mutants catalyzed aza-Michael addition.

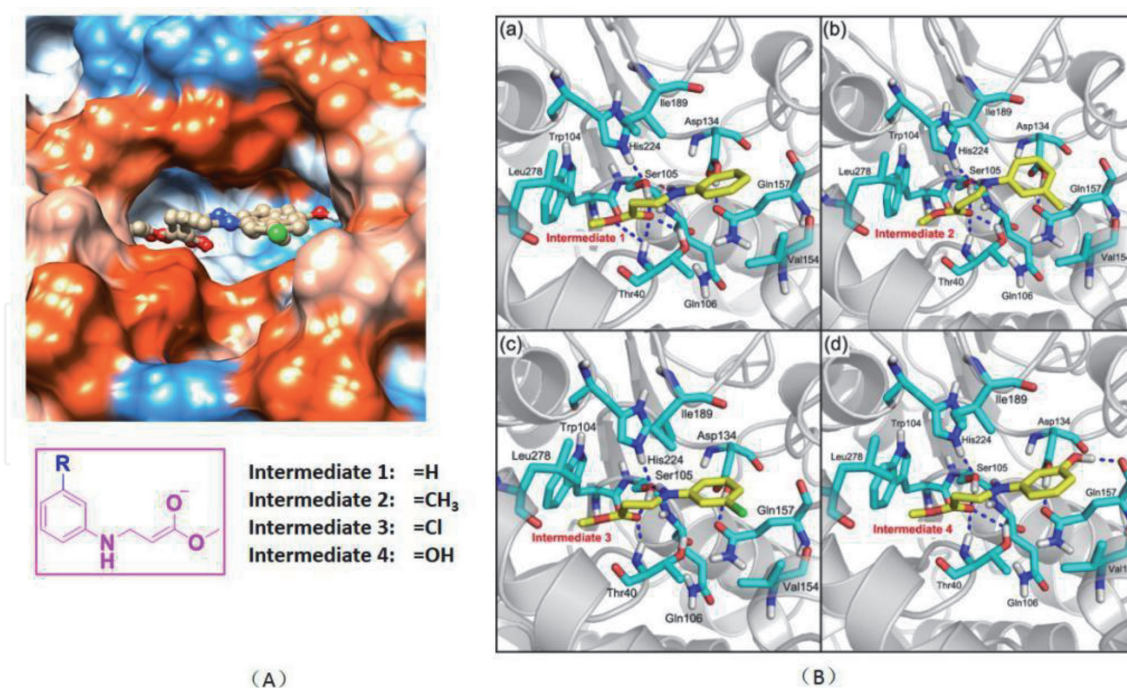


Figure 13.
Molecular docking simulation of CALB with four different reaction intermediates. (A)Hydrophobic matching of the four reaction intermediates with CAL B. The cavity represents the CAL B catalytic pocket which is able to bind and orient the substrates. The blue surface represents hydrophilic while the orange surface represents hydrophobic. The substrates are shown in the pocket in ball-and-stick representation with the atom of substrate coloured according to their atom types (carbon, grey; nitrogen, blue; oxygen, red; chlorine, green). (B)Three-dimensional model of the binding between four aza-Michael addition intermediates (1, 2, 3 and 4) and the CAL B active site. The protein is shown in grey with interacting residues shown as a sky blue stick model. The intermediate is shown as a yellow stick model, and the blue dotted lines indicate the hydrogen bonds between the intermediate and the active site of CAL B.

that the binding modes of the four intermediates with the CALB catalytic chamber are basically the same.

In order to determine the catalytic activity of CALB, three mutants and wild-type CALB were expressed in *E. coli* and were purified to catalyze the aza-Michael addition reaction. The results showed that aza-Michael activity could be dramatically decreased by the mutation of active sites: neither mutant S105 A nor mutant H224 could catalyze the reaction.

However, the mutant I189 A still had a weak catalytic effect on this reaction. Based on these experimental results, the molecular docking was carried out, and the mechanism of aza-Michael addition catalyzed by CALB was studied, and a reasonable reaction mechanism was proposed (**Figure 14**). This helped to explain the effect of substrate structure on the reaction. The substituents of substrates affect the interaction with CALB active sites. Some substituents enhance the binding of substrates and facilitate the reaction. In the whole process, the Ser105 and His224 residues played an important role in proton transfer. Without these two residues, the proton transfer would be blocked, and the aza-Michael addition could not be possible. Besides, the Ile189 residue forms hydrophobic interaction with the benzene ring of the substrate, which makes the substrate more stable in the active cavity.

The biocatalytic thia-Michael reaction is an attractive strategy to develop C—S bond-forming reactions. In 2012, Kieľbasinski and co-workers have reported the use of a number of lipases including PPL, MJL, CALB, and PSL in the addition of benzenethiol to racemic phenyl vinyl sulfoxide or 2-phosphono-2,3-didehydrothiolane S-oxide in organic solvents at room temperature (**Figure 15**) [28]. The addition of piperidine to phenyl vinyl sulfoxide in chloroform is carried out in both enzymatic and non-catalytic processes, while in the former, the reaction rate is 2.5 times faster. Conversely, the conjugate addition of phenylmercaptan with phenyl vinyl sulfoxide is only carried out in the presence of enzymes and ethanol as solvent. In any case, the product is not enantiomerically enriched. However, in the

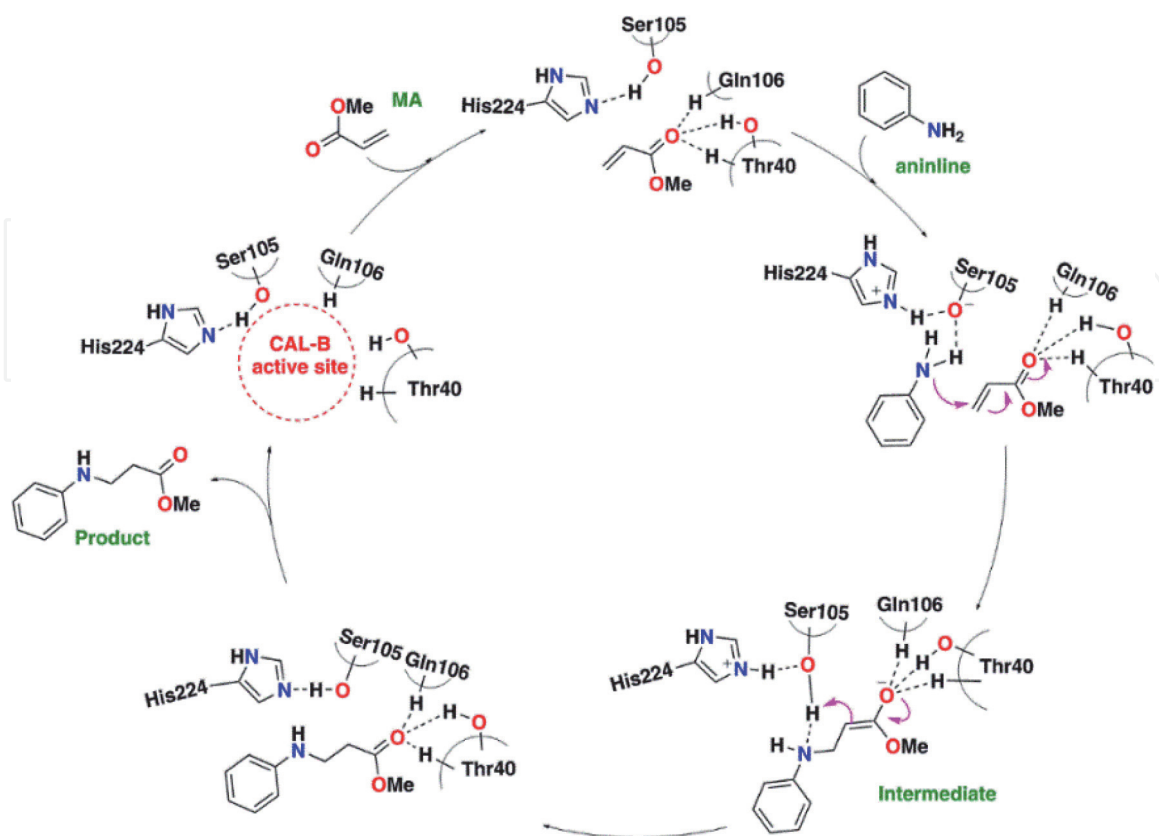
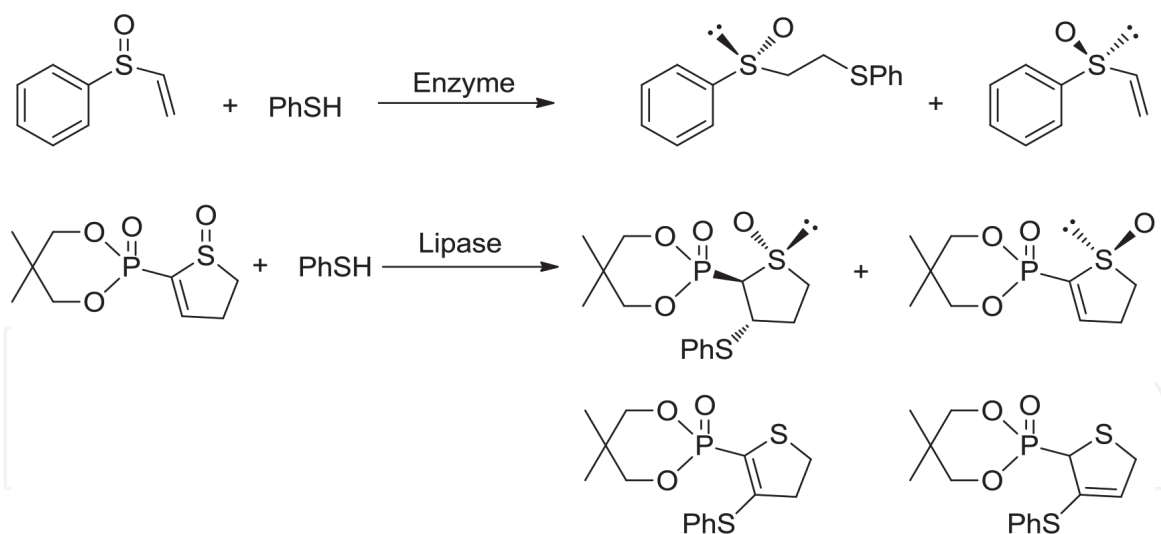


Figure 14.
 Proposed reaction mechanism of aza-Michael addition catalyzed by CALB.

**Figure 15.**

The Michael addition of benzenethiol to racemic phenyl vinyl sulfoxide or 2-phosphono-2,3-didehydrothiolane S-oxide.

presence of various lipases, the addition of phenylmercaptan to a better Michael receptor, cyclic sulfonyl alkylphosphonate, in some cases resulted in up to 25% optical purity of the product and the recovered substrate.

Then, some mechanistic considerations are presented in the studies. The authors proposed sulfoxide oxygen atoms are bound to the “oxygen anion pore” of the enzyme activity site by hydrogen bond. Conversely, histidine catalyzed by binary enhances the nucleophilicity of sulfur centers in phenylmercaptan molecules. Although the interaction of the latter is the same as Michael’s addition of mercaptan to enols, the H-binding of sulfoxide oxygen atom must be different from that of carbonyl oxygen atom, which results in the lower catalytic efficiency of the enzyme for the reaction. It is well known that oxygen anion holes bind to the transition state better than the ground state. When lipase catalyzes ester hydrolysis, the intermediate oxygen anion is tetrahedral. Although the sulfoxide group is tetrahedral, which indicates that the bonding of sulfoxide group should be uniform, compared with the oxygen anion, the sulfoxide group has no negative charge on the intermediate oxygen atom, which significantly reduces the strength of hydrogen bond. In addition, for the Michael addition of nucleophilic reagents, the intermediate oxygen anion is planar, which reduces the space requirement and makes it more suitable for the oxygen anion pore than the tetrahedral sulfoxide intermediate (**Figure 16**).

In 2014, Domingues and co-workers firstly reported the reaction between cinnamaldehyde and thiophenol. Several hydrolases such as PPL, lipozyme, chymosin, and papain have demonstrated different levels of activities, and PPL has found application on the multigram scale (**Figure 17**) [29]. These reactions were carried out at room temperature, and good or excellent sulfur Michael adducts were obtained. The scheme describes the use of EtOH as a solvent and fewer enzymes. The chymosin and papain were used as biocatalysts for organic reactions for the first time.

2.2 Carbon-carbon bond formation Michael addition

C—C bond-forming reactions are one of the mainstays of organic chemistry. In this field the hydrolase-catalyzed Michael reaction also has numerous applications in synthetic chemistry.

