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Trends in lipase-catalyzed asymmetric access to enantiomerically pure/enriched compounds

Ashraf Ghanem*

Biomedicinal Chemistry Unit, Biological and Medical Research Department (MBC-03-95), King Faisal Specialist Hospital and Research Centre, PO Box 3354, Riyadh 11211, Saudi Arabia

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Abstract—Over the last few years, there has been a dramatic increase in the number of publications in the field of lipase-catalyzed reactions performed in common organic solvents, ionic liquids or even non-conventional solvents. A fairly large percentage of these publications have emerged from organic chemists who have recognized the potential of biocatalysis as a viable and popular technique in organic synthesis. Considerable research has shown that reactions catalyzed by enzymes are more selective and efficiently performed than many of their analogues in the organic chemistry laboratory. This review article focuses on some of the recent developments in the rapidly growing field of lipase-catalyzed asymmetric access to enantiomerically pure/enriched compounds. The literature search is dated back to the last five years and covers some comprehensive examples.

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* Tel.: +966 1 4647272x39914; fax: +966 1 4427858; e-mail addresses: ghanem@kfshrc.edu.sa; ashraf_ghanem@yahoo.com

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1. Introduction

1.1. Enzymes: historical development

Living cells are extraordinary complex systems where a vast number of chemical reactions occur.[1] The catalysts that enable such chemical reactions to occur are called enzymes. Without their existence, the chemistry performed by living cells, called metabolism, would happen at too slow rate for the organism to stay alive.[2]

The history of enzymes and applied biocatalysis goes back thousands of years to ancient Egypt. Thus, enzymes from microorganisms were used in ancient cooking, baking, brewing, alcohol production and cheese making. This has been demonstrated in an old Egyptian papyrus showing methods used to preserve food and alcoholic drinks. Of particular interest is the two-part process used to produce beers, where the grains are deliberately sprouted and heated to provide sugar and flavour. The cooking made the grain more susceptible to attack by the enzymes that convert starch into sugars. This batch was then mixed with sprouted, but unheated, grains in water. Finally, yeast was added to the combination of sugar and starch in solution, and this was fermented to make beer (Fig. 1).

During the 18th and 19th century, applied biocatalysis has been developed on a more scientific basis. In 1833, Payen and Persoz (from France) isolated an enzymatic complex from malt, naming it 'diastase' (a mixture of amylases).[3] A few years later (1835), the Swedish chemist, Jöns Jacob Berzelius, described the first enzymatic hydrolysis of starch using diastase, which has been used in the production of dextrins from the 1830s onwards.[4] In 1836, Theodor Schwann (from Germany) isolated a substance responsible for albuminous digestion in the stomach and named it 'pepsin', the first enzyme prepared from animal tissue. The fermentation of sugar to ethanol by yeast is a process that has been known for very long time. In 1839, the German chemist, Justus von Liebig, developed a mechanistic explanation for the role of yeast in the fermentation process. He viewed the yeast present in the fermentation mixture as a decomposing matter that emitted certain vibrations ['the sugar atoms suffer a displacement; they rearrange themselves in such a way as to form alcohol and carbon dioxide (spontaneous reaction)'].[4]

About two decades later, the French chemist and biologist, Louis Pasteur (1858), proved that fermentation occurs only in the presence of living cells.[5] This divergence in the understanding of the role of yeast in the fermentation process caused a heated debate between Liebig and Pasteur.[6] In 1897, the German chemists, Eduard Buchner and Hans Buchner, discovered that a cell-free extract of yeast could cause alcoholic fermentation. The yeast cell produces the enzyme, and the enzyme brings about fermentation.[7] In 1883, Johan Kjeldahl from Copenhagen developed a method

Figure 1. Enzyme-catalyzed beer manufacturing in ancient Egypt.

for detecting nitrogen, which has been extensively used in the determination of protein in food. His method was based upon the determination of nitrogen existing in amino acids, which constitute proteins, and was the basis for the development of quantitative enzymology and general biotechnology.[6]

1.2. Progress and commercial achievements

In 1891, the Japanese biochemist, Jokichi Takamine, developed in the USA the commercial production of Koji from the fungus, *Aspergillus oryzae*, and called it 'takadiastase'. Koji is prepared from steamed rice into which a mixture of mould fungi is inoculated.[8] Three years later (1894), the German chemist, Emil Fischer, developed the lock-and-key theory based on the properties of glycolytic enzymes and considered it as a precondition for the potential of an enzyme to have a definite chemical effect on the substrate. In this way, he recognized that a vital function of enzymes also depends on the stereometric configuration (geometrical form) of the enzyme molecules (e.g., the position of the atoms relative to one another). Fischer was the first one to determine the molecular structures of glucose (or grape sugar) and fructose and to synthesize them from glycerol in 1890.[9] In 1903, Victor Henri concluded in Paris that an enzyme combines with its substrate to form an enzyme–substrate complex as an essential step in enzymatic catalysis. Leonor Michaelis of Germany and Maud Lenora Menten of Canada elaborated this idea, in 1913, where the general theory of enzyme action was expressed mathematically. They postulated that the enzyme E first combines with its substrate S to form an enzyme–substrate complex ES in a relatively fast reversible reaction: $E+S \rightarrow ES$. The complex then breaks down in a second, slower reversible reaction to yield the reaction product P and the free enzyme: $ES \rightarrow P+E$.

In 1914, Burnus, the first new detergent product was launched in the market by Röhm and his wife from Germany. The enzyme was so effective that only a small quantity was required. Burnus was originally sold in tablet form as a stain remover, one tablet being mixed with 10 l of water. Afterwards, it was modified and sold as a washing powder in 50 g boxes.[10] In 1926, James B. Sumner of the USA demonstrated that enzymes are proteins and performed the first crystallization of an enzyme. At the same time, the Danish scientist, K. Lindestroem-Lang, investigated the ionization of proteins and laid down a basic formalism for the purification of enzymes. In 1953, James Dewey Watson of Indiana, USA and Francis Harry Compton Crick of Cambridge, UK proposed the double-helix structure of DNA. They shared the Nobel Prize in 1962 with Maurice Hugh Frederick Wilkins, whose diffractograms were used for their proposal.

George Wells Beadle, Edward Lawrie Tatum and Joshua Lederberg (USA) received the Nobel Prize in 1958, Beadle and Tatum for concluding that the characteristic function of the gene was to control the synthesis of a particular enzyme and Lederberg for his discoveries concerned with the genetic recombination and the organization of the genetic material of bacteria.[11]

The years 1963–1974 represent the start of the commercial utilization of enzymes, e.g., alcalase protease in detergents, launching of glucoamylase free of transglucosidase, allowing starch to be broken down into glucose. Since this time, all glucose production has switched from traditional acid hydrolysis to enzymatic hydrolysis. From 1984 to the present time, recombinant DNA technology has brought a revolution to the field of the development of new enzymes. Molecular screening and protein engineering started to be efficient processes in finding many more of nature's own enzyme variants.[12]

1.3. Enzymes in organic solvents

Enzymatic catalysis in non-aqueous media significantly extends the conventional aqueous-based biocatalysis.[13] Water is a poor solvent for nearly all applications in industrial chemistry, since most organic compounds of commercial interest are very sparingly soluble and are sometimes unstable in aqueous solutions. Furthermore, the removal of water is tedious and expensive due to its high boiling point and large heat of vaporization. Side reactions like hydrolysis, racemization, polymerization and decomposition are often accompanying such processes. Chemists realized these limitations on the use of enzymes in aqueous media and started to develop enzymatic procedures in organic solvents a long time ago. Biocatalytic transformations in organic solvents offer the following advantages:

- Better overall yield and the recovery of the product is facilitated by the use of low-boiling-point organic solvents.
- Non-polar substrates are converted at a faster rate, due to their increased solubility.[13]
- Microbial contamination is negligible in the case of using living cells in biotransformations.
- Deactivation and/or substrate or product inhibition is minimized.
- Side reactions such as unfavourable hydrolysis are largely suppressed.
- Immobilization of enzymes is not required; the enzyme can be recovered by simple filtration.
- Denaturation of enzymes is minimized in organic solvents (except ethanol).
- Thermodynamic equilibria are shifted to favour synthesis over hydrolysis.

The use of enzymes in organic solvents, however, has some drawbacks:

- Their decreased catalytic activities (due to the heterogeneous system), which are generally several orders of magnitude lower than those in aqueous solution.
- Many enzymatic reactions are prone to substrate or product inhibition, which deactivates the enzymes at higher substrate or product concentration, leading to a decrease in the reaction rate and enantioselectivity.

1.4. Lipases

Lipases (E.C.3.1.1.3) are ubiquitous enzymes[14] belonging to the family of serine hydrolases and can be found in animals, plants, fungi and bacteria. In the industrially developed countries, the edible lipids present in the human diet consist mainly of triacylglycerols (TAGs), from 100 to about 150 g per day, i.e., 30% of each individual's daily caloric intake,

and these TAG molecules cannot cross the intestinal barrier. A series of hydrolytic and absorption stages are therefore necessary to produce the chemical energy resources present in the hydrocarbon chains of biologically usable TAGs. The lipases in the digestive tract therefore play a particularly important role in nutrition processes, both in humans and in higher animals.[2]

Lipases were previously defined in kinetic terms, based on the 'interfacial activation' phenomenon, i.e., the increase in activity, which occurs when a partially water-soluble substrate becomes water insoluble.[15] This process was not observed among esterases. The determined 3D structures of lipases show an α/β-hydrolase fold as well as a nucleophilic elbow where the catalytic serine is located.[16] Some, but not all, lipases show a 'lid' controlling access to the active site. The above structural features, however, including the presence of a lid as well as the 'interfacial activation' phenomenon, are not suitable criteria for classifying specific esterases as lipases.

Since enzymes are usually named after the type of reaction they catalyze, lipases can be pragmatically redefined as carboxylesterases acting on long-chain acylglycerols, in other words, they are simply fat-splitting 'ferments'. Accordingly, lipases are often termed triacylglycerol hydrolases. Since the reaction is reversible, they can also catalyze the formation of acylglycerols from free fatty acids and glycerol (Fig. 2).

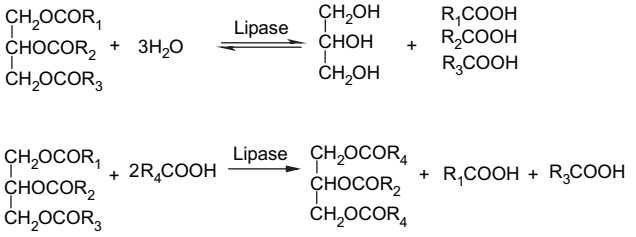

Figure 2. Examples of lipase-catalyzed reactions of triacylglycerols.

Since they belong to the class of serine hydrolases and do not require cofactors, they can be used in either free or immobilized form. They are readily available, inexpensive, highly stable, and accept a broad range of substrate structures, while retaining a high stereoselectivity in their action. They act at the lipid–water interface and, therefore, they do not require water-soluble substrates. This provides lipases with an inherent affinity for hydrophobic media, which distinguishes them from other hydrolytic enzymes, and their efficiency in conducting transformations in organic solvents, safety, ease of handling and the mild conditions under which they operate (typically, room temperature and around neutral pH) render them a useful tool in organic synthesis.[13,14] In addition to all these practical aspects, they are, like most enzymes, environmentally benign and completely biodegradable. Unlike chemical catalysts that are applied in moles, lipases and all enzymes are applied in units. Via an activity test, the specific activity of lipase in units per weight (solid enzymes) or volume (enzyme solution) using standard reactions can be determined. Besides all of the techniques used to measure the activity of lipases,[2,17] a common example of such reactions is using a tributyrin solution and a pH stat (Fig. 3). This solution was automatically titrated with

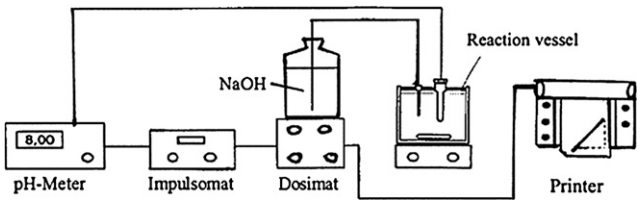

Figure 3. Schematic representation showing a pH stat.

NaOH solution to the desired pH before lipase was added. The consumption of the NaOH was recorded as a function of time. The lipase activity is then expressed in units (U). One unit corresponds to the liberation of 1 μmol fatty acids per minute. The specific activity of the lipase is determined in U/mg.

2. Biocatalysis in non-aqueous media

2.1. Lipase in organic solvents: insights and limitations

Lipases, the workhorses of biocatalysis, continue to serve as enantioselective catalysts for synthesis. They have been widely used in three main types of asymmetric transformations, namely kinetic resolution of racemic carboxylic acids or alcohols, enantiotopic group differentiations of *meso* dicarboxylic acids or *meso* diols and enantiotopic group differentiation of prochiral dicarboxylic acid and diol derivatives.[13,14]

A maturing understanding of these enzymes allows researchers to use them predictably on more complex structures and to attempt more difficult to resolve substrates. Conventially, lipase-catalyzed reactions were carried out in emulsion systems, where the reactions take place at the interface of oil droplets. Upon the discovery of Kilbanove[18] that lipases can successfully catalyze reactions under almost anhydrous conditions in an organic medium, the range of possible chemical reactions has widened far beyond hydrolysis reactions.[13,14] Thus, lipase can be used for ester synthesis from carboxylic acids and alcohols. In order to shift the equilibrium towards complete ester formation, water should be removed using molecular sieves or a vacuum. Transesterifications (acyl transfer reactions) can be similarly conducted in a water-free medium and can be divided into alcoholysis, acidolysis and interesterification (ester–ester interchange). Of these reactions, the direct esterification and alcoholysis involving racemic alcohols have been frequently used in lipase-catalyzed access to the enantiomerically pure alcohols. As has been reported, conditions favouring rapid and irreversible reactions are necessary for the achievement of high enantioselectivity in a kinetic resolution process. The optical purity of the resulting product and the remaining unreacted substrate diminishes as the reverse reaction advances, due to the reversible nature of the esterification and the interesterification in biocatalytic kinetic resolutions and the same enantioselection of the catalyst in both directions. Several strategies have been used to overcome these problems by suppressing the reversibility or ensuring the irreversibility of the processes, although the slow reaction rate will remain an obstacle in such reactions. The uses of enol esters, such as vinyl or isopropenyl esters, are the most

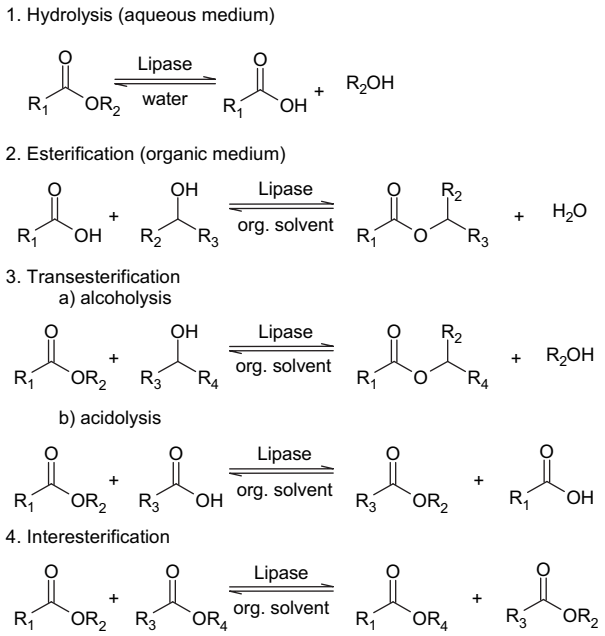

Figure 4. Some reactions catalyzed by lipases.

common in the transesterification process. The vinyl alcohol formed as a by-product undergoes keto-enol tautomerization, yielding the corresponding carbonyl compound (acetaldehyde or acetone), making the reaction practically irreversible. Thus, these transesterifications are much faster, compared to reactions using free carboxylic acids or simple esters such as ethyl acetate.

The direction of the reaction is dependent upon the adequate solvent medium used, e.g., aqueous or organic solvents (Fig. 4). More recently, the use of lipases in non-aqueous media has been extended to include supercritical fluids,[19] and ionic liquids[20] with tunable solvent properties. The use of these solvents reduces the quantities of waste volatile compounds, which is an important step in the direction of 'green chemistry'.

2.2. Lipase in supercritical carbon dioxide: unique properties in enantioselective molecular transformations

2.2.1. Properties of supercritical carbon dioxide. The advantage of the use of biocatalysts in organic solvents discussed above encouraged researchers to discover new non-toxic media having similar properties to organic solvents. Among the developed media, supercritical fluids have unique properties, which can be applied to a wide range of novel chemical processes.[21] Among many fluids, supercritical carbon dioxide ($scCO_2$), defined as CO_2 above its critical point, has the added benefits of an environmentally benign nature, non-flammability, low toxicity and ready availability, and it exhibits similar properties to organic solvents. It differs from ordinary solvents in having a combination of gas-like properties (i.e., low viscosities and high diffusivities which render them favourable for mass transfer) and liquid-like properties (i.e., solubilizing power).[22] Moreover, these properties are tunable by the manipulation of pressure and temperature (Fig. 5). Small variations in pressure or temperature lead to significant changes in density and density-dependent solvent properties such as dielectric constant, solubility parameters and partition coefficient, as shown in Figure 6. These render them more attractive as 'green designer' solvents and promising reaction media for environmentally benign chemical processes.[22]

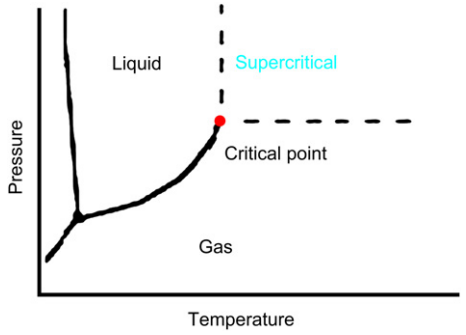

Figure 5. Phase diagram for CO_2 (critical point: 73 atm (7.38 Mpa) at 31 °C).

2.2.2. Lipase-catalyzed reactions in $scCO_2$. The first reports on enzyme-catalyzed reactions in $scCO_2$ appeared in 1985 by Randolph et al.,[24] Hammond et al.,[25] and Nakamura et al.,[26] followed by Mori and Okahata[27] and Kamat et al.[28] Recently, the beneficial effects of $scCO_2$ for lipase-catalyzed reactions using flow- and batch-type reactors have been demonstrated by Matsuda et al. (Fig. 7).[22]

Of particular interest is the use of lipase in $scCO_2$ to resolve P-chiral hydroxymethanephosphinates **1** as heterogenic

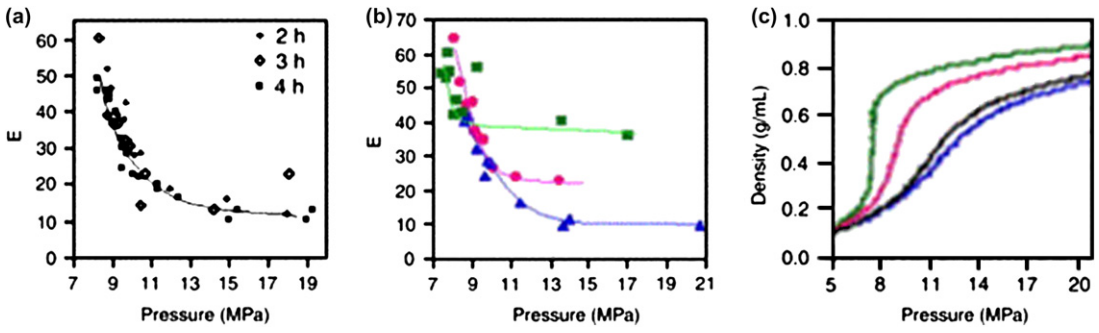

Figure 6. Tunability of solvent properties of $scCO_2$ by temperature and pressure in the enantioselective acetylation of 1-(*p*-chlorophenyl)-2,2,2-trifluoroethanol by the lipase Novozym. (a) Reaction at 55 °C for 2, 3, or 4 h. (b) Reaction at 31 °C (green squares), 40 °C (magenta circles) and 60 °C (blue triangles). (c) Density versus pressure[23] for CO_2 at 31 °C (green), 40 °C (magenta), 55 °C (black) and 60 °C (blue). *E*=enantioselectivity (see Section 4.3).