In 2011, the asymmetric C—C Michael addition catalyzed by lipozyme TLIM (immobilized lipase from *Thermomyces lanuginosus*) in organic medium in the presence of water was reported for the first time by Guan et al. The biocatalytic reaction

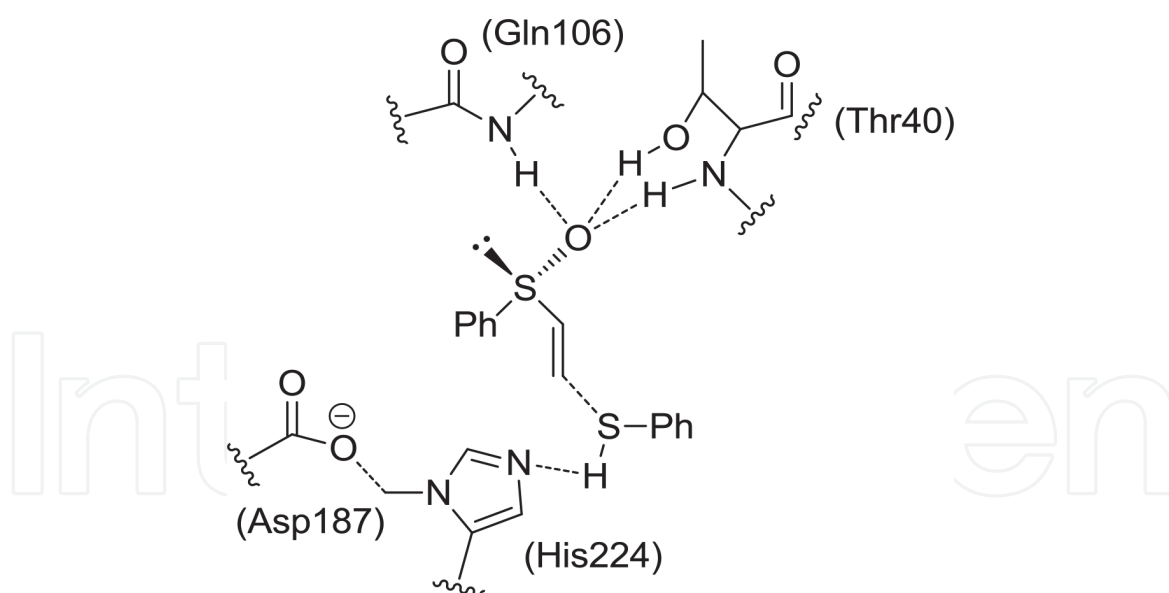


Figure 16.
 Assumed mechanism of the conjugate addition of benzenethiol to racemic phenyl vinyl sulfoxide.

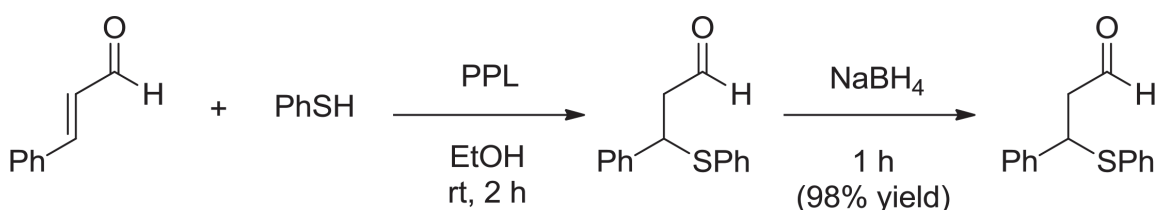


Figure 17.
 PPL-catalyzed C—S bond-forming reaction between cinnamaldehyde and thiophenol.

is suitable for adding large amounts of 1,3-dicarbonyl compounds and cyclohexanone to aromatic and heteroaromatic nitroolefins and cyclohexenone in DMSO in the presence of water under mild reaction conditions (**Figure 18**) [30]. The enantioselectivities up to 83% ee and yields up to 90% were achieved.

Then, the same research group explored porcine pancreatic lipase (PPL) which was used as a biocatalyst to catalyze the Michael addition of 4-hydroxycoumarin to α,β -unsaturated enones in organic medium in the presence of water to synthesize warfarin and derivatives in 2012 (**Figure 19**) [31]. The products were obtained in moderate to high yields (up to 95%) with none or low enantioselectivities (up to 28% ee). The influence of reaction conditions including solvents, temperature, and molar ratio of substrates was systematically investigated. It was the first time warfarin and derivatives were prepared using a biocatalyst.

Sometimes Michael adducts are not the final targeted compounds. We studied lipase-catalyzed Michael addition between nitrostyrene and acetylacetone in DMSO in the presence of water under mild reaction conditions in 2011 [32]. Two possible adducts can be isolated: the routine Michael addition product and cyclic product

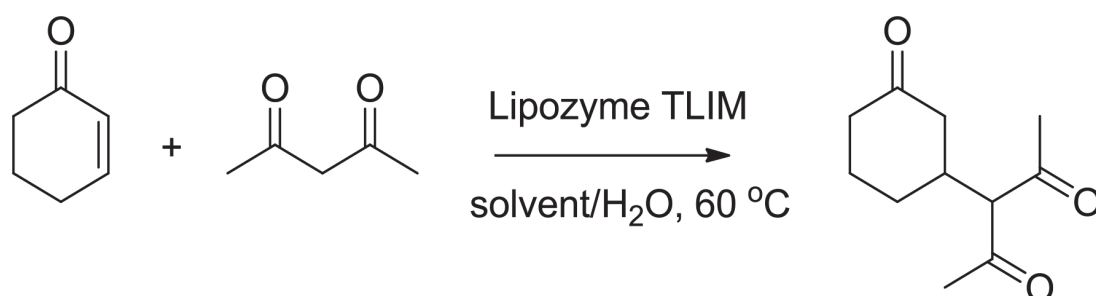
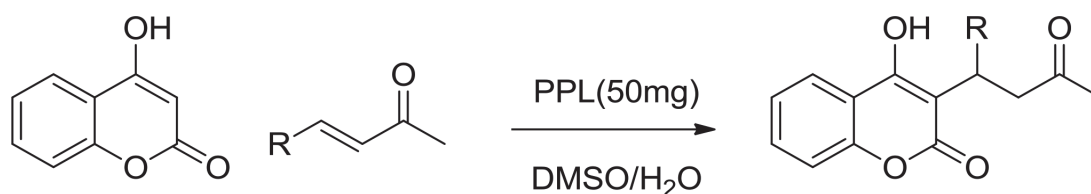
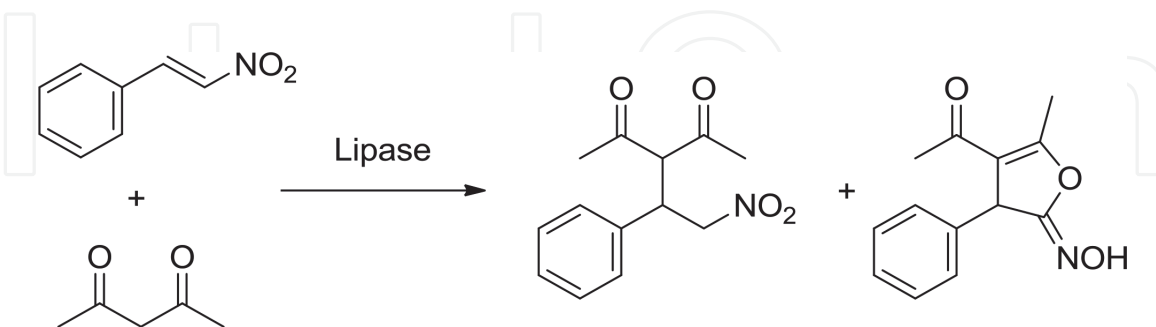


Figure 18.
 Michael reaction of cyclohexenone and acetylacetone catalyzed by lipozyme TLIM in different solvents.

**Figure 19.**

The Michael addition of 4-hydroxycoumarin 1 to α,β -unsaturated enones for the formation of warfarin and derivatives.

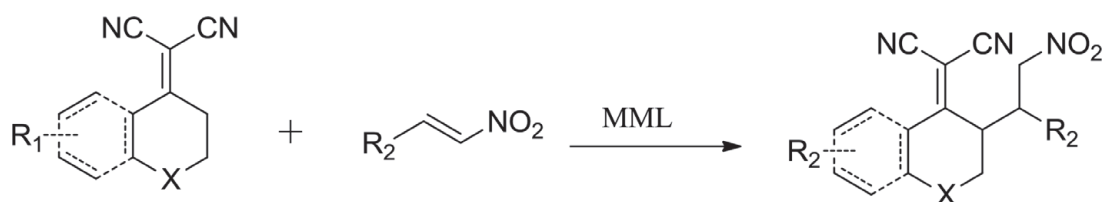
**Figure 20.**

Lipase-catalyzed reaction of nitrostyrene and acetylacetone.

(**Figure 20**). With the aim to get cyclic product in more efficient manner, the catalytic activities of several lipases were firstly tested in mixed ethanol/water solvents. Among the tested lipases, PPL showed the best activity. And according to the single-crystal X-ray diffraction analysis of cyclic products, the reaction was confirmed to give the product oximes with Z-stereoselectivity.

Then, our group reported for the first time lipase-catalyzed direct vinylogous Michael addition reactions of vinyl malononitriles to nitroalkenes (**Figure 21**) [33]. A series of nitroalkenes reacted with vinyl malononitriles to produce the corresponding products with moderate to high yields in the presence of Lipozyme[®] (immobilized lipase from *Mucor miehei*). The excellent diastereoselective products were produced in all reactions in acetonitrile at 30°C for 48 h. The enzyme has only a very slight loss of catalyst efficiency after being reused for seven consecutive cycles of the reaction in the previously determined optimized conditions.

The reaction mechanism was studied by computational simulation approach using dock. Based on the proposed catalytic mechanism of Michael reaction, two docking process of the substrates with the amino acids of the active site were performed. The calculation results explained the experimental results that the lipase possessed specific substrate selectivity. To further elucidate the different catalytic effects of RML, CALB, and CRL, structural characteristics of their active site were analyzed, respectively (**Figure 22**). The docking results showed that once vinyl malononitrile was occupied by nitrostyrene, it could not be docked with the active site of CRL. It indicates that the active site is too narrow to bind both two substrates at the same time, so it could not catalyze the direct Michael addition reaction. As for CALB, the active site seems big enough for both nitrostyrene and vinyl malononitrile, but the docking results showed that nitrostyrene blocked proton

**Figure 21.**

Lipase-catalyzed direct vinylogous Michael addition reaction.

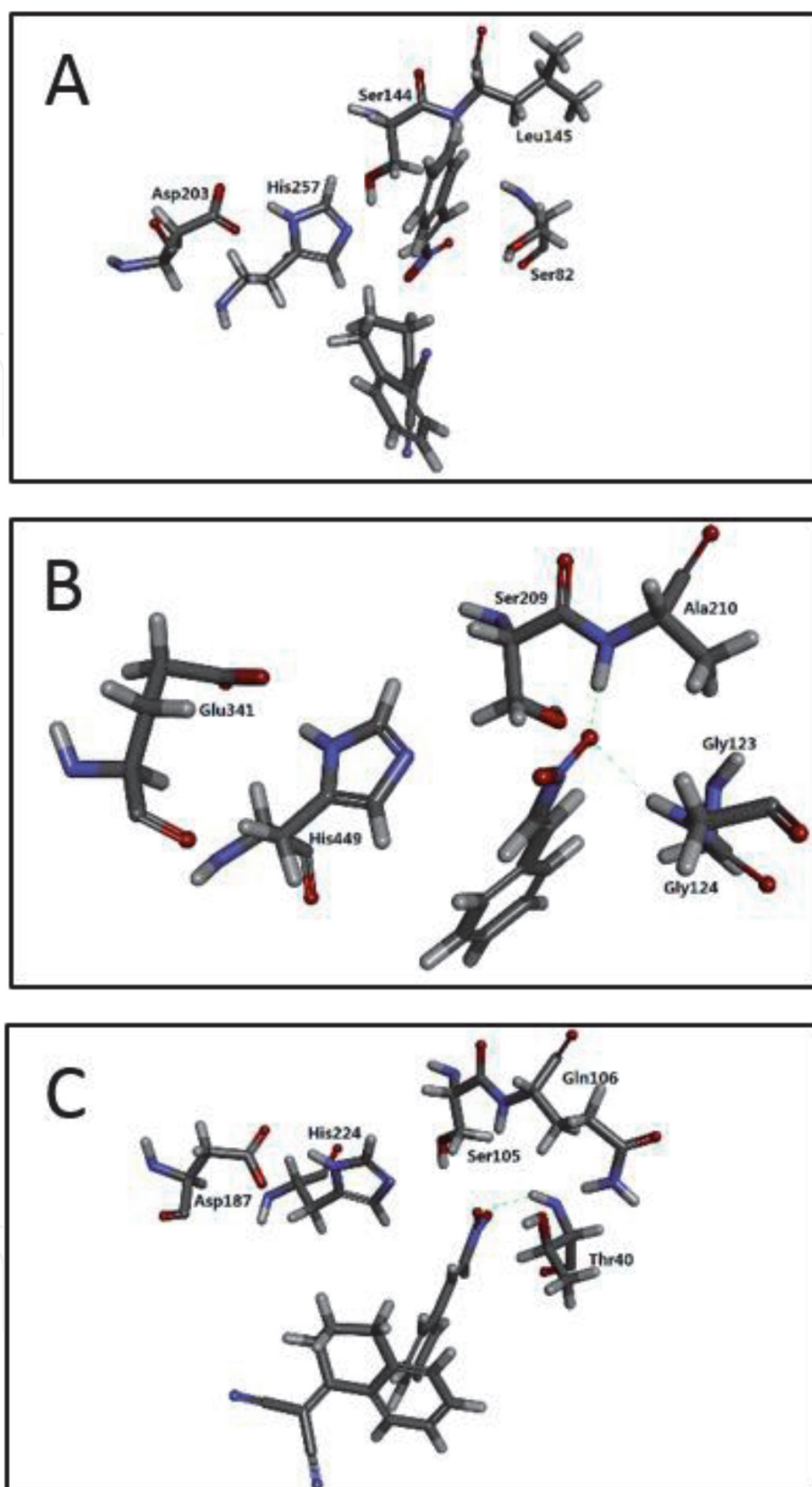


Figure 22.
 The comparison of RML, CRL, and CALB: (A) the docking result of RML and substrates, (B) the docking result of CRL and substrates and (C) the docking result of CALB and substrates.

transferring from vinyl malononitrile to histidine, which may make it unable to catalyze the direct vinylogous Michael addition.

Reaction mechanism of the lipase-catalyzed direct vinylogous Michael addition reaction has been proposed (**Figure 23**). First, nitroalkenes bind to oxygen anion pores and were stabilized by three hydrogen bonds with Leu145 and Ser82.

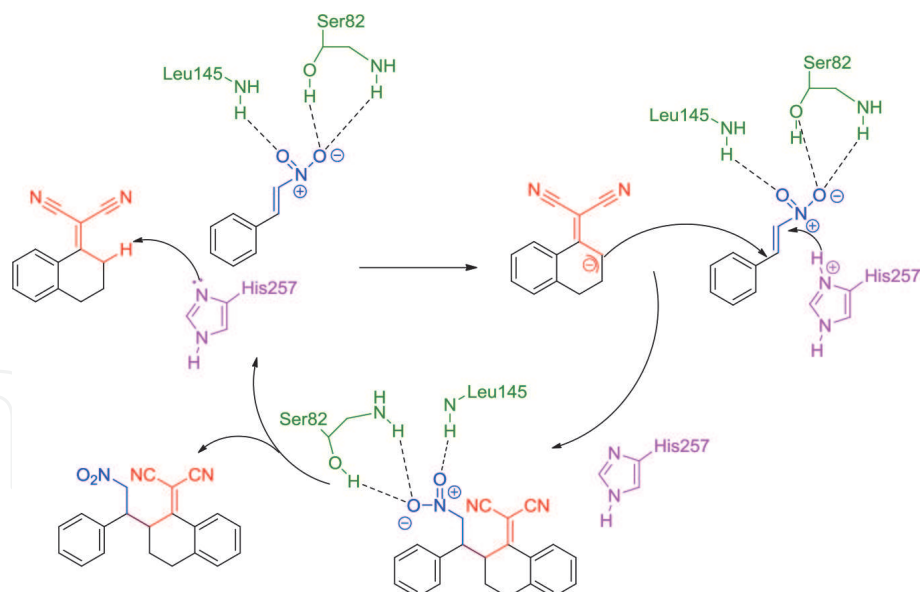


Figure 23.
The proposed mechanism of lipase-catalyzed vinylogous Michael addition.

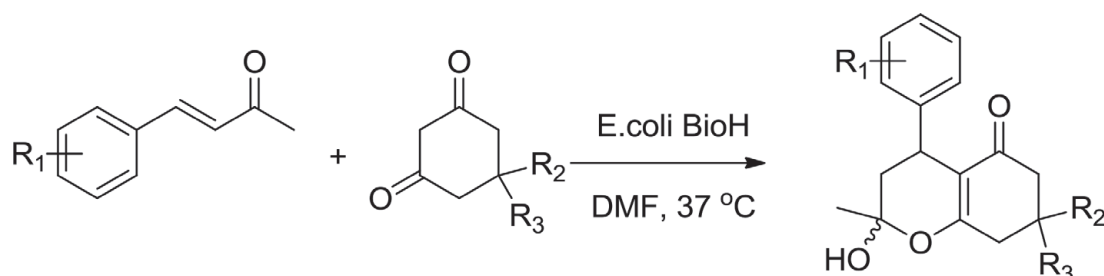


Figure 24.
BioH esterase-catalyzed Michael addition-cyclization cascade reaction.

The protons were then transferred from vinyl malononitrile to His 257 to form a transition state. Subsequently, the protons were transferred from the imidazole group of His 257 to nitroolefins, and the carbon of nitroolefins were attacked by nucleophilic carbon molecules to form products.

In 2014, Ye et al. reported the preparation of 2-hydroxy-2-methyl-4-(4-nitrophenyl)-3,4,7,8-tetrahydro-2H-chromen-5(6H)-one by Michael addition-cyclization cascade reaction of p-nitrobenzalacetone with 1,3-cyclohexanedione in anhydrous media, and control experiments were conducted (**Figure 24**) [34]. The high yield was observed with *Escherichia coli* BioH esterase in DMF at 37°C. In order to preliminarily explore the mechanism of the reaction, site-directed mutagenesis was performed on the hydrolysis catalytic triad of BioH, and the results indicated “alternate-site enzyme promiscuity.” Using a series of substituted phenylacetone and 1,3-cyclodiketone as reactants, the yield could reach 76.3%.

3. Aldol reaction

The aldol reaction has long been recognized as one of the most useful tools for organic chemists. The ability to form carbon-carbon bonds can generate a broad range of both natural and novel poly-hydroxylated compounds. Thus, it is the most important and valuable reaction for the preparation of pharmaceuticals, fine chemicals, and natural products. Aldolases have evolved to catalyze the metabolism and catabolism of highly oxygenated metabolites and are found in many biosynthetic pathways of

carbohydrates, keto acids, and some amino acids [35]. Aldolases bind their respective donor substrates with high specificity and generally will not accept any other donors, even if their structures are similar to the natural donor. The advantages of using aldolases are very high stereospecificity and environmentally benign reaction conditions [36]. However, the limited number of substrates as well as the high cost of these biocatalysts has led researchers to consider other more stable enzymes [37].

In 2003, Berglund and co-workers firstly reported the serine hydrolase *Candida antarctica* lipase B to have catalytic activity for aldol reactions [38]. Our group reported the first lipase-catalyzed asymmetric aldol reaction in 2008 [39]. However, these aldol reactions in earlier studies involving hydrolases just showed moderate activities and selectivities; some more efficient promiscuous aldol reaction have been researched and presented in the last decade.

3.1 Aldol reaction

In 2012, Guan et al. firstly demonstrated that lipase from porcine pancreas, type II (PPL II), has been observed to catalyze the direct asymmetric aldol reaction of heterocyclic ketones with aromatic aldehydes at 30°C in CH₃CN/H₂O (**Figure 25**) [40]. PPL II has good catalytic activity and good adaptability to different substrates. Its enantioselectivity can reach 87% ee and enantioselectivity 83:17 (anti/syn). Then PPL II has aldolase function in organic solvents.

In the same year, the same group also reported the similar asymmetric aldol reaction of aromatic and heteroaromatic aldehydes with cyclic and acyclic ketones in acetonitrile in the presence of a phosphate buffer by chymopapain, which is a cysteine proteinase isolated from the latex of the unripe fruits of *Carica papaya* [41]. Chymopapain exhibited the best catalytic activity and moderate stereoselectivity in DMSO, and the enzyme showed the best enantioselectivity of 79% ee in CH₂Cl₂ with low diastereoselectivity (**Figure 26**). In consideration of both diastereo- and enantioselectivities, the group chose MeCN as a suitable solvent for the asymmetric direct aldol reaction, which gave the best dr of 77:23 and a moderate ee of 76% among the tested solvents. Then, in order to further optimize the direct asymmetric aldol reaction catalyzed by papain, the effects of water content,

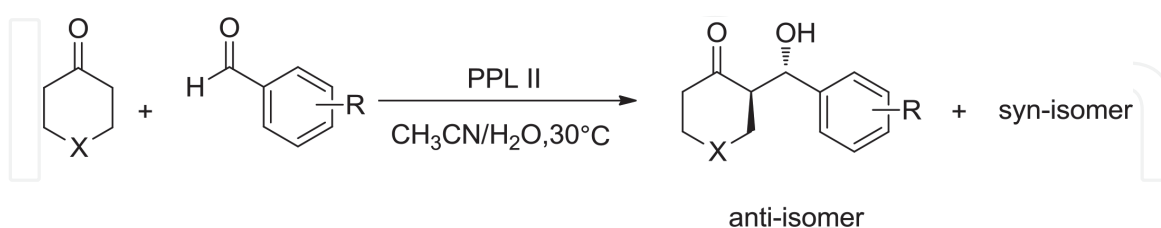


Figure 25.
 Lipase-catalyzed direct asymmetric aldol reaction of heterocyclic ketones with aromatic aldehydes.

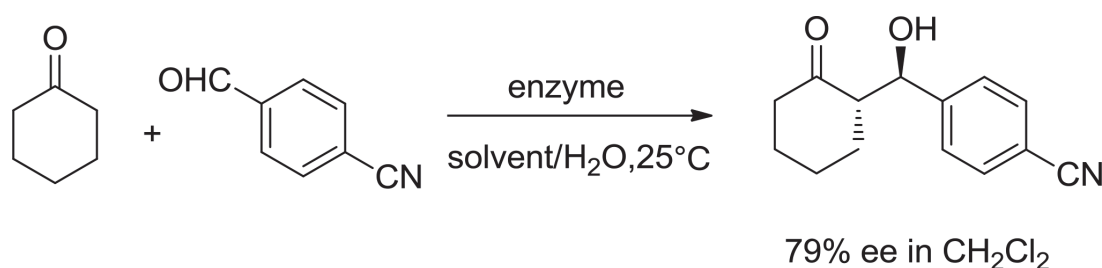


Figure 26.
 The asymmetric aldol reaction of 4-cyanobenzaldehyde and cyclohexanone.

reaction temperature, and the amount of buffer on the enzymatic reaction were investigated. The reaction of 4-cyanobenzaldehyde with cyclohexanone was used as a model reaction.

The authors proposed a mechanism for the chymopapain-catalyzed aldol reaction (**Figure 27**). The catalytic triad of Cys, His, and Asn formed the active site of chymopapain. Firstly, the carbonyl of the substrate ketone is coordinated in the oxygen anion pore of Asn-His binary and active center. Secondly, a proton is transferred from the ketone to the His residue to form enolate ion. Thirdly, another substrate aldehyde accepts the proton from imidazolium cation and forms a new carbon-carbon bond with ketones. Finally, the product is released from the oxyanion hole and separates from the active site.

In 2013, our group firstly reported the asymmetric aldol reaction between aromatic aldehydes and cyclic ketones by PPL (**Figure 28**) [42]. The results showed that a small amount of water could promote the catalytic activity of PPL at 37°C. A wide range of aromatic aldehydes reacted with cyclic ketones to provide the corresponding aldol products with high yields (up to 99%) and moderate to good stereoselectivity (up to 90% ee and 99:1 dr).

In the same year, a simple and convenient synthesis route of series α,β -unsaturated aldehydes was formed by combining the two catalytic activities of the same enzyme with the one-pot method of aldehyde-alcohol reaction and in situ acetaldehyde formation (**Figure 29**) [43]. Lipase from *Mucor miehei* has conventional and promiscuous catalytic activities for the hydrolysis of vinyl acetate and aldol condensation with in situ-formed acetaldehyde.

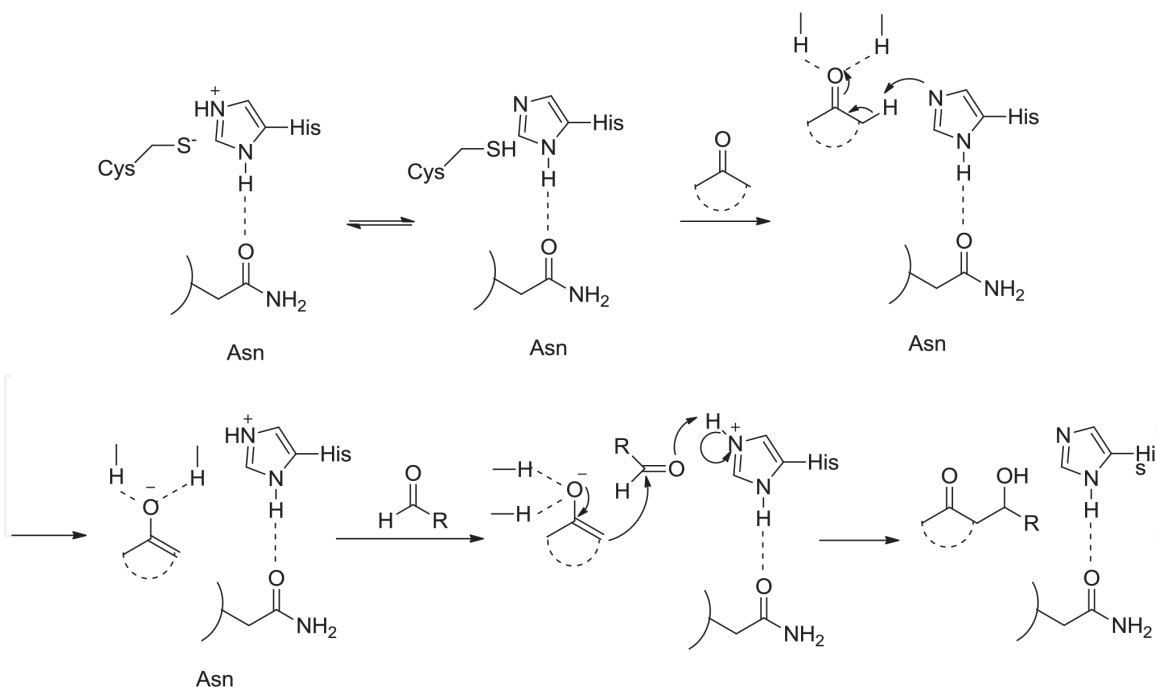


Figure 27.
Proposed mechanism for the chymopapain-catalyzed aldol reaction.