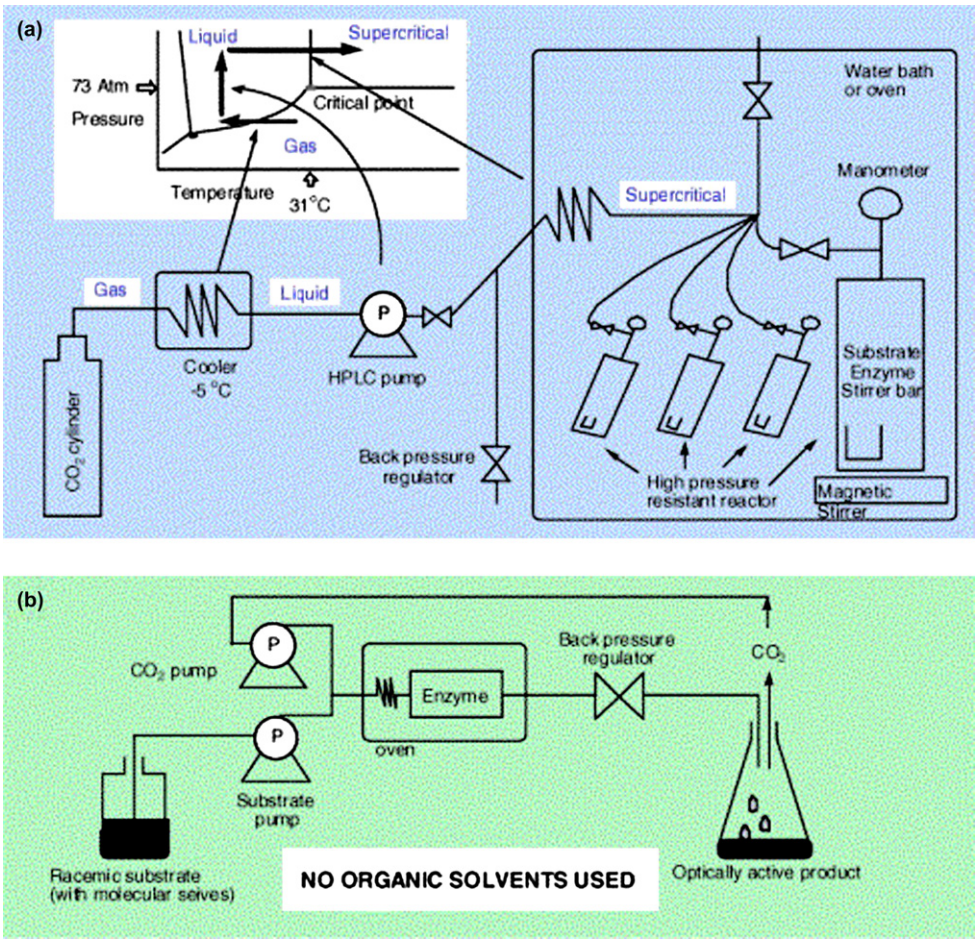

Figure 7. Experimental apparatus for enzymatic reactions in $scCO_2$: (a) batch-type reactor; (b) flow-type reactor.[19]

substrates of primary alcohols bearing a remote stereogenic heteroatom centre (Fig. 8).[29] Among the many lipases tested, *Candida antarctica* lipase (CAL) was the best enzyme in conducting the enantioselective transesterification using vinyl acetate as an acyl donor in $scCO_2$ to afford **2** leaving **1** in enriched form. Both the reactivity and stereoselectivity of the reaction could be largely improved by changing the pressure. Thus, at pressures up to 8 MPa, i.e., below the critical density, the reaction did not proceed at all. Upon altering the pressure, however, the reaction conversion could be largely improved. The author concluded that it was important to tune the pressure so that the density of CO_2 exceeds the critical density. The reaction was the fastest when the pressure was closer to the critical pressure: at 11 MPa, the reaction rate reached its maximum and then gradually decreased as the pressure was increased to 15 MPa. The optimal conditions were obtained at 13 MPa, at which point the reaction stopped at ca. 50% conversion and the enantioselectivity was the highest. Thus, the control of the reaction outcome by changing the solvent properties is a very special characteristic of supercritical fluids.

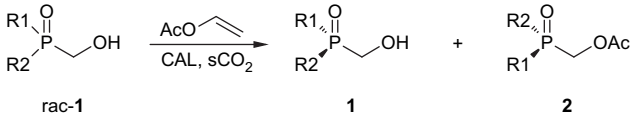

Figure 8. Lipase-catalyzed enantioselective transesterification of P-chiral hydroxymethanephosphinates.[29]

Other examples of the lipase-catalyzed reaction in $scCO_2$ have been reviewed. Some examples consisting of the lipase-catalyzed kinetic resolution of **3**, **5** and **7** using vinyl acetate as acyl donor in $scCO_2$ to afford (*R*)-**4**, (*R*)-**6** and (*R*)-**8**, respectively, leaving (*S*)-**3**, (*S*)-**5** and (*S*)-**7** in optically pure form are mentioned below (Fig. 9).[19]

2.3. Lipase in ionic liquids: effects on structure and activity

A considerable amount of work has been performed to understand solvent effects on the structure and function of enzymes in order to select more stable and efficient biocatalysts.[30] Most organic media have a number of generic disadvantages, however, including toxicity to the environment and potential explosion hazards, due to their volatile and flammable nature.[31]

2.3.1. Properties of ionic liquids. Ionic liquids, also known as molten salts, are solutions composed entirely of ions.[32–34] They are relatively polar solvents and promote the dissolution of a vast array of pharmaceutical intermediates and final drug substance (target) molecules.[35] The replacement of conventional solvents in biocatalytic processes by ionic liquids could therefore overcome many of the disadvantages associated with organic solvents. The ability to readily alter the physicochemical properties of these solvents by simple structural modifications to the cations or changes in the

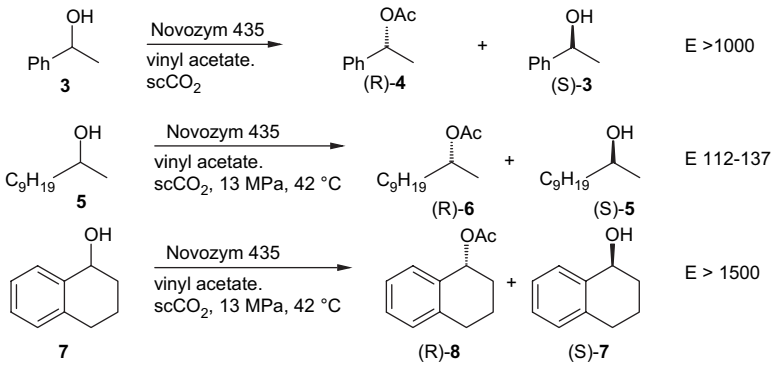

Figure 9. Lipase-catalyzed enantioselective transesterification in $scCO_2$ flow system.[19]

anions render them of considerable interest to organic chemists.[20]

A vast number of publications have recently shown the potential of carrying out enzymatic bioconversions in ionic liquids and there are already a number of reviews on this topics.[36,37]

2.3.2. Lipase in ionic liquids. The first report on enzyme catalysis in ionic liquids concerned the synthesis of *Z*-aspartame by the protease, thermolysin.[38] This report was followed by publications concerning other proteases[39] and also galactosylation reactions.[40] Sheldon et al. first demonstrated lipases to be active in anhydrous ionic liquids.[41] They examined the activity of *C. antarctica* lipase B (CAL-B) in ionic liquids for alcoholysis, aminolysis and perhydrolysis, discovering similar reaction rates, compared to the reactions performed in organic solvents such as propan-2-ol and butan-1-ol. Subsequently, other groups have investigated lipases in ionic liquids, due to the widespread use of these enzymes in industry.[42] These workers have generally reported good enzyme activity and stability.[43] Park and Kazlauskas investigated the regioselective acylation of glucose in ionic liquids and obtained much higher yields and selectivities than in commonly used organic solvents,[44] due to the high solvation properties of ionic liquids for both hydrophobic and hydrophilic reactants. Furthermore, they demonstrated the influence of an additional purification regime, following ionic liquid synthesis, to remove impurities and enhance enzyme activity. This is of particular significance when silver salts (e.g., $AgBF_4$ and $AgPF_6$) are used to prepare ionic liquids, silver being a well-known enzyme inhibitor.[44] Other groups have demonstrated the importance of controlling the water content in ionic liquids, in order to achieve a higher conversion.[45] Recently, Roberts et al. reported the CAL-B-catalyzed resolution of 2,3,4,5-tetrahydro-4-methyl-3-oxo-1*H*-1,4-benzodiazepine-2-acetic acid methyl ester **9**, a key lotrafiban intermediate, in six ionic liquids including $[BMIM][PF_6]$ and $[BMIM][N(SO_2CF_3)_2]$ to afford (2*S*)-2,3,4,5-tetrahydro-4-methyl-3-oxo-1*H*-1,4-benzodiazepine-2-acetic acid **10** leaving the unreacted (2*R*)-2,3,4,5-tetrahydro-4-methyl-3-oxo-1*H*-1,4-benzodiazepine-2-acetic acid methyl ester and (*R*)-**9** in optically pure form (Fig. 10).[36] The results have been compared with the industrial process operated in *tert*-butanol.[46] The authors concluded that replacing the organic solvent with an ionic liquid under otherwise identical conditions reduced the rate of conversion. Exploiting the increases in the solubility of the substrate in ionic liquids and the ability to operate at higher temperatures, however, increased the overall rate of the reaction by 4-fold, while maintaining the same overall yield of 47%. In each case, the ee of the product was 99%.[36]

Sheldon et al. studied the effect of ionic liquids on the activity of Novozym 435 (an immobilized version of CAL-B) on a model example consisting of the CAL-B-catalyzed alcoholysis (transesterification) of ethyl butanoate **11** with 1-butanol to give **12** and compared the results with that carried out in *tert*-butyl alcohol (Fig. 11).[37]

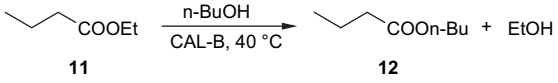

Figure 11. Transesterification of ethyl butanoate with 1-butanol.

The results revealed that, when the reaction was performed in ionic liquids containing a nitrate, ethylsulfate or lactate anion, in which the CAL-B is soluble, the reaction rate was at least 10-fold slower. The authors concluded that the

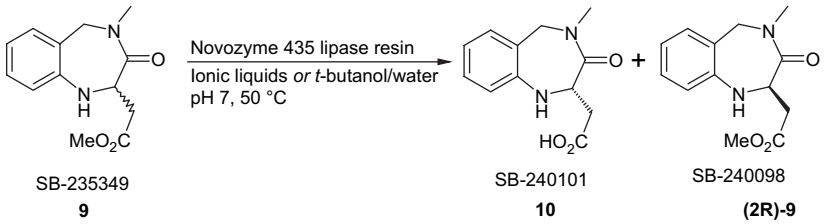

Figure 10. Lipase-catalyzed enantioselective resolution of 2,3,4,5-tetrahydro-4-methyl-3-oxo-1*H*-1,4-benzodiazepine-2-acetic acid methyl ester **9** (SB-235349) to (2*S*)-2,3,4,5-tetrahydro-4-methyl-3-oxo-1*H*-1,4-benzodiazepine-2-acetic acid **10** (SB-240101) leaving the unreacted (2*R*)-2,3,4,5-tetrahydro-4-methyl-3-oxo-1*H*-1,4-benzodiazepine-2-acetic acid methyl ester (2*R*)-**9** (SB-240098). SB-240101 is a key intermediate in the synthesis of the glycoprotein antagonist, lotrafiban.[36]

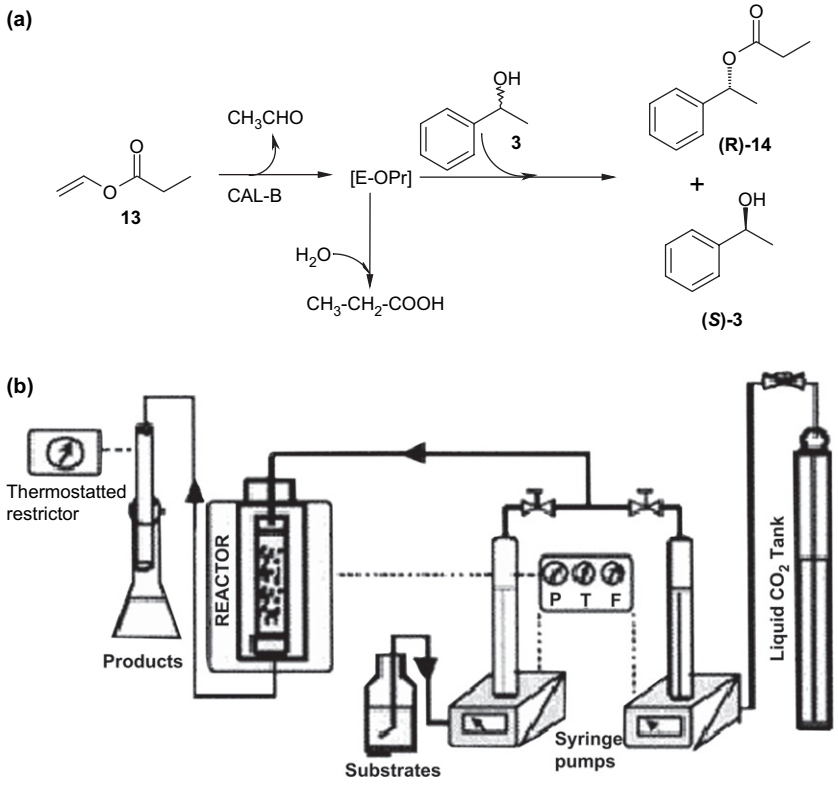

Figure 12. (a) Lipase-catalyzed kinetic resolution of *rac*-1-phenylethanol **3**, (b) Experimental set-up of the bioreactor with ionic liquids and supercritical carbon dioxide ($scCO_2$).[47]

ionic liquids interact with the protein sufficiently and strongly to effect its dissolution, but also induce a structural change that leads to a loss of activity.[37]

Lozano et al. have combined ionic liquids with supercritical carbon dioxide in lipase catalysis.[47] Thus, free and immobilized *C. antarctica* lipase B (CAL-B) dispersed in ionic liquids (1-ethyl-3-methylimidazolium bistriflimide and 1-butyl-3-methylimidazolium bistriflimide) were used as catalysts for the continuous kinetic resolution of *rac*-1-phenylethanol **3** in supercritical carbon dioxide at 120 or 150 °C and 10 Mpa to afford (*R*)-**14** leaving (*S*)-**3**. Excellent activity, stability and enantioselectivity levels were observed in the continuous operation (Fig. 12).

2.4. Alternatives: hydrofluorocarbon solvents in biotransformations

2.4.1. Properties. Another group of potential solvents for lipase-catalyzed reactions that has received little attention to date is that of pressurized liquids with normal boiling points below, and critical temperatures above, room temperature. These fluids are easily handled at moderate pressures and their relative volatility should allow the ready removal of solvent residues from the products, in contrast to the situation with many ionic liquids.[48] Such fluids can be readily compressed and reliquified in a closed system, thereby allowing the solvents to be recycled and re-used with minimal losses into the environment, the latter property rendering them suitable solvents for greener chemical processes. Hydrofluorocarbons (HFCs) are generally of low toxicity, they do not have an ozone depletion potential and they are not classified as volatile organic compounds.[49] Many of them such as 1,1,1,2-tetrafluoroethane (*R*-134a) and 1,1,1,2,3,3,3-heptafluoropropane (*R*-227ea) are even non-flammable and others are used as replacements for the chlorofluorocarbons (CFCs) and hydrochlorofluorocarbons (HCFCs) in the refrigeration industry and are manufactured globally on a large scale to high purity. In addition, both *R*-134a and *R*-227ea are manufactured according to current good manufacturing practice (CGMP) standards for use in metered-dose inhaler applications in the pharmaceutical industry.[48] All these interesting properties render them environmentally benign and economically feasible alternatives to conventional organic solvents, supercritical fluids and ionic liquids.

2.4.2. Lipase-catalyzed reactions in low-boiling hydrofluorocarbon solvents. Micklefield et al. reported the first investigation of biotransformation in such solvents.[48] Thus, the lipase-catalyzed kinetic resolution of racemic 1-phenylethanol **3** and the desymmetrization of *meso*-2-cyclopentene-1,4-diol **16** were investigated in liquid phase, low-boiling hydrofluorocarbon (HFC) solvents such as 1,1,1,2-tetrafluoroethane (*R*-134a), 1,1,1,2,3,3,3-heptafluoropropane (*R*-227ea) and difluoromethane (*R*-32). In comparison to anhydrous *n*-hexane and methyl *tert*-butyl ether (MTBE) and under identical conditions, the acylation of 1-phenylethanol **3** in HFCs was superior both in terms of rate and degree of conversion observed to afford (*R*)-**15** leaving (*S*)-**3**. On the other hand, in comparison with THF, the lipase-catalyzed desymmetrization reaction of *meso*-2-cyclopentene-1,4-diol **16** with vinyl acetate in the same HFCs yielded the monoacetate product **17** and the diacetate **18** in around a quarter of the time when using *Pseudomonas cepacia* lipase. For Novozyme 435, the use of HFCs similarly increased the yield of **17** with dramatically improved

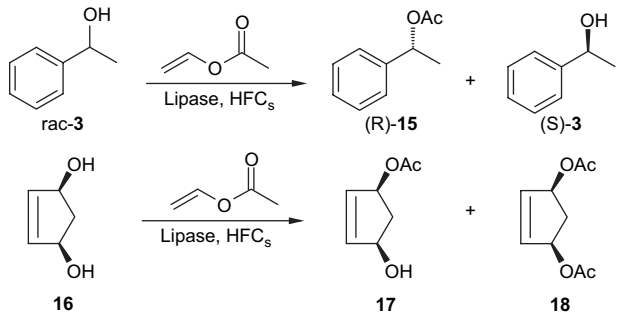

Figure 13. Lipase-catalyzed kinetic resolution of racemic 1-phenylethanol **13** and desymmetrization of *meso*-2-cyclopentene-1,4-diol **16**.

rates and increased the enantioselectivity from 40% ee in THF or 91% ee in THF/Et_3N to >99% ee in all of the HFCs (Fig. 13).[48]

3. Asymmetric access to enantiomerically pure/enriched compounds catalyzed by lipases

3.1. Lipase-catalyzed kinetic resolution of racemates

According to the IUPAC recommendation, kinetic resolution (KR) is defined as follows: 'The achievement of partial or complete resolution by virtue of unequal rates of reaction of the enantiomers in a racemate with a chiral agent (reagent, catalyst, solvent, etc.)'.[50] In the simplest cases of KR, the substrate enantiomers interact with a chiral reagent or catalyst to generate two diastereomeric transition states. The Gibbs free energies of these competing transition states define the rate constants for conversion of the fast reacting and slow-reacting enantiomers, and the ratio k_{fast}/k_{slow} (see Section 4) controls the product distribution. The history of kinetic resolution has been recounted many times and has recently been reviewed.[51] It is of particular interest to recall that the first examples of kinetic resolution catalyzed by lipases were reported by Dakin, who described the hydrolysis of racemic ethyl mandelate with crude pig liver lipase.[52] His results revealed that enzymes are not perfectly enantioselective and that the relative reactivity issues and percentage conversion must be taken into account.

3.1.1. Kinetic resolution of important functional group categories.

3.1.1.1. Alcohols. As the most versatile class of substrates for synthetic applications, alcohols have received the most attention in the context of lipase-catalyzed kinetic resolution. Here, we report on the lipase-catalyzed kinetic resolution of primary, secondary and tertiary alcohols.