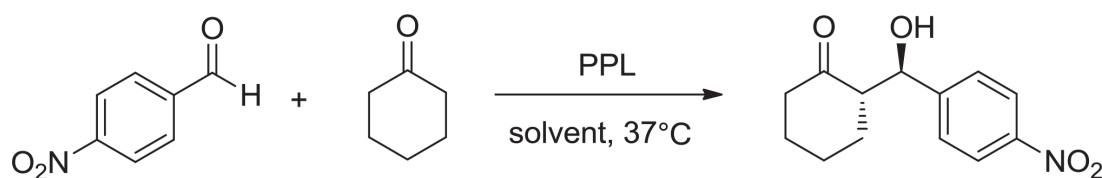


Figure 28.
The asymmetric aldol reaction between aromatic aldehydes and cyclic ketones by PPL.

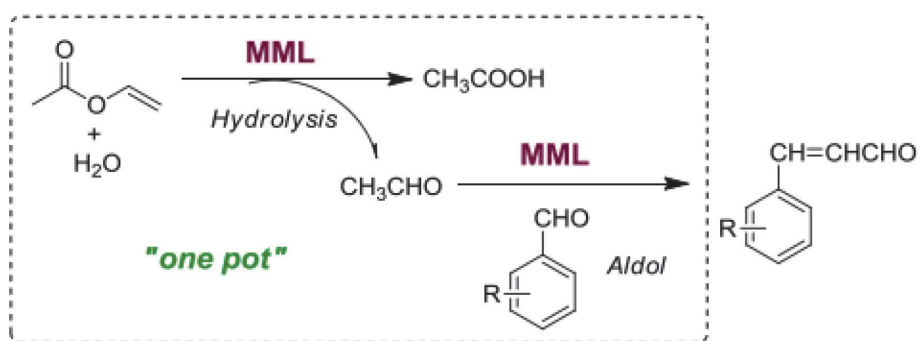


Figure 29.
 MML-catalyzed aldol condensation using an in situ-generated acetaldehyde.

In 2014, Majumder and Gupta found that the properties of lipase-catalyzed reaction products of acetylacetone with 4-nitrobenzaldehyde depend on the source of lipase and reaction medium (**Figure 30**) [44]. *Mucor javanicus* lipase was found to give 70% aldol and 80% enantiomeric excess in anhydrous t-amyl alcohol.

Gao and Guo et al. demonstrated the catalytic promiscuity of an acyl-peptide releasing enzyme from *Sulfolobus tokodaii* (ST0779) for aldol addition reaction for the first time, and accelerated activity was observed at elevated temperature (**Figure 31**) [45]. The turnover number k_{cat} (s^{-1}) of this thermostable enzyme at 55°C is 7.78-fold higher than that of PPL at its optimum temperature of 37°C, and the molecular catalytic efficiency k_{cat}/K_m ($M^{-1} s^{-1}$) adds up to 140 times higher than PPL.

The authors proposed a mechanism for the ST0779-catalyzed aldol reaction between acetone and 4-nitrobenzaldehyde (**Figure 32**). Based on the structure simulation of ST0779, the aldol reaction catalyzed by ST0779 with acetone and p-nitrobenzaldehyde as model reaction was proposed. Because of its thermodynamic superiority and high affinity, acetone first enters the active site and then is accommodated by the active site residues Ser439 and His 555. Proton transfer forms a transition state of enol salts, which is stable by Ser439. Asp523 is involved in stabilizing the positive charge of His 555-protonated imidazole ring. In the next

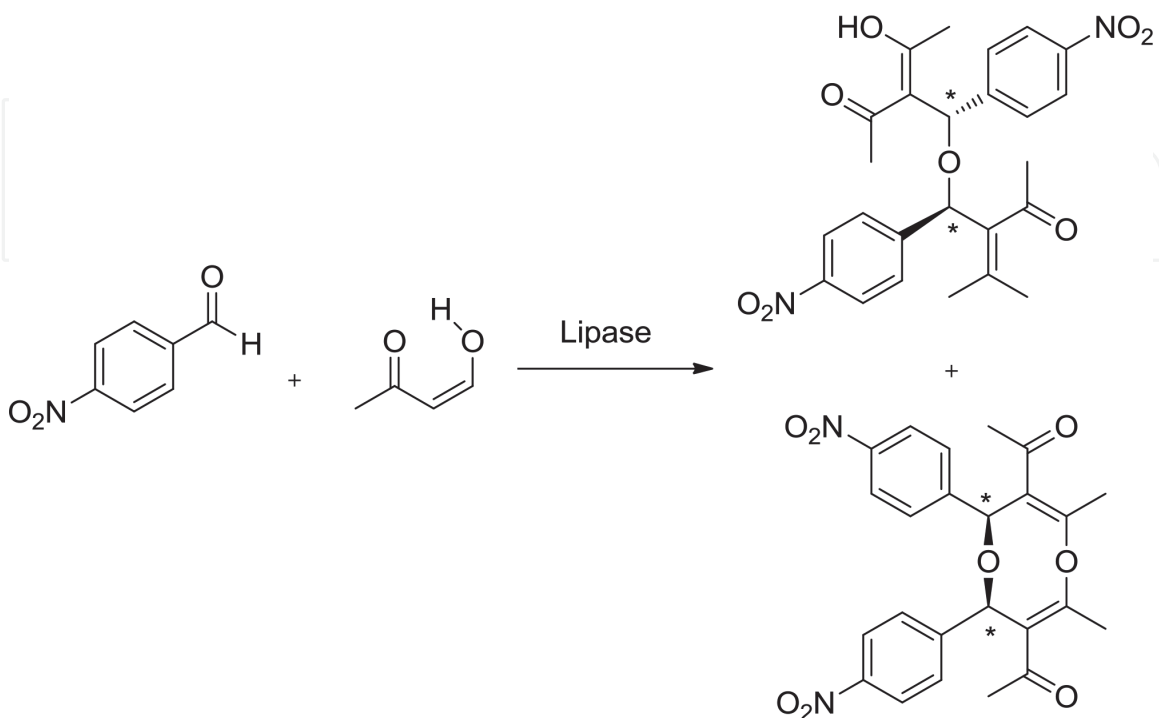
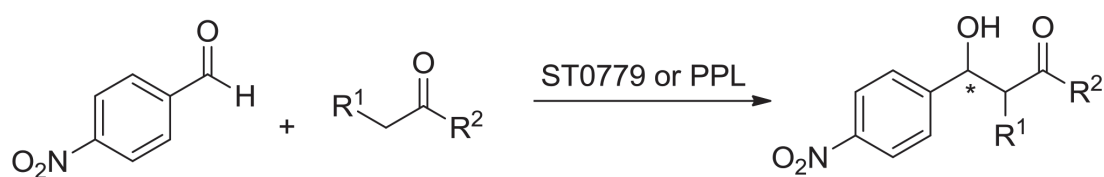


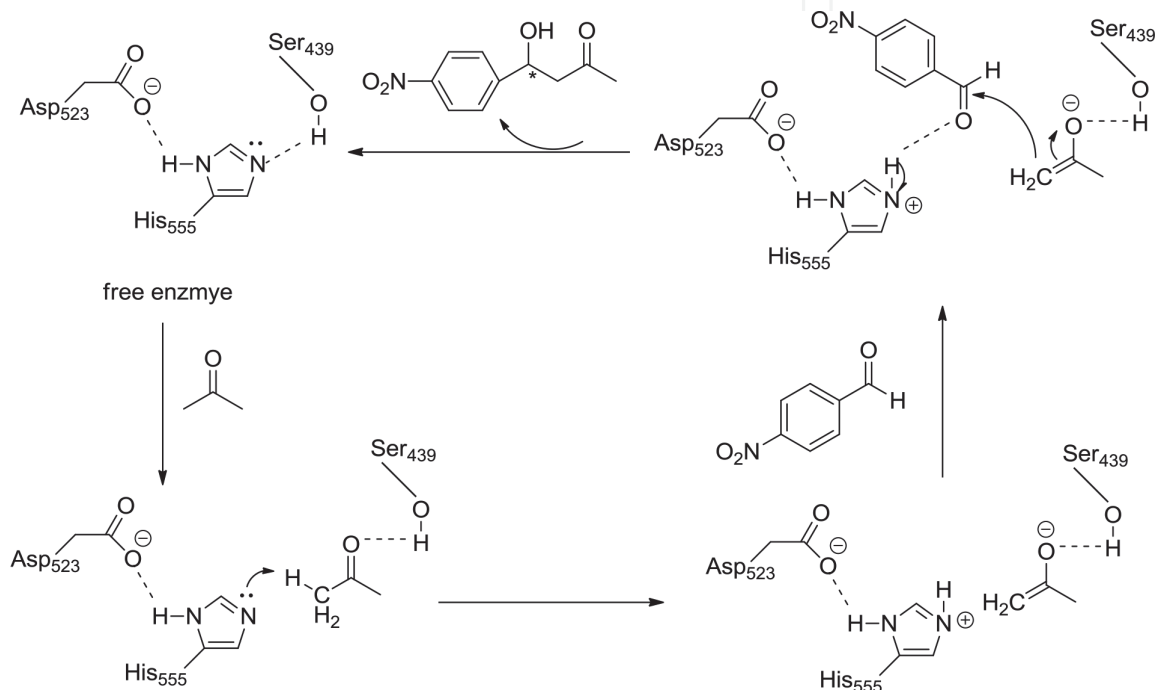
Figure 30.
 The lipase-catalyzed promiscuous reaction: formation of acyclic and cyclic 2:2 adducts.



1. $\text{R}^1=\text{H}$, $\text{R}^2=\text{CH}_3$ (acetone)
2. $\text{R}^1=\text{CH}_3$, $\text{R}^2=\text{CH}_3$ (2-Butanone)
3. $\text{R}^1\text{-R}^2=-\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2-$ (cyclohexanone)

Figure 31.

Aldol reactions catalyzed by ST0779 or PPL.

**Figure 32.**

Proposed mechanism for ST0779-catalyzed aldol reaction between acetone and 4-nitrobenzaldehyde.

step, the oxygen of carbonyl group in 4-nitrobenzaldehyde is protonated by protons from His 555 imidazole ring, and the carbon atoms in the same carbonyl group are neutrally attacked by oleic acid carbon to form a new C—C bond. Finally the aldol product is released from the enzyme, and the enzyme is freed for a new reaction.

In 2016, Wu et al. demonstrated a one-pot bienzymatic cascade in organic media to synthesize chiral β -hydroxy ketones for the first time (**Figure 33**) [46]. The decarboxylative aldol reaction catalyzed by an immobilized lipase from *Mucor miehei* (MML) and the synthesis of β -hydroxy ketone catalyzed by a lipase A or B from *Candida antarctica* (CALA or CALB) were combined in this one-pot protocol, reducing the purification step between the two reactions. (S)- β -hydroxy ketones and acylated (R)- β -hydroxy ketones could be obtained under mild reaction condition. The ee values of most chiral compounds were in a range of 94–99%, while the total yields of both chiral products were all above 85%. This enzymatic one-pot chain method is still very effective, not only can it be amplified to the level of grams but also the catalyst was recovered three times.

In 2019, Gao et al. demonstrated the construction of an unencapsulated remote-controlled nanobiocatalytic system. The system used three enzyme-conjugated gold nanorod composites (EGCs) to control reaction rates in real time by self-assembling

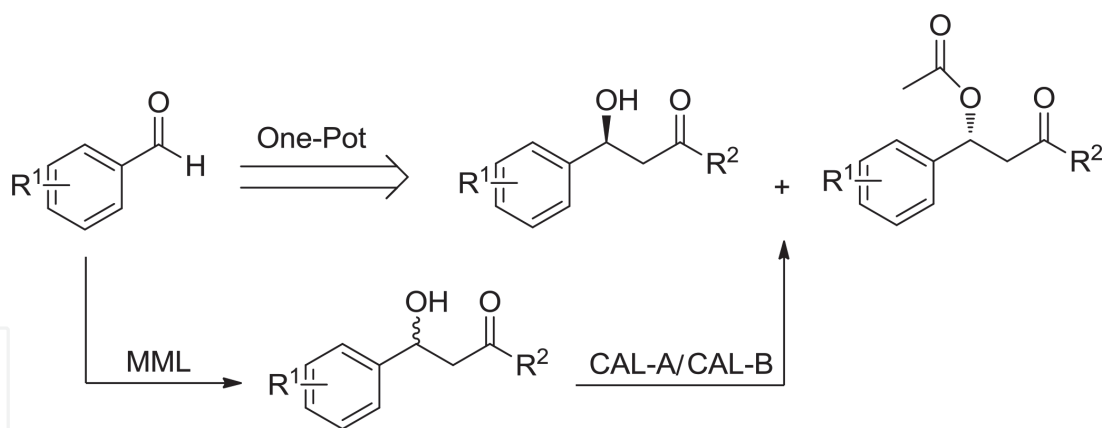


Figure 33.
 One-pot cascade for synthesis of chiral β -hydroxy ketone derivatives.

enzymes formed by the combination of enzymes at different optimal temperatures and gold nanorods (GNRs) [47]. By using the photothermal effect of GRS to transfer energy quickly, coupled with the real-time and long-range regulation of enzyme activity, improving the thermal stability of the enzyme and effective catalysis of the aldol reaction can be achieved. The increase in energy inside GRS, stimulated by distant near-infrared (NIR) stimuli, leads to increased enzyme activity. The results show that the method of internal heating that transfers energy more directly to the enzyme-catalyzed site is a faster and more effective energy transfer method. The results also show that the catalytic effect of the remote-controlled nanocatalytic system at lower temperature is the same as that of the free enzyme at higher temperature, but it has the advantages of improving the stability of the enzyme and extending its service life. Specifically, PPL EGCs at room temperature exhibit the same catalytic effect as achieved by free PPL at 40°C, while ST0779EGCs and APE1547 EGCs at 33°C exhibit a higher catalytic effect than their corresponding free enzymes at 63°C. In addition, EGCs have superior catalytic efficiency and product yield compared with aldol addition in free enzyme systems.

3.2 Henry (nitroaldol) reaction

The nitroaldol or Henry reaction is one of the most useful carbon-carbon bond-forming reactions and has wide synthetic applications in organic chemistry. This reaction provides access to valuable racemic and optically active β -nitro alcohols, which are very useful in organic synthesis as precursors for pharmaceutical and biological purposes. In recent years, efficient nonconventional biocatalytic approaches have been reported [48].

Guan et al. firstly reported transglutaminase was used to catalyze Henry reactions of aliphatic, aromatic, and heteroaromatic aldehydes with nitroalkanes (**Figure 34**) [49]. The reactions were carried out at room temperature, and the corresponding nitroalcohols were obtained in yields up to 96%.

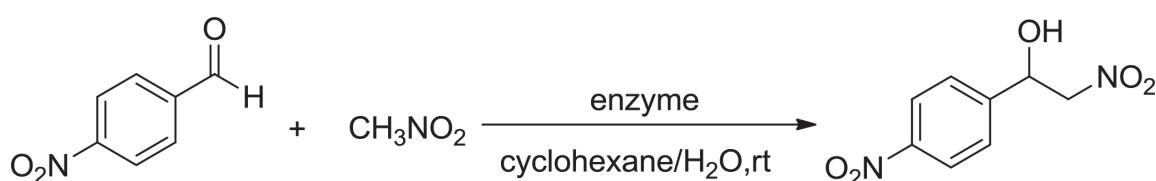


Figure 34.
 Enzyme-catalyzed Henry reaction of 4-nitrobenzaldehyde and nitromethane.

Then, the same group reported glucoamylase from *Aspergillus niger* (AnGA) catalyze Henry reactions of aromatic aldehydes and nitroalkanes in 2013 [50]. The reactions were carried out at 30°C in the mixed solvents of ethanol and water, and the corresponding β -nitro alcohols were obtained in yields of up to 99%. Experiments demonstrated that AnGA could be inhibited by the product of the Henry reaction at 80°C. This enzymatic Henry reaction has a broad substrate scope and could be facily enlarged to gram scale. Based on the experiments with denatured and inhibited AnGA, and the comparison of natural activity and promiscuous activity, the possible mechanism was also discussed (**Figure 35**). Glu400, as a base, deprotonates the α -carbon of the nitroalkane providing intermediate I. At the same time, Glu179, as an acid, donates a proton to the carbonyl oxygen of the aldehyde generating intermediate II. Then, the α -carbon of I, as a nucleophile, attacks the carbonyl of II forming a new carbon-carbon bond. Finally, the product (β -nitro alcohol) is released from the active site.

On the other hand, Lin and co-workers demonstrated the Henry reaction can also be catalyzed in a neat organic solvent. When using the D-aminoacylase from *Escherichia coli* as the promiscuous biocatalyst, DMSO was found to be the best solvent at 50°C [51]. 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 (**Figure 36**) [52]. Both alcohols and acetates were isolated in good yields and high enantiomeric excess (>84% ee_s; >96% ee_p; E > 150).

In 2013, lipase A from *Aspergillus niger* was used in the Henry reaction between aromatic aldehydes and a large excess of nitroalkanes in an organic/water medium (**Figure 37**) [53]. The yield of corresponding β -nitro alcohols at 30°C reached 94%.

Gotor and co-workers reported the inexpensive carrier protein bovine serum albumin (BSA) as catalyst was firstly used in the condensation of an appropriate aldehyde with 1-nitroalkanes in aqueous media (**Figure 38**) [54]. By optimizing the reaction conditions, the yield of corresponding nitroalcohols at 30°C reached 91%.

Similarly, two other well-known lipases, *Pseudomonas cepacia* lipase and CALB, were found to catalyze the Henry reaction [55]. Nevertheless, spectroscopic experiments showed that the immobilization protocols contribute to the change in the secondary structure of the enzyme, which leads to improved conversion rates.

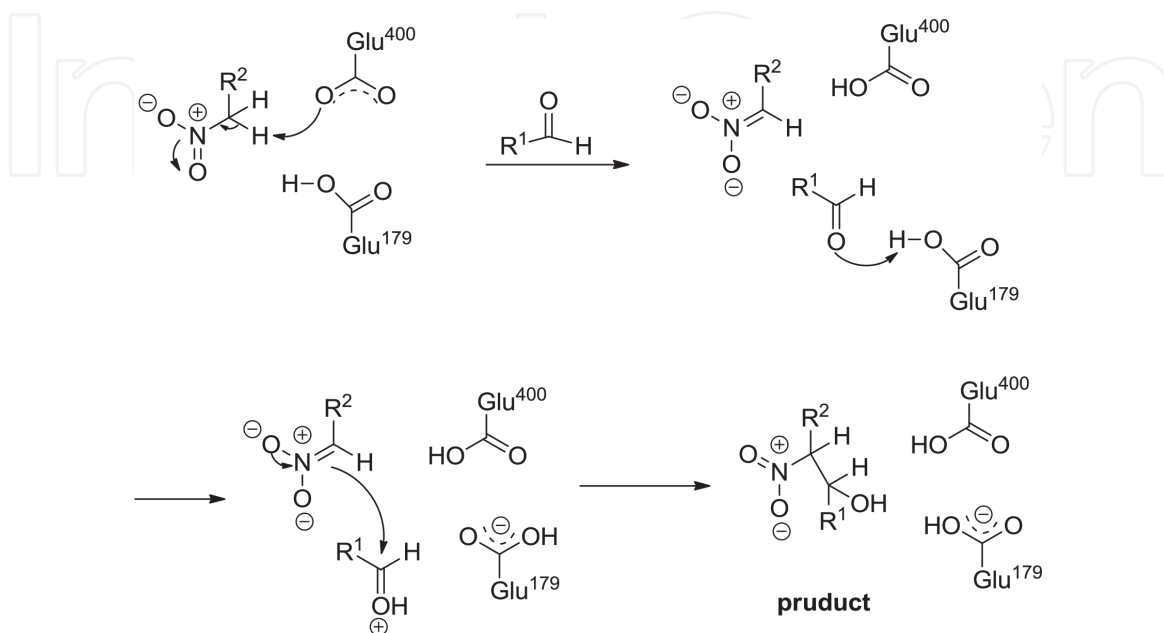


Figure 35.
Possible mechanism of the AnGA-catalyzed Henry reaction.

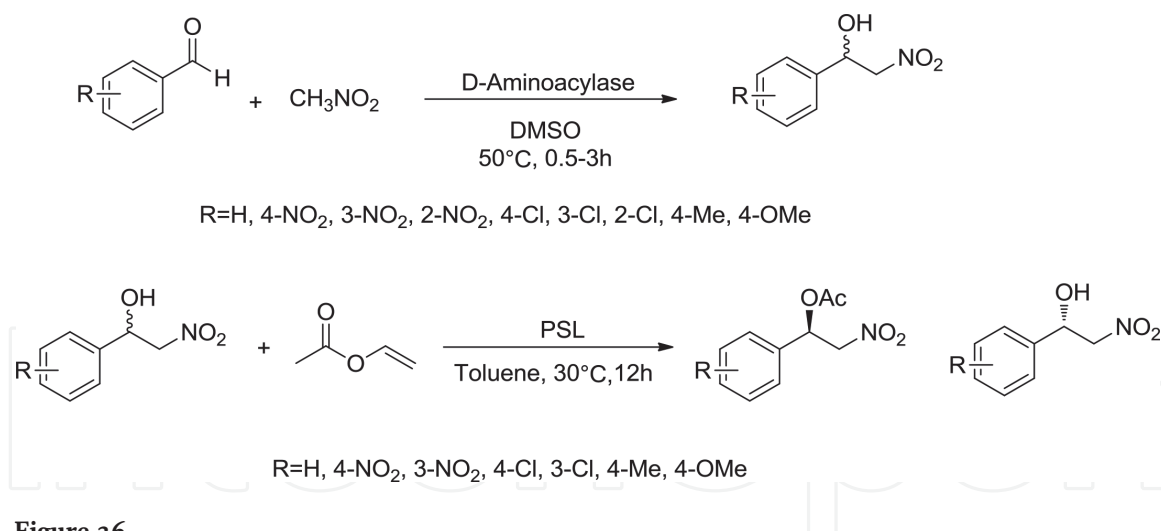


Figure 36.

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

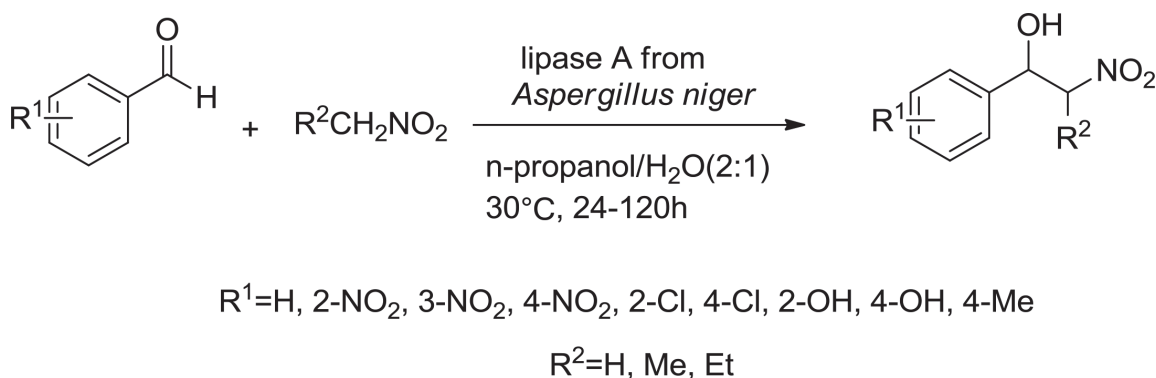


Figure 37.

Nitroaldol reaction between aromatic aldehydes and nitroalkanes catalyzed by lipase A from *Aspergillus niger*.

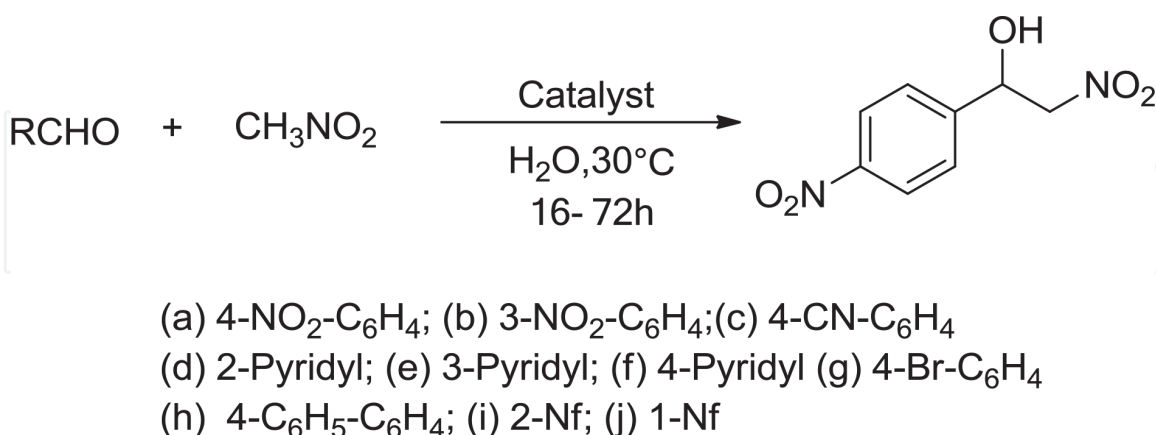


Figure 38.

Catalytic nitroaldol addition between different aromatic aldehydes and nitromethane.

3.3 Aldol (nitroaldol) reaction in untraditional solvent

Solvents for a biocatalysis reaction have experienced several generations of development. Traditional organic solvents (water miscible or water immiscible), in the form of cosolvents or second phase, can provide solutions for the above-described challenges. However, organic solvents inevitably face their own

disadvantages, such as high volatility, difficulty in preparation, and inhibition of the activity of biocatalysts. So the untraditional solvents such as buffer solvent, ionic liquids (ILs), and deep eutectic solvents (DESs) have attracted the interest of many groups.

In 2013, our group demonstrated bovine pancreatic lipase (BPL) was first used to catalyze the aldol reaction and acidic buffer was first used for promiscuous enzymatic aldol reaction (**Figure 39**) [56]. The highest yield (99.0%), the best dr of 96:4, and a moderate ee of 66% were observed with aromatic aldehyde and ketone by BPL in phosphate-citrate buffer (pH 5.6, 5.0 mL) at 30°C.

Porto et al. demonstrated the lipase from *Rhizopus niveus* (RNL) catalyzed by unspecific protein catalysis the aldol reactions between cyclohexanone and aromatic aldehydes in organic solvents with water or aqueous buffer solution (**Figure 40**) [57]. The reactional conditions strongly influenced the yield (0–99%) and enantioselectivities in the anti-products (6–55% ee). The aldol products with enantioselectivities in the anti-product were observed for inactive enzyme and in denaturing conditions. Therefore, the reactions in the evaluated conditions were proceeded by unspecific protein catalysis with moderate enantioselectivities and not by promiscuous activity.