3.1.1.1.1. Primary alcohols. While lipase-catalyzed enantioselective access to enantiomerically pure secondary alcohols is a very efficient tool in organic synthesis, the kinetic resolution of racemates of primary alcohols by the same method is more difficult to achieve. This is due to the lower enantioselectivity of lipases towards chiral primary alcohols. To enhance the enantioselectivity of lipases towards primary alcohols, Sakai et al. reported a low-temperature method. Thus, for the kinetic resolution of 2,2-dimethyl-1,3-dioxolane-4-methanol using lipase AK from *Pseudomonas fluorescens*, the *E* value increased from 9 to 55 by lowering the reaction temperature from 22 to −40 °C, despite the large amount of lipase and the long reaction time needed.[53,54] Recently, this low-temperature method was extended to a new synthetic approach for the four stereoisomers of 2-hydroxymethyl-3-methyl-3-phenyl-aziridine, (±)-**19** (2*R*,3*S*) and (±)-**21** (2*R*,3*R*), as an interesting class of primary aziridine alcohols having two stereogenic centres at the β- and γ-carbons, via a lipase-catalyzed resolution using vinyl acetate as the acyl donor without N-protection. Thus, porous ceramic (Toyonite)-immobilized lipase (PS-CII) was used in the resolution of (2*R*,3*S*)-3-methyl-3-phenyl-2-aziridinemethanol, (±)-**19**, at low temperature, giving the synthetically useful (2*R*,3*S*)-**19** and its acetate (2*S*,3*R*)-**20** with (2*S*)-selectivity (*E*=55 at −40 °C), while a similar reaction of (2*R*,3*R*)-3-methyl-3-phenyl-2-aziridinemethanol, (±)-**21**, gave (2*S*,3*S*)-**21** and its acetate (2*R*,3*R*)-**22** with (2*R*)-selectivity (*E*=73 at −20 °C) (Fig. 14).[55]

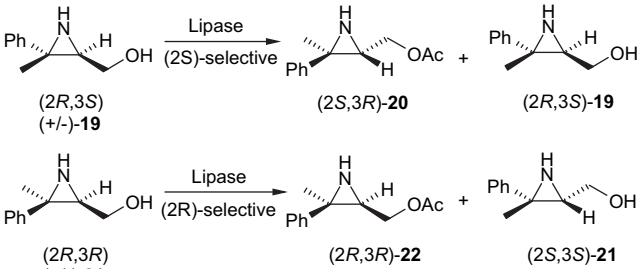

Figure 14. Lipase-catalyzed resolution of (2*R*,3*S*)- and (2*R*,3*R*)-2-hydroxymethyl-3-methyl-3-phenyl-aziridine, (+/−)-**19** and (+/−)-**21**, respectively, using vinyl acetate as acyl donor.[55]

Kazlauskas et al. reported another approach in which an accurate choice of the acyl chain length and solvent increased the enantioselectivity of PCL from *E*=17 to 70 for the hydrolysis of esters of primary alcohols (e.g., 2-phenoxy-1-propyl-heptanoate) when changing the substrate from 1-acetate to 1-heptanoate.[56] In an alternative approach, Kawanami et al. investigated the substrate-tuning method for the lipase-catalyzed enantioselective transesterification of *trans*-2,5-substituted pyrrolidine derivatives **23** to afford (+)-**24** leaving (−)-**23** (Fig. 15) and glycerol derivatives **25** to afford (*S*)-**26** leaving (*R*)-**25** (Fig. 16) using vinyl acetate

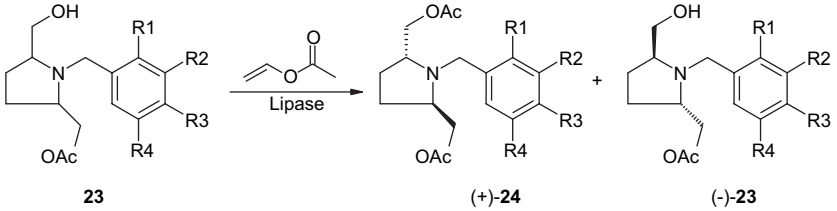

Figure 15. Lipase-catalyzed enantioselective transesterification of *trans*-2,5-disubstituted pyrrolidines **23**.

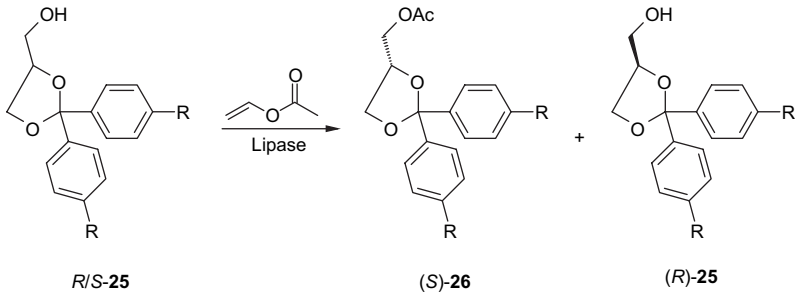

Figure 16. Lipase-catalyzed enantioselective transesterification of various substituted diphenyl-1,2-ketals of glycerol **25**.

as the acyl donor. The results revealed that, by altering the protecting group, the *N*-3,5-dimethylbenzyl groups and the bis(4-bromophenyl) ketal were found to enhance the enantioselectivity up to E=108 and 57, respectively. Furthermore, Kawanami et al. showed a cutaway view of the active-site cleft of PCL and the supposed transition-state model for the fast-reacting (*S*)-bis(4-bromophenyl) ketal of glycerol (Fig. 17).[57,58]

Using the same method of modification of the substrate structure, Kawanami extended the work to include 2-benzyloxy- and 2-silyloxy-1-propanols **27** to afford (*S*)-**28** leaving (*R*)-**27** and **29** to afford (*S*)-**30** leaving (*R*)-**29** (Figs. 18 and 19), which are useful chiral building blocks for the synthesis of optically active and bioactive compounds.[59]

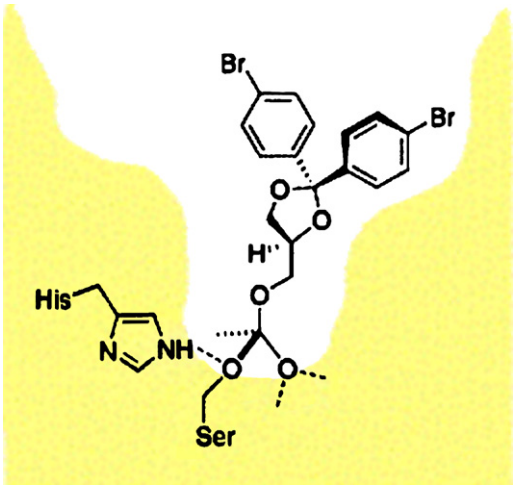

Figure 17. Proposed model of the fast-reacting (*S*)-enantiomer for PCL and a cutaway view of the active site of the cleft of PCL. A larger hydrophobic pocket is located far away from the cutaway view and an alternate hydrophobic pocket points towards this site.[59]

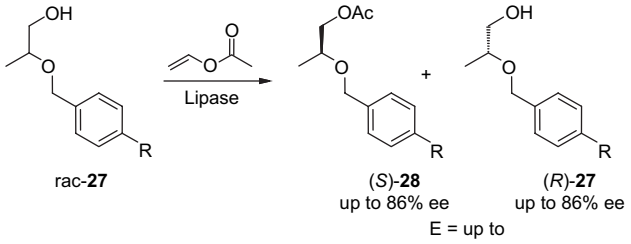

Figure 18. Lipase-catalyzed enantioselective transesterification of 2-benzyloxy-1-propanols **27**.[59]

Among all of the lipases tested in the literature, lipase from *P. cepacia* (PCL) showed efficiency and high enantioselectivity towards a broad range of primary alcohols.[60] Nordin et al.[61] studied the enantioselectivity of lipase from *P. cepacia* towards a series of primary 2-methyl-substituted alcohols **31**–**36** using vinyl acetate as the acyl donor in transesterifications in organic solvents (Fig. 20).

In terms of enantioselectivity, the best results were found for 3-aryl-2-methylpropan-1-ols with an enantiomeric ratio E over 100 in most cases, whereas other 3-substituted primary 2-methylpropan-1-ols **37**–**49** generally displayed lower enantioselectivities, e.g., 3-cycloalkyl-2-methylpropan-1-ols (E∼20) and 2-methylalkan-1-ols (E∼10) (Fig. 21).[62]

Moving the aryl group closer to, or further away from, the stereogenic centre resulted in low enantioselectivities, e.g., 2-arylpropan-1-ols (E<10), 2-methyl-4-(2-thienyl)butan-1-ol (E=12), 2-methyl-5-(2-thienyl)pentan-1-ol (E=3.2) and 2-methyl-6-(2-thienyl)hexan-1-ol (E=3.8).

Furthermore, Kawasaki et al. reported a highly enantioselective kinetic resolution of a primary alcohol **50** by lipase-catalyzed transesterification to afford (*S*)-**51** leaving (*R*)-**50** as a key step to prepare 5,6-dehydrosenedigitalene, a member of the norsesquiterpenes (Fig. 22). Among several acyl donors used, vinyl 3-(4-trifluoromethylphenyl)propanoate was the best in terms of enantioselectivity (E=331).[63]

A practical method for the synthesis of chiral pyridazinones bearing a pyrazolopyridine ring via lipase-catalyzed resolution of 2-(acyloxymethyl)-4,5-dihydro-5-methylpyridazin-3(2*H*)-one derivatives **52** was reported by Yoshida et al. (cf. Fig. 23).[64]

The importance of allenes is well established in the field of natural products. A considerable effort has been made to

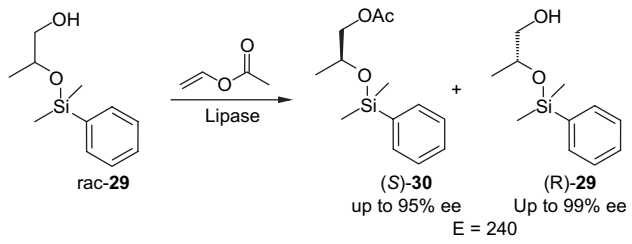

Figure 19. Lipase-catalyzed enantioselective transesterification of a 2-silyloxy-1-propanol **29**.[59]

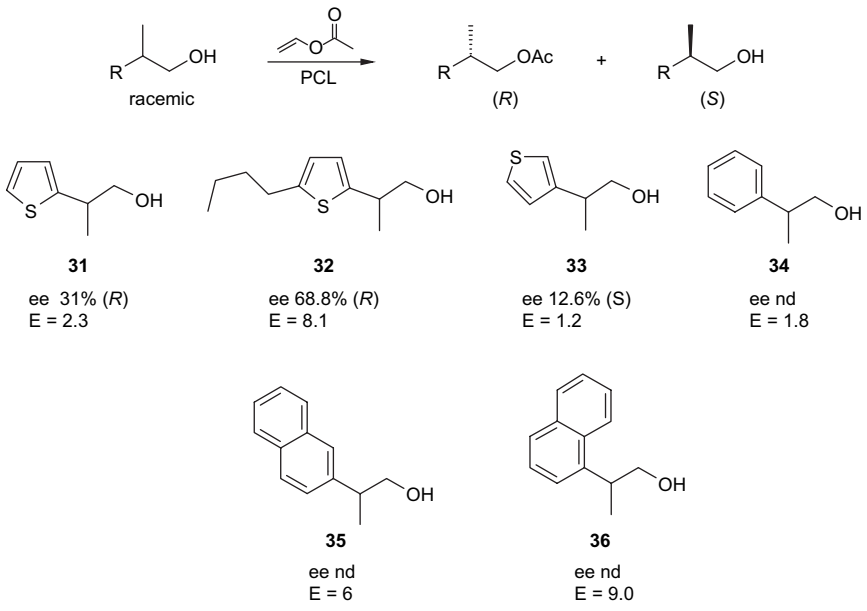

Figure 20. Lipase-catalyzed kinetic resolution of racemic 2-substituted 2-methylethanols by transesterification with vinyl acetate.[61]

access chiral allenes in enantiomerically pure/enriched forms. Among the methods used, lipase-catalyzed resolution of allenic alcohols is considered as one of the most fascinating in biochemical transformations. Primary allenic alcohols with axial chirality have been resolved by lipase from *Candida rugosa* (CRL) using the hydrolysis mode.[65] Thus, the enantioselective hydrolysis of racemic esters **54** and **56** (Fig. 24) was performed in phosphate buffer (pH 7.2) and *n*-hexane at room temperature to afford the alcohols (*R*)-**55** and **57** in enantiomerically pure/enriched form.

Examples of other primary alcohols **58**–**77**, which have also been successfully used as substrates for other lipases, are shown in Fig. 25.[56,66–73]

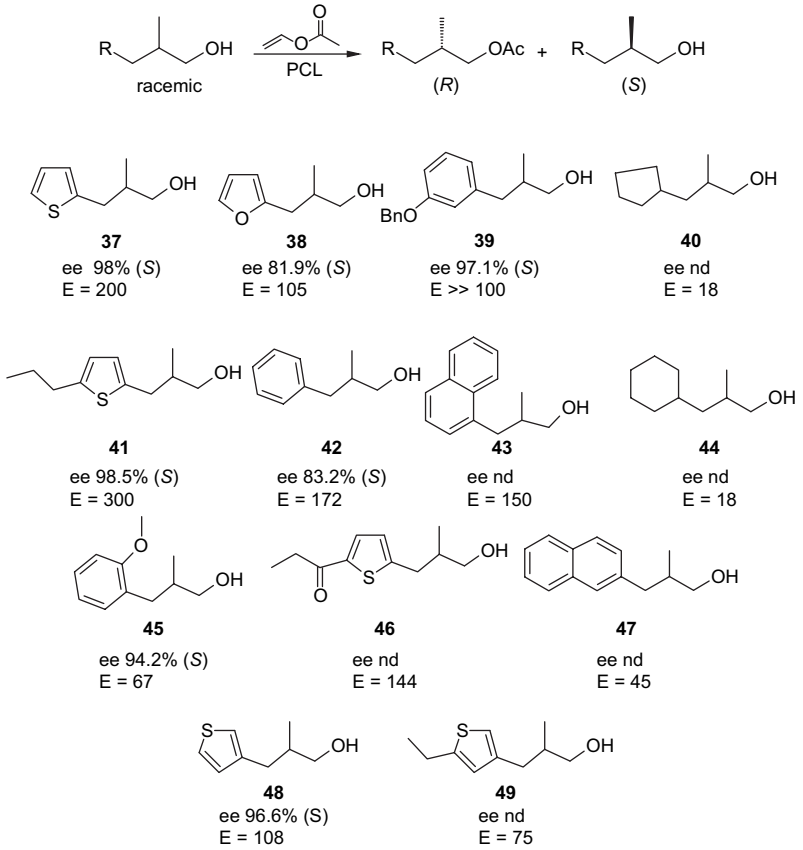

Figure 21. Lipase-catalyzed kinetic resolution of racemic 3-substituted 2-methylpropan-1-ols by transesterification with vinyl acetate.[62]

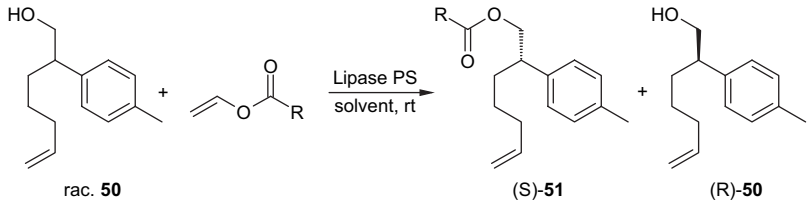

Figure 22. Lipase-catalyzed kinetic resolution of *rac*-**50**.

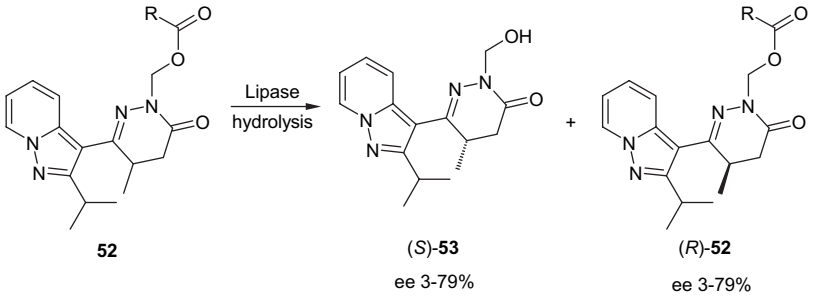

Figure 23. Lipase-catalyzed resolution of 2-(acyloxymethyl)-4,5-dihydro-5-methylpyridazin-3(2*H*)-one derivatives **52**.

Apart from the transesterification and hydrolysis mode used in the above examples, direct enzymatic esterification catalyzed by immobilized *C. antarctica* lipase B (CAL-B) and *Rhizomucor miehei* lipase (RML) was investigated in the kinetic resolution of a set of primary alcohols with a stereogenic centre at the next carbon atom, e.g., 2-methoxy-2-phenylethanol, 2-phenyl-1-propanol and 1-phenyl-1,2-ethanediol. Thus, the esterification reactions with different acids used as acyl donors (e.g., pentanoic, 4-oxopentanoic, 4-pentenoic, *trans*-2 and -3-pentenoic, hexanoic, octanoic, decanoic and *cis*-9-octadecanoic acids) were performed in a solvent-free system with the removal of water at low pressure. The results revealed that CAL-B was superior to RML in both reaction rates and enantioselectivity and 4-oxopentanoic acid gave the best results.[74]

3.1.1.1.2. Secondary alcohols. Secondary alcohols are frequently used as excellent targets in lipase-catalyzed kinetic resolutions.[75] This is due to their utility in organic synthesis and their higher enantioselectivity in resolutions, compared to those in primary and tertiary alcohols, which are difficult to achieve.[76–86]

Numerous examples can be found in the literature and only a few selected examples are included in this survey.

Of particular interest are allylic enynes and propargylic alcohols **78** and **80** (Fig. 26), which have been successfully resolved by lipase (Novozyme 435)-catalyzed transesterification with vinyl acetate in organic solvents to afford (*R*)-**79** and (*R*)-**81**, respectively. The unreacted substrates (*S*) were recovered with high ee (up to 99%), while the esters (*R*) were obtained in >99 and 96% ee, respectively.[87]

Instead of two reaction steps, Kamal et al. reported a one pot, lipase-catalyzed synthesis of enantiopure secondary alcohols **3** and **82–89** starting from a carbonyl compound.[88] Thus, the reduction of acetophenones with sodium borohydride in the presence of neutral alumina in *n*-hexane followed by enantioselective acylation catalyzed by lipases was performed in one pot (Fig. 27).