Ionic liquids are the first enzyme-compatible untraditional media developed by the green and sustainable concept (given their low vapor pressure). Numerous reactions, e.g., hydrolytic and redox reactions as well as formation of C—C bond, have been successfully performed in such ILs-containing media. We demonstrated PPL was used to catalyze asymmetric cross aldol reactions of aromatic and heteroaromatic aldehydes with various ketones in ionic liquid ([BMIM][PF₆]) for the first time in 2014 (**Figure 41**) [58]. PPL exhibited high catalytic activity and

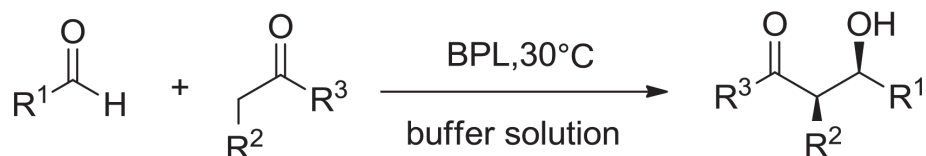


Figure 39.
The BPL-catalyzed asymmetric aldol reaction in buffer solution.

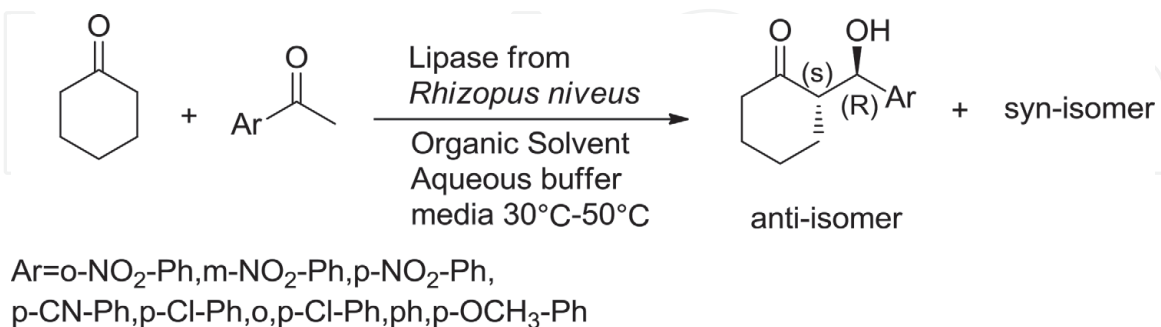


Figure 40.
Aldol reactions by lipase from *Rhizopus niveus*.

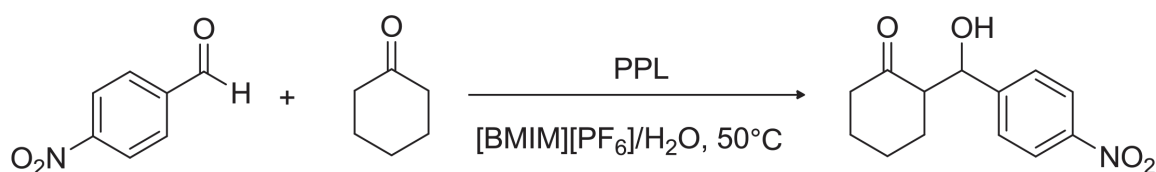


Figure 41.
The PPL-catalyzed asymmetric cross aldol reaction in ionic liquid.

excellent stereoselectivity in this efficient and recyclable room-temperature ionic liquid in the presence of moderate water. High yields of up to 99%, excellent enantioselectivities of up to 90% ee, and good diastereoselectivities of up to >99:1 dr were achieved.

Despite the excellent performance of ILs in biocatalysis, more doubts about their ungreenness and environmental influence have been gradually presented. Deep eutectic solvents, the recognized alternative of ILs, first came to the public vision in 2001. Since then, research on DESs faced a prosperous increase in many fields, such as extraction, materials synthesis and biotransformation, and biocatalysis.

Gotor-Fernández and co-workers reported a promiscuous lipase-catalyzed aldol reaction has been performed for the first time in DESs in 2016. The aldol reaction between 4-nitrobenzaldehyde and acetone was examined in-depth, with excellent compatibility being found between PPL and DESs (choline chloride/glycerol mixtures) for the formation of the aldol product in high yields (**Figure 42**) [59]. The system was compatible with a series of aromatic aldehydes and ketones including acetone, cyclopentanone, and cyclohexanone.

At the same year, Tian et al. explored the Henry reaction catalyzed by lipase AS using deep eutectic solvents as a reaction medium (**Figure 43**) [60]. The studies had shown that adding 30 vol% water to DES could increase the catalytic activity of enzymes. The final yield of the lipase AS-catalyzed Henry reaction was 92.2% in a DES-water mixture within only 4 h. In addition, the lipase AS activity was improved by approximately threefold in a DES-water mixture compared with that in pure water. The methodology was also extended to the aza-Henry reaction. The enantioselectivity of both Henry and aza-Henry reactions was not found.

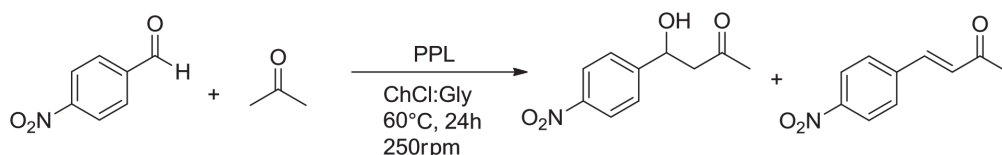
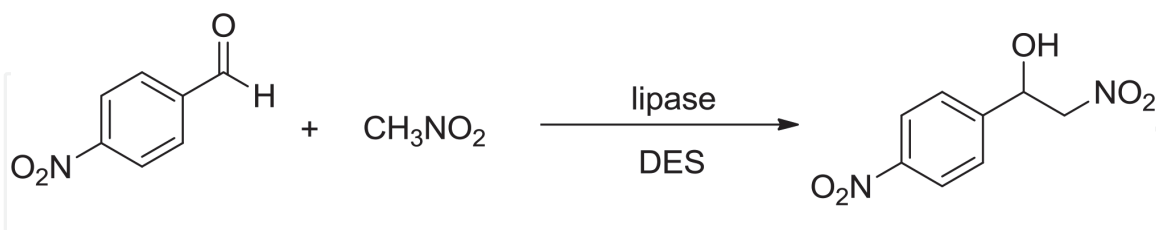


Figure 42.
The PPL-catalyzed asymmetric cross aldol reaction in DESs.



Lipase= lipase from *Aspergillus niger*

DES=ChCl/Gly, ChCl/EG, ChCl/U with different molar ratio

Figure 43.
Lipase AS-catalyzed Henry reaction in DES.

4. Multicomponent reactions (MCRs)

Multicomponent reactions have attracted sustained attention because they represent a powerful tool for the construction of complex molecular structures with evident advantages, such as simplified workup procedures, high overall yields, and versatile product libraries. Recently, hydrolases have allowed the development of

multiple transformations and mainly served for the synthesis of heterocyclic compounds with high complexity in high yields. In this section, we will focus on the hydrolase-catalyzed multicomponent reaction in a one-pot transformation.

4.1 Mannich reaction

The Mannich reaction is a typical and the first example for the hydrolase-catalyzed multicomponent reaction, which is atom-economic and a powerful synthetic method for generating carbon-carbon bonds and nitrogenous compounds. An unprecedented “one-pot,” direct Mannich reaction of ketone, aldehyde, and amine catalyzed by lipase was described first in 2009 [61]. Lipase from *Mucor miehei* (MML) efficiently catalyzed the Mannich reaction (**Figure 44**).

To assess the generality of the lipase-catalyzed Mannich reaction, we extended other substrates such as cyclohexanone, butanone, and 1-hydroxy-2-propanone in more benign reaction system (ethanol/water) catalyzed by the lipase from *Candida rugosa* (CRL) [62]. It was found that a wide range of aromatic aldehydes could effectively participate in the CRL-catalyzed Mannich reaction to give the corresponding β -amino carbonyl compounds (**Figure 45**). The reaction was favored by the electron-withdrawing substituents of the aldehydes.

In 2012, Guan et al. reported the enzyme-catalyzed, direct, three-component asymmetric Mannich reaction using protease type XIV from *Streptomyces griseus* (SGP) in acetonitrile (**Figure 46**) [63]. This characteristic makes it important to develop an enzyme-catalyzed asymmetric Mannich reaction as a more sustainable

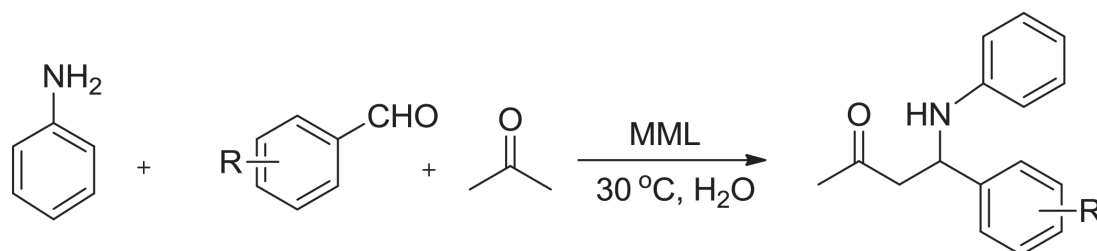


Figure 44.
The first lipase-catalyzed direct Mannich reaction.

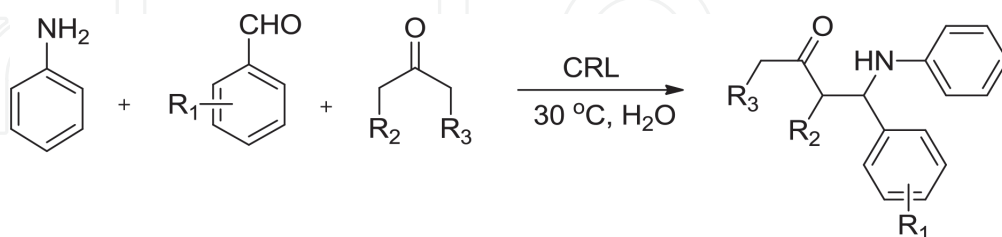


Figure 45.
Lipase-catalyzed direct Mannich reaction of various aryl aldehydes and ketones with aniline.

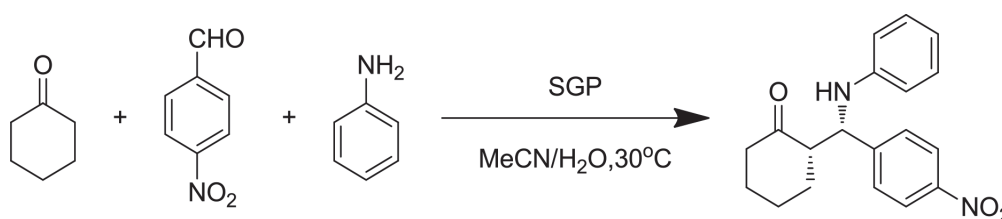


Figure 46.
SPG-catalyzed direct Mannich reaction.

complement to chemical catalysis. The control experiments with the denatured enzyme and non-enzyme proteins indicated that the specific natural fold of SGP was responsible for its stereoselectivity in the Mannich reaction. A wide range of substrates were accepted by the enzyme, and yields of up to 92%, enantioselectivities of up to 88% ee, and diastereoselectivities of up to 92:8 dr were achieved. As an example of enzyme catalytic promiscuity, this work broadens the scope of SGP-catalyzed transformations.

In the same year, Lin et al. inspired by chemical cofactors or mediators expect some small molecules to similarly improve the enzymatic Michael addition of unactivated carbon nucleophiles [64]. They found that the CALB/acetamide co-catalyst system can effectively catalyze the Michael addition between less-activated ketones and aromatic nitroolefins. This is of particular interest because neither CALB nor acetamide can independently catalyze the reaction to any significant extent. The CALB/acetamide catalyst system is also effective for other C—C bond-forming reactions with varying degrees of success, for example, CALB-catalyzed Mannich reaction (**Figure 47**). After adding acetamide as a co-catalyst, the yield increased by 50% (from 25 to 38%). The synergistic catalytic system of the lipase and the small molecule organic catalyst will greatly expand the application prospect of the enzyme in organic synthesis.

After 2 years, Guan et al. reported the use of acylase I from *Aspergillus melleus* in the asymmetric Mannich reaction (**Figure 48**) [65]. Compared to the current chemical technologies, this enzymatic reaction is more environmentally friendly and sustainable by using biocatalysts from inexpensive renewable resources. The activity and stereoselectivity of AMA can be improved by adjusting the solvent, pH, water content, temperature, substrate molar ratio, and enzyme loading. A wide range of substrates can be accepted by AMA, achieving enantioselectivities up to 89% ee, diastereoselectivities up to 90:10 dr, and yields up to 82% in the mixture of MeCN and phosphate buffer pH 8.1 (85:15 v/v 1 mL) at 30°C. This work not only provides new examples of enzyme-catalyzed reliability and potential synthetic methods of organic chemistry but may also help to better understand the metabolic pathways of nitrogen-containing compound biosynthesis.

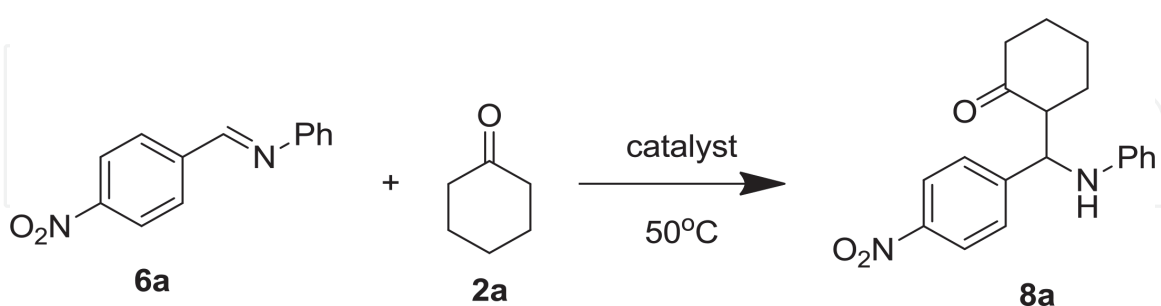


Figure 47.
Mannich reaction of (*E*)-*N*-(4-nitro-benzylidene)aniline with cyclohexanone.

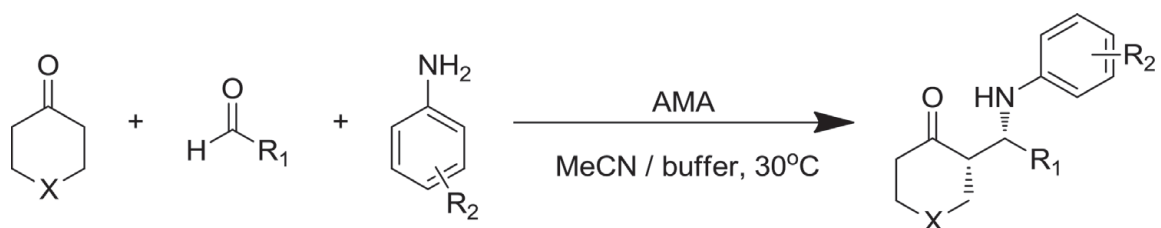


Figure 48.
The AMA-catalyzed Mannich reactions.

4.2 Biginelli reactions

In 2013, Sinha et al. reported bovine serum albumin promoted simple and efficient one-pot procedure for synthesis of 3,4-dihydropyrimidin-2(1H)-ones including potent mitotic kinesin Eg5 inhibitor monastrol under mild reaction conditions (**Figure 49**) [66]. After the reaction conditions are optimized, the yields reached up to 82% in EtOH at 60°C. The catalyst recyclability and gram-scale synthesis have also been demonstrated to enhance the practical utility of process.

Followed by our group, we reported trypsin as a multifunctional catalyst for synthesis of 3,4-dihydropyrimidin-2(1H)-ones by the Biginelli reaction of urea, β -dicarbonyl compounds, and in situ-formed acetaldehyde (**Figure 50**) [67]. This one-pot multistep reaction consists of two relatively independent reactions, both of which are catalyzed by trypsin. First is the transesterification of ethyl acetate and isobutanol at 60°C to produce in situ acetaldehyde, followed by in situ-generated acetaldehyde, urea, and β -dicarbonyl compounds for Biginelli reaction. The first reaction continuously provides a substance for the second reaction, effectively reducing the volatilization loss, oxidation, and polymerization of acetaldehyde and avoiding the negative influence of excess acetaldehyde on the enzyme. Under optimal conditions, a wide range of substrates participate in the reaction and provide the target product in high yield.

In 2017, CALB-catalyzed for synthesis of 3,4-dihydropyrimidin-2(1H)-ones by a tandem multicomponent reaction in one pot (**Figure 51**) has been reported [68]. Several control experiments were performed using acetaldehydes directly to explore the possible mechanism of this procedure. Moreover, owing to the distinct modularity and highly efficient features of the MCR, it assembles libraries of structurally diverse products and provides an exceptional synthesis tool for the discovery of the minimal deep-blue luminogen in the solid state, namely, a single ring. A few of the compounds show deep-blue emissions which only contain a single ring. This is an important application of green biocatalytic promiscuity for constructing a wide variety of new materials.

4.3 Hantzsch reaction

Lin et al. reported a three-component (aldehyde, 1,3-dicarbonyl compound, and acetamide) Hantzsch-type reaction in anhydrous solvent, which gave 1,4-dihydropyridines in moderate to good yields (**Figure 52**) [69]. The group used acetamide as a new source of ammonia. Initially the yield of the reaction with CALB

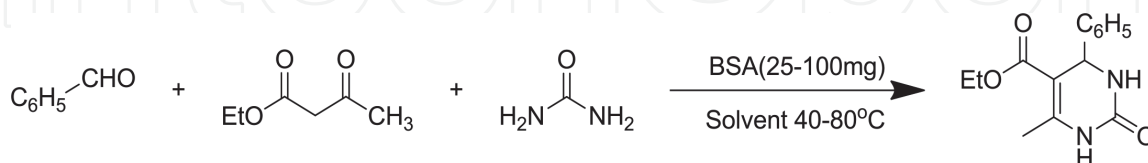


Figure 49.
BSA-catalyzed Biginelli reaction.

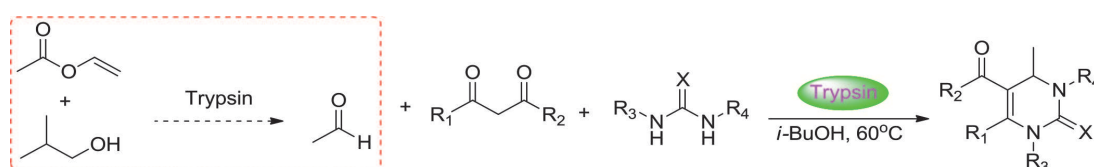


Figure 50.
Trypsin-catalyzed Biginelli reaction using an in situ-generated acetaldehyde.

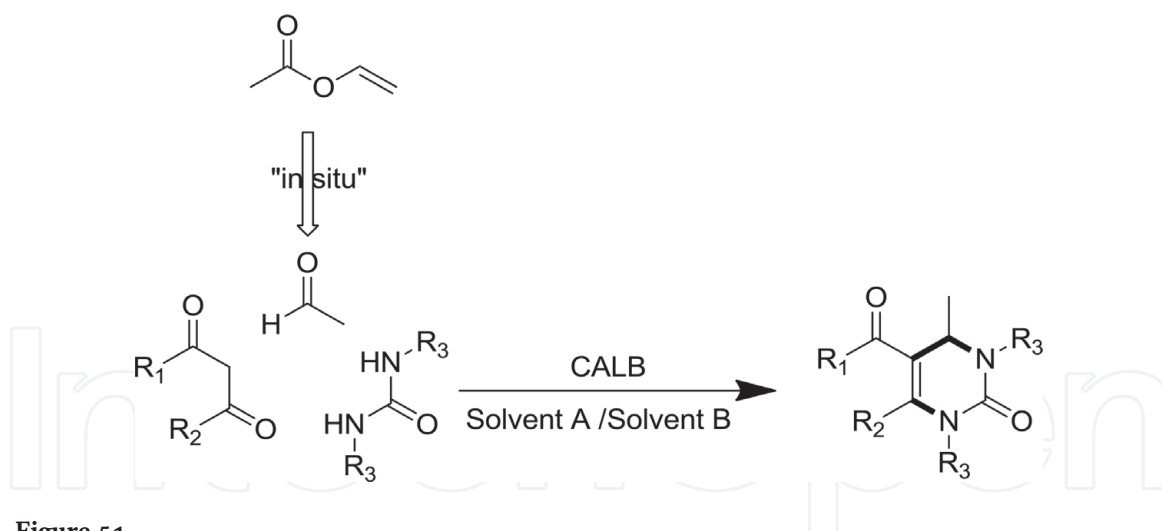


Figure 51.
 CALB-initiated tandem Biginelli reaction.

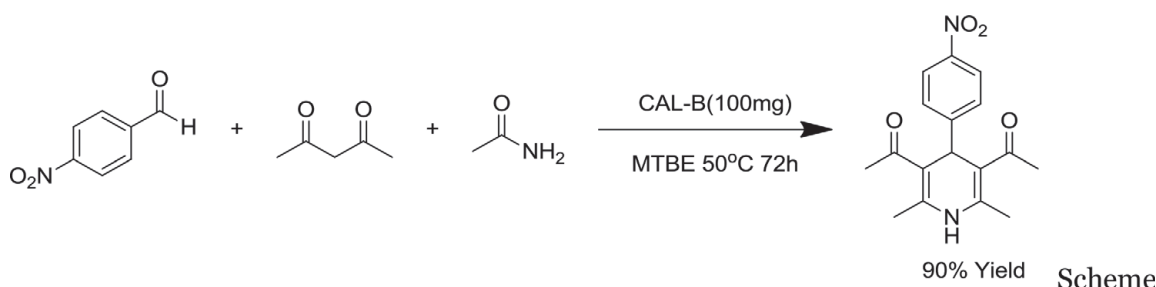


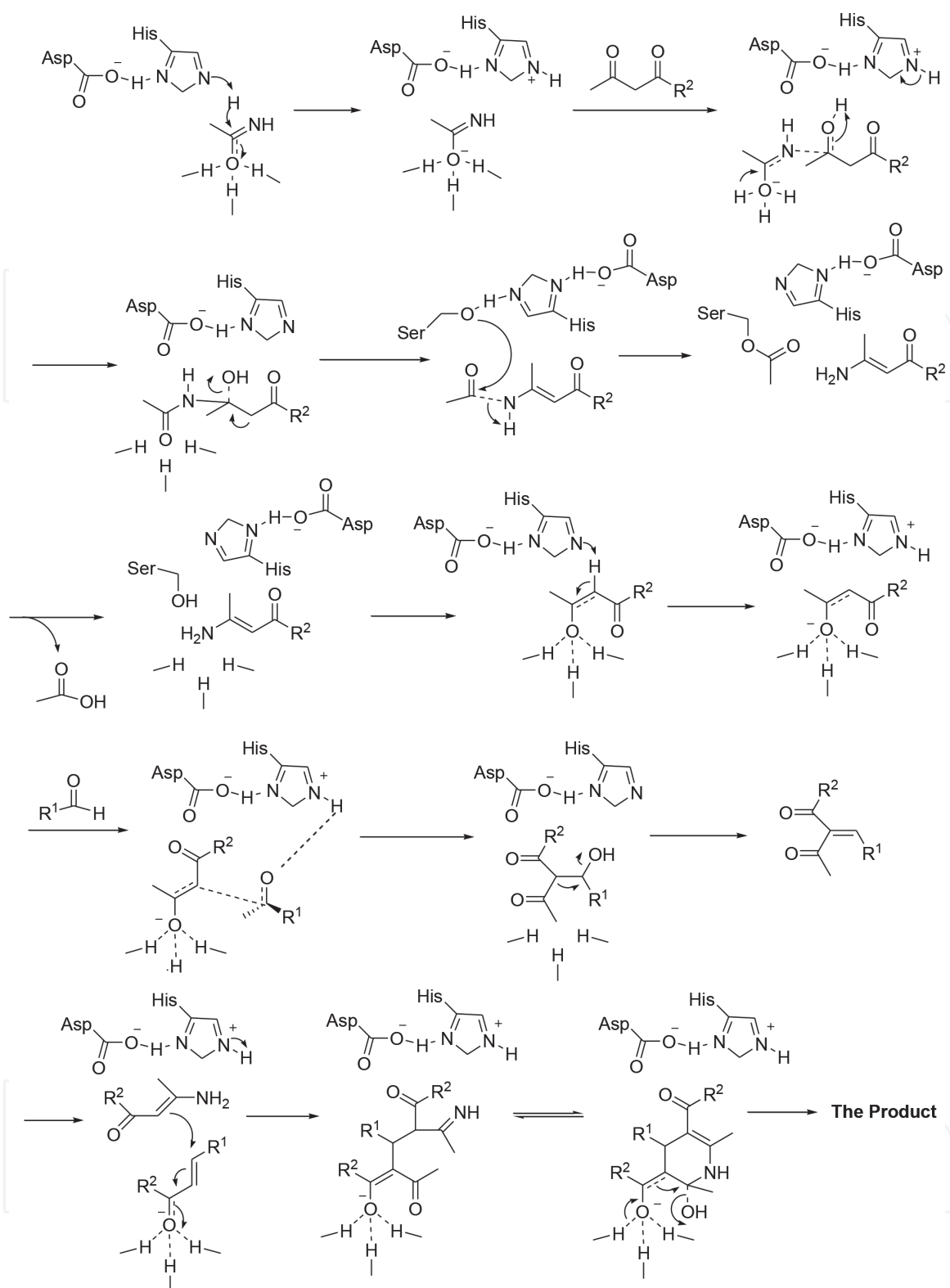
Figure 52.
 Lipase-catalyzed Hantzsch-type reaction.

was very low (only 25%). The yield of the product was slightly improved using a mixed solvent (the ratio of MTBE to acetylacetone was 6:4), and the molar ratio of 4-nitrobenzaldehyde acetamide was 1:4 at 50 mg/ml lipase. When the lipase concentration (100 mg/mL) was increased, the yield increased sharply.

They proposed a reasonable mechanism of the reaction, wherein Asp-His dyad and oxyanion hole in the active site stabilized acetamide (**Figure 53**). This activated acetamide reacted with 1,3-dicarbonyl compounds to form an intermediate, which upon subsequent hydrolysis by CALB formed an enamine intermediate. During this period, a CALB-catalyzed Knoevenagel condensation reaction of the 1,3-dicarbonyl compound with aldehyde formed a separate intermediate (α,β -unsaturated carbonyl compound). Subsequently, the intermediate that is stabilized by the catalytic center of the lipase forms the final product (1,4-dihydropyridine) by Michael addition and intramolecular condensation.