Starting from the readily available 2-hydroxycyclobutanone (acyloin) **90** (Fig. 28) or the corresponding acetals **92** (Fig. 29), Hazelard et al. reported the preparation of optically active 2-hydroxycyclobutanone and derivatives **92**, by enantioselective transesterification with various lipases in organic solvents. Due to the racemization drawback, alcohol **90** was recovered in racemic form (25–45% yield), while the (*R*)-ester **91** was obtained in up to 89% ee, depending on the lipase, the acyl donor and the solvent used.[89]

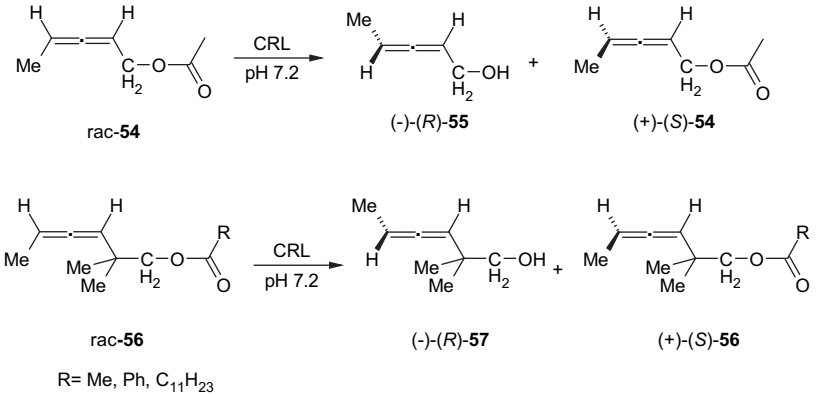

Figure 24. Lipase-catalyzed enantioselective access to enantiomerically pure/enriched allenic alcohols.[65]

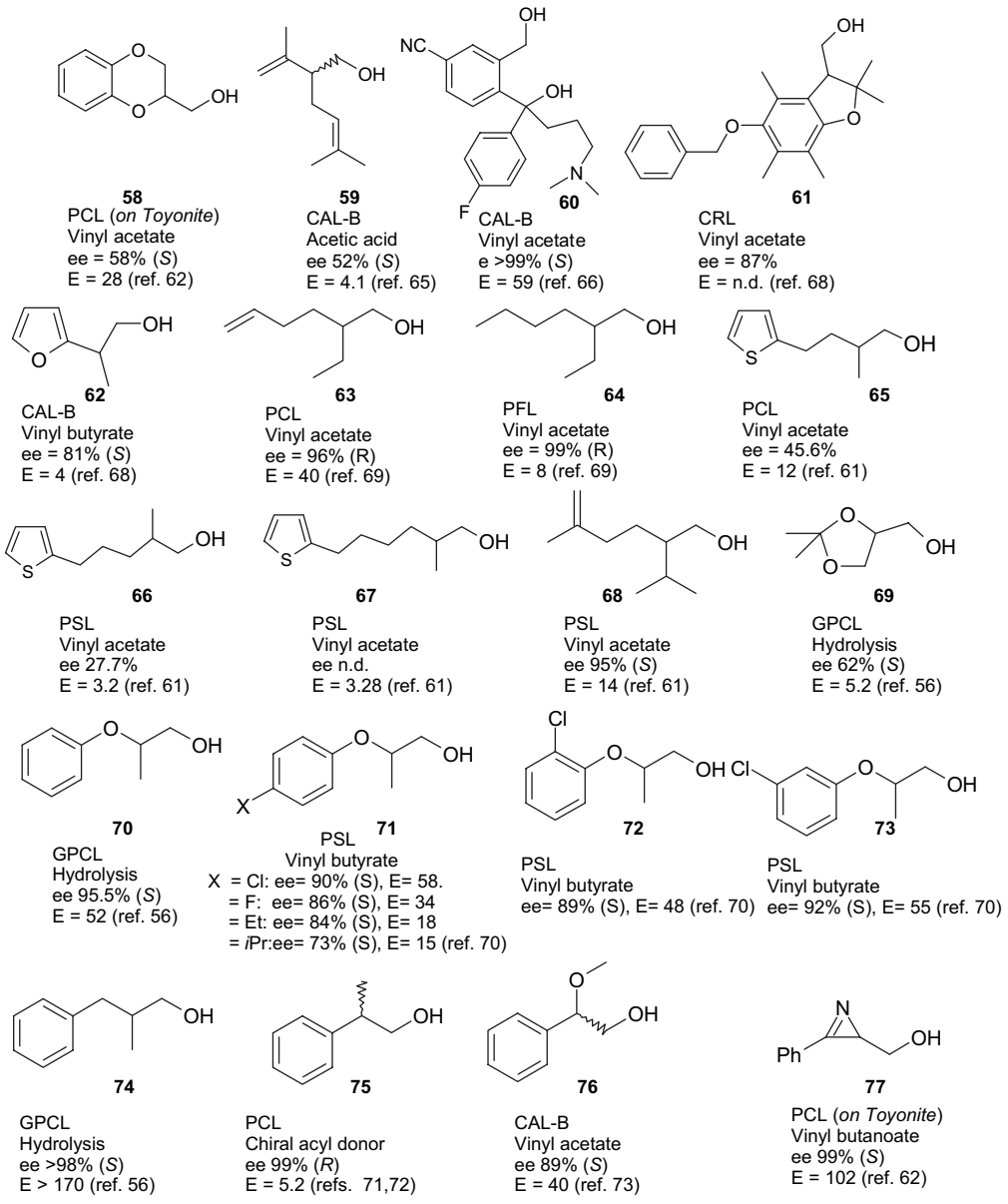

Figure 25. Selected examples of some primary alcohols resolved by lipases (see Refs. 56, 61, 62, 65, 66, 68–73).

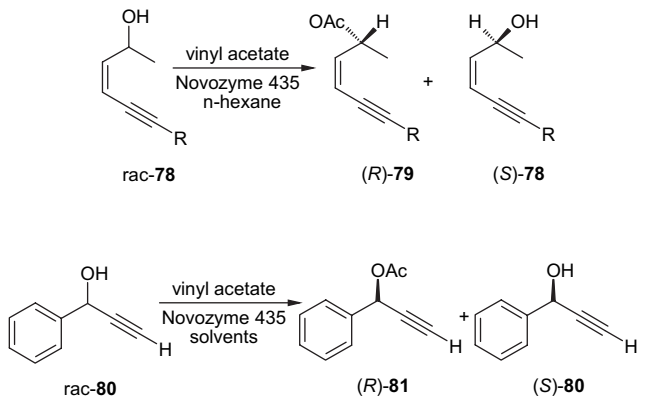

Figure 26. Lipase-catalyzed enantioselective transesterification of allylic enynes **78** and propargylic alcohol **80**.

The hydroxy methyl acetal **92** was cleanly converted using lipase CAL-B into the acetate, (*R*)-(+)-2-acetoxycyclobutanone methyl acetal (*R*)-**93**, in 42% yield (ee>97%), while the unreacted alcohol, (*S*)-(+)-hydroxycyclobutanone methyl acetal (*S*)-**92**, was recovered in 39.5% yield (ee=99.9%) and high enantiomeric ratio ($E>700$). On the other hand, the hydroxy ethyl acetal **94** was slowly converted into the corresponding acetate (*R*)-**95** with 98% ee, while the remaining hydroxy acetal (*S*)-**94** was only recovered with 64% ee with $E=190$.[89]

A new route to protected acyloins and their enzymatic kinetic resolution with lipases has been reported. Thus, the racemic acyloin esters **96** (Fig. 30) were hydrolysed using different lipases in phosphate buffer (pH 7) at ambient temperature to afford the free acyloins (*S*)-**97**.

Other secondary alcohols containing benzofuran, azide, alkylthio, carboxylic acid ethyl ester and α-methylene-β-

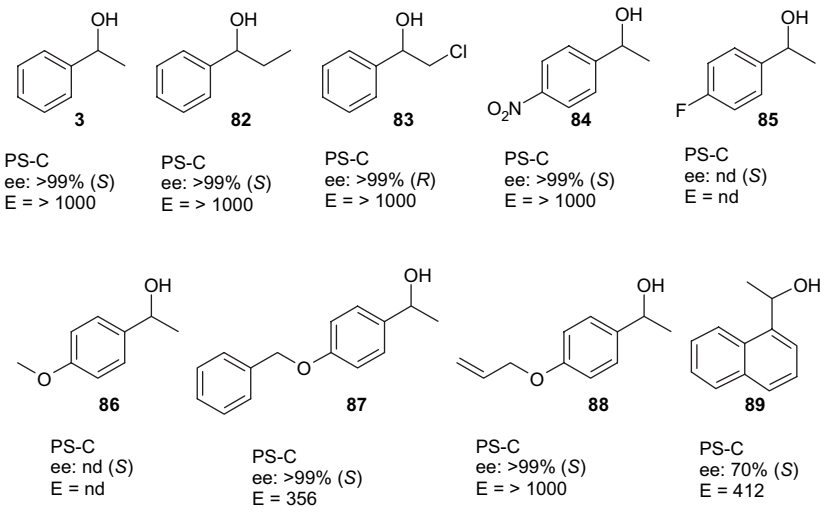

Figure 27. Selected examples of a one pot, lipase-catalyzed synthesis of enantiopure secondary alcohols using isopropenyl acetate as acyl donor.[88]

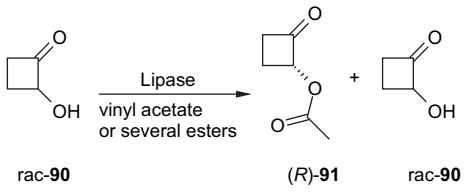

Figure 28. Asymmetric access to 2-hydroxycyclobutanone (acyloin) **90** using lipase in the transesterification mode.

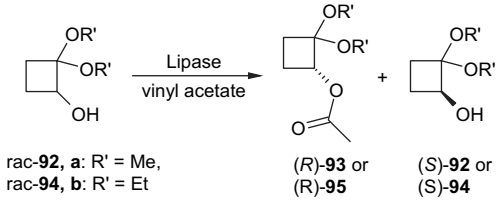

Figure 29. Lipase-catalyzed enantioselective transesterification of a hydroxy methyl (or ethyl) acetal.

hydroxy ester moieties **98–118** have been successfully resolved (Fig. 31).

Of particular interest, the rapid screening of different hydrolases for the enantioselective hydrolysis of esters of difficult to resolve substrates such as pentalactone **115**, 1-methoxy-2-propanol **116**, 3-butyn-2-ol **117** and 3-hydroxy-tetrahydrofuran **118** was studied by Baumann et al.[105] The screening was performed in a pH-indicator-based format in microtiter plates.

Biaryls and arylpyridines form the basic structure of many biologically active compounds,[106] and are found in new

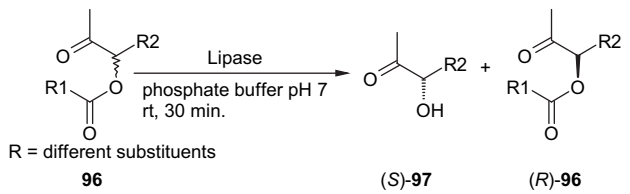

Figure 30. Lipase-catalyzed enantioselective hydrolysis of protected acyloins **96**.

materials such as electroluminescent conjugated polymers[107] and semiconductors.[108] Furthermore, their optically active forms have found widespread application as ligands in catalytic asymmetric synthesis.[109] These interesting applications prompted Rebolledo et al. to investigate the lipase-catalyzed resolution of 1-biaryl- and 1-(pyridylphenyl)alkan-1-ols **119**, **121** and **123**.[110] Thus, lipase B from *C. antarctica* (CAL-B) was used in the enantioselective transesterification of some 1-biaryl-2-yl-, -3-yl- and -4-yl-ethanols and -propan-1-ols, as well as 1-(*o*-, *m*- and *p*-pyridylphenyl)ethanols, **119**, **121** and **123**, with vinyl acetate as the acyl donor to afford (*R*)-**120**, (*R*)-**122** and (*R*)-**124**, respectively (Figs. 32–34). In the case of the resolution of *meta*- and *para*-substituted substrates (Fig. 32), these were transformed within several hours (conversion degrees ranging from 23 to 50%), but the reaction rates for propan-1-ol derivatives were slower than those for ethanol derivatives. The transesterifications of *ortho*-substituted alcohols took several days (Fig. 33) and yielded (*S*)-**121** with 31% ee and (*R*)-**122** in an enantiomerically pure form with enantioselectivity $E>200$. These reactions were, however, accompanied by a chemoenzymatic side reaction consisting of the formation of another acetate **125** derived from the hemiacetal between **121** and acetaldehyde released from vinyl acetate (Fig. 34). This side reaction was suppressed in the presence of isopropenyl acetate as the acyl donor, and the conversion degrees for transesterification ranged between 20 and 40% after 10 days with an enantioselectivity $E>200$.[110]

Of particular interest to polymer chemists is the polyester synthesis via a lipase-catalyzed reaction developed by Gross et al.[111] Thus, the extraordinary activity of lipase has been used to build up and modify high-molecular-weight polymers through the formation of ester bonds under mild conditions in a one-pot step without the need of a solvent. The high selectivity of lipase streamlines the reactions by eliminating the need for protection and deprotection of reactive side groups. The natural role of lipases is to cleave the C–O ester bonds in triglycerides, but, in polyester synthesis, the lipase works in reverse, forming C–O ester bonds instead of cleavage.[112] Gross et al. reported several examples of polyester synthesis, among which are the solvent-free condensations of polyols (having three or more

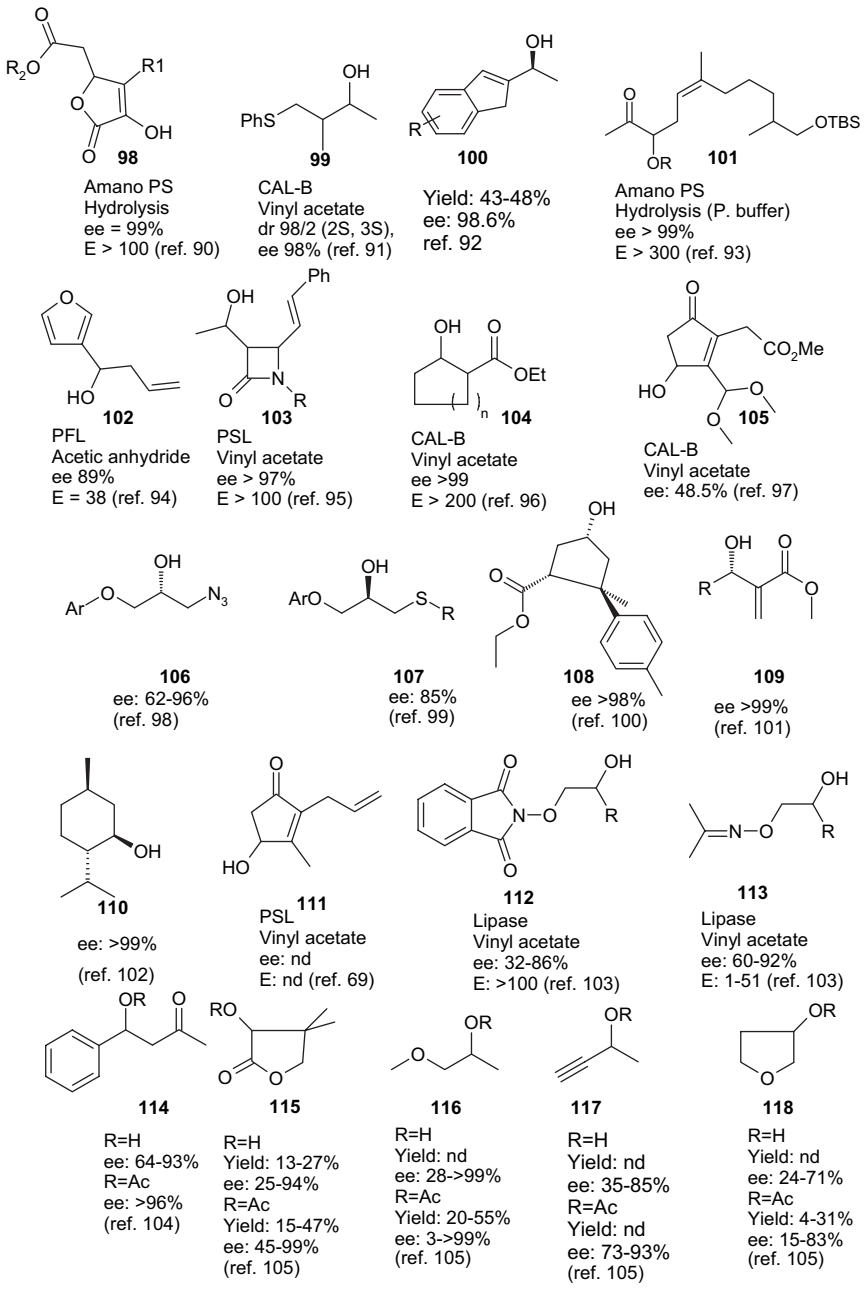

Figure 31. Selected examples of lipase-catalyzed kinetic resolution of secondary alcohols and difficult to resolve substrates (see Refs. 69, 90–105).

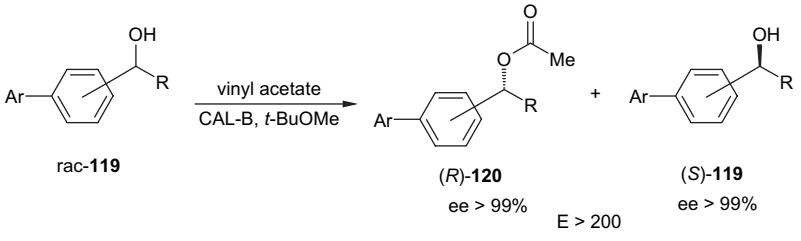

Figure 32. Lipase-catalyzed resolution of *para*- and *meta*-biaryl alcohols *rac*-**119** and *rac*-**119**.[110]

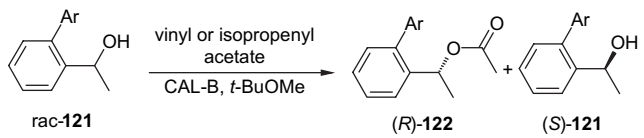

Figure 33. Lipase-catalyzed resolution of *o*-biaryl alcohols *rac*-**121**.[110]

hydroxyl group) as well as lactone and cyclic carbonate ring-opening polymerizations. Furthermore, the authors showed that the lipase, Novozym 435, added to the melt of high-molecular-weight polyesters catalyzes rapid transesterification reactions to form block and random copolymers.[113]

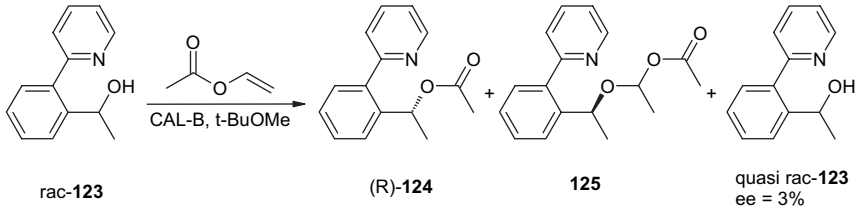

Figure 34. Lipase-catalyzed resolution of biaryl alcohol **123** showing the chemoenzymatic side reaction consisting of the formation of another acetate **125** derived from the hemiacetal between **123** and acetaldehyde coming from vinyl acetate.[110]

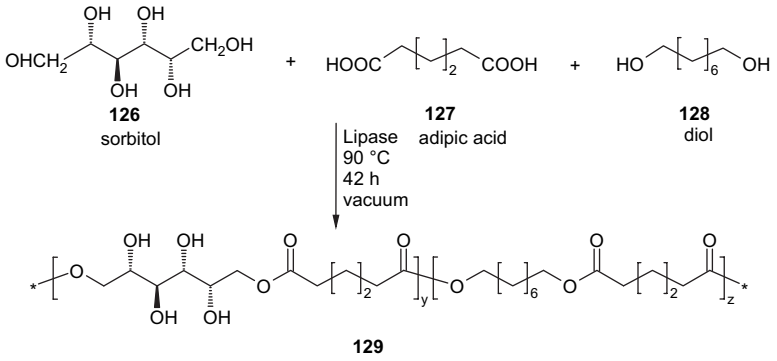

Figure 35. Lipase-catalyzed polymerization of sorbitol **126**.[114]

In the case of polyol polyesters, the condensation of binary, ternary, or more complex mixtures of diacids **127**, diols **128**, and reduced sugar polyols **126** (Fig. 35) without solvents results in the formation of polyesters such as poly(sorbityl adipate) **129**.[114] These polymers can be produced by chemical methods, but multistep protection–deprotection is needed to avoid crosslinking between secondary alcohol groups. The reactions normally require temperatures above 200 °C, which does not allow the use of monomers with sensitive substituents such as vinyl groups.[114] Using the same technique, the lipase-catalyzed polymerization of glycerol **130** to form terpolyesters such as **131** has been reported (Fig. 36).

In case of the lactone and cyclic carbonate ring-opening polymerizations, Gross et al. showed the rapid lipase-catalyzed caprolactone **133** ring opening to polycaprolactone **134** in the presence of carbohydrate initiator **132** (Fig. 37). A high conversion was reached in as little as four hours with molecular weights near 45,000 when using CAL-B.[111]

One of the most important advantages of lipase catalysis over traditional chemical catalysis that is evident in the above-mentioned examples of polymer synthesis is that lipases are not oxygen sensitive and benefit from the presence of water in reactions. In contrast to lipase