In 2017, our group reported a series of 1,4-dihydropyridines was produced via facile enzymatic Hantzsch reactions in one pot, using acetaldehydes/aromatic aldehydes prepared in situ (**Figure 54**) [70]. After screening several parameters on a model reaction, the tandem process afforded 1a in 80% yield. This approach provided an opportunity to discover novel libraries of AIEEgens that contain the minimum requirement necessary for AIEE behavior, namely, a single ring.

Meanwhile, we found that certain 1,4-DHPs could stain the mitochondria in live cells with high selectivity but without obvious guiding units (such as cationic groups). Taking one of the 1,4-DHPs as an example, we found that it exhibited excellent photostability and storage stability and that it could be utilized in applications such as real-time imaging, long-term tracking of mitochondrial morphological changes, and viscosity mapping (**Figure 55**). We believe that the use of biocatalysis

**Figure 53.**

Proposed mechanism of lipase-catalyzed Hantzsch-type reaction of an aldehyde with acetamide and 1,3-dicarbonyl compounds.

could be simplified with workup procedures and could provide avenues for discovering a wide variety of new materials.

In recently, Ye et al. reported solvent-free quick synthesis of 1,4-DHP calcium antagonists felodipine, nitrendipine, nifedipine, and nemadipine B and their derivatives by Lipozyme[®] RM IM-catalyzed multicomponent reactions of aromatic aldehyde, alkyl acetoacetate, and alkyl 3-aminocrotonate under ball milling conditions (**Figure 56**) [71]. The product was obtained in moderate yield (up to 86.8%), and the effects of the reaction conditions were investigated, including catalyst loading,

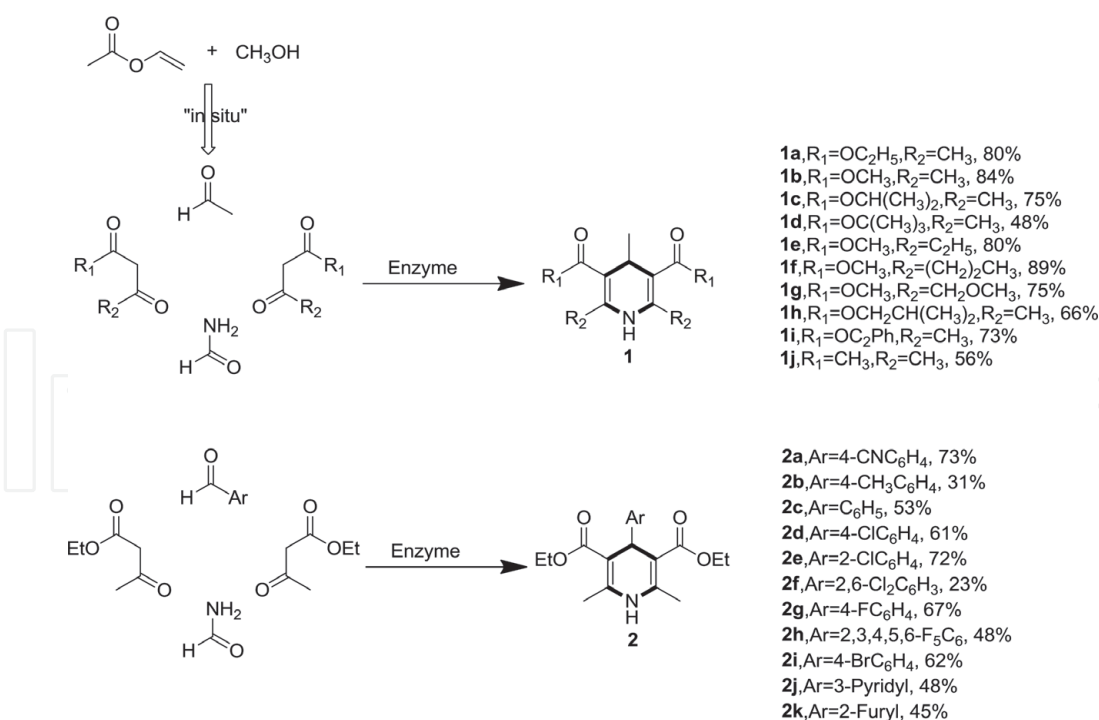


Figure 54.
 Synthesis of **1** and **2** through one-pot multicomponent reactions.

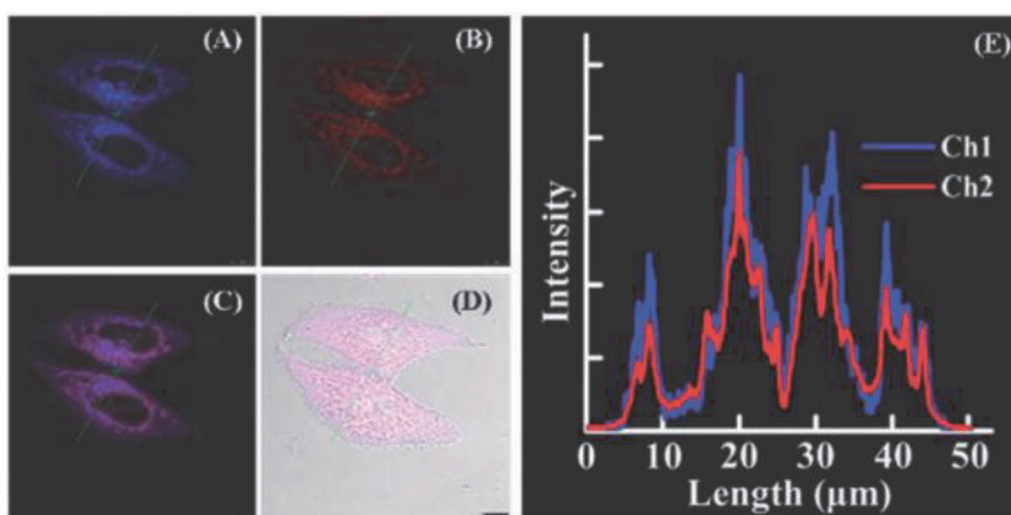


Figure 55.
 Confocal fluorescence images of HeLa cells stained with **2h** and MitoTracker[®] Deep Red FM (MTDR). (A) Fluorescent image of **2h** (10.0 μM) in HeLa cells, collected at 410–460 nm and λ_{exc} = 405 nm. (B) Fluorescent image of MTDR (1.0 μM), collected at 660–740 nm and λ_{exc} = 633 nm. (C) Merged image of A and B. (D) Overlay of fluorescence and bright-field image. (E) Intensity profiles of linear regions of interest (ROI) across the HeLa cells. Scale bar: 7.5 μm.

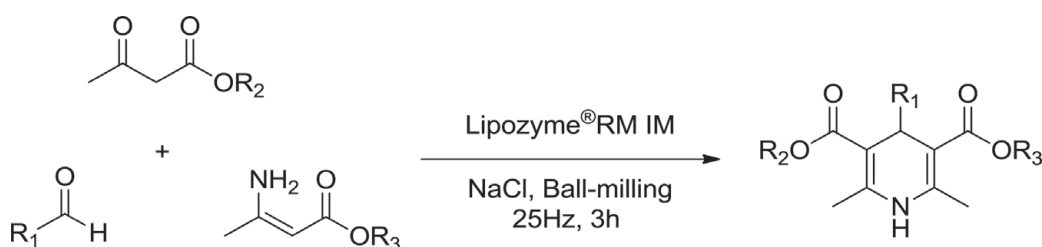


Figure 56.
 Lipozyme[®] RM IM-catalyzed rapid synthesis of 1,4-DHP calcium antagonists and derivatives under ball milling conditions.

grinding aid, and milling frequency. The reaction features environmentally friendly, simple, and efficient operation. A major feature distinguishing this enzyme promiscuity from previously reported work is the use of mechanochemical ball milling techniques that overcome disadvantages such as long reaction times and the use of hazardous organic solvents. This work demonstrates the potential application of mixed enzyme-catalyzed reactions for drug synthesis under ball milling conditions.

4.4 Ugi reaction

In 2013, Berlozecki et al. reported for the first time an enzyme-catalyzed Ugi reaction that has many advantages over previous reactions, such as good reaction at room temperature and extensive solvent selection (**Figure 57**) [72]. In this three-component reaction, the aldehyde, amine, and isocyanide are condensed to form a dipeptide. Of all the selected lipases, Novozym 435 had the highest yield of 75%.

Recently, Thomas et al. reported the concatenation of the Ugi four-component synthesis, and the CALB-catalyzed aminolysis of the intermediary formed Ugi methyl ester products furnishes a novel consecutive five-component reaction for the formation of triamides (**Figure 58**) [73]. This one-pot method is compatible with metal catalysis methods such as copper-catalyzed alkyl azide ring addition and Suzuki cross-coupling or both in a one-pot process.

The mild reaction conditions make this sequence superior to the stepwise process with isolation of the Ugi product and even more favorable than other base-catalyzed or microwave-assisted aminolyses. This efficient scaffold forming

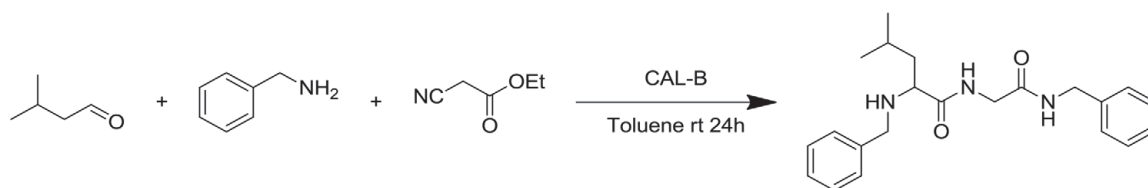


Figure 57.
CALB-catalyzed Ugi reaction.

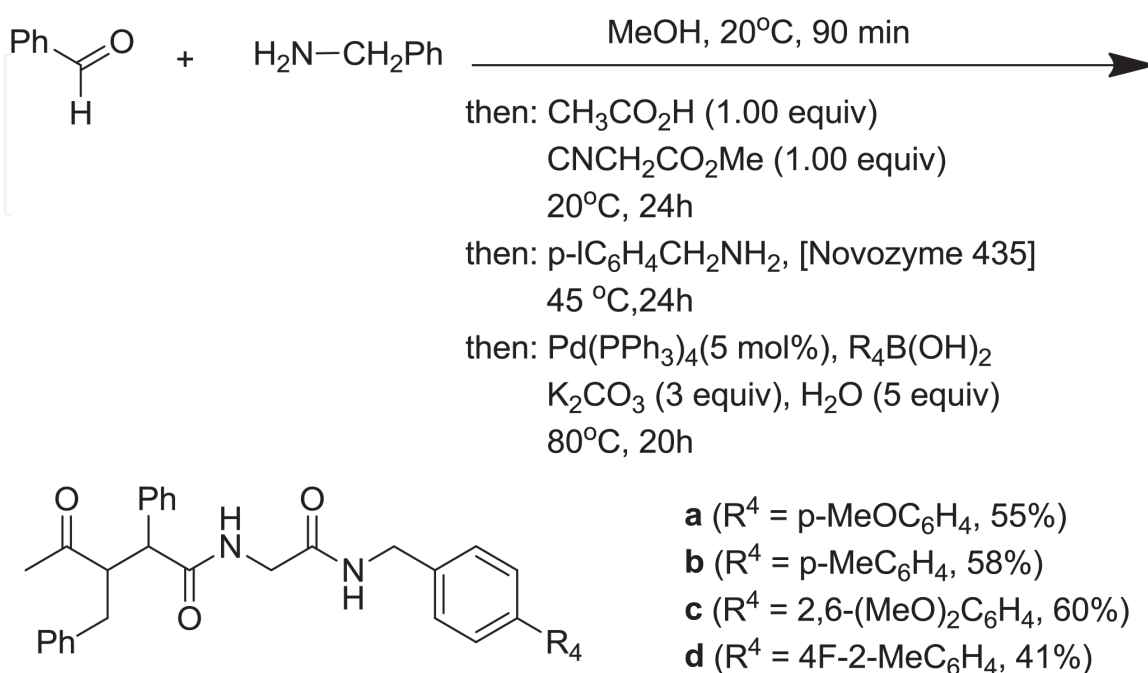


Figure 58.
Consecutive six-component U-4CR-CALB-catalyzed aminolysis-Suzuki cross-coupling sequence of biaryls.

processes are particularly favorable for creating compound libraries for medicinal chemistry lead finding and for functional chromophores in materials sciences.

5. Summary

This chapter has reviewed some examples of various types of hydrolase catalytic promiscuous reactions and their applications in the past decade. Several different types of hydrolases catalyzed carbon-carbon or carbon-heteroatom formation reactions have been discussed: aldol reactions, Michael reactions, and multicomponent reactions.

From these examples, it is clear that enzymes that display catalytic promiscuity can provide new opportunities for organic synthesis. Exploiting enzyme catalytic promiscuous reactions might lead to new, efficient, and stable catalysts with alternative activity and could provide more promising and green synthetic methods for organic chemistry. The development of protein engineering and enzyme engineering can extend the application of metagenome libraries and find enzymes with specific promiscuous behavior. We believe the progress in the area of biocatalytic promiscuity will greatly extend the useful applications of enzymes.

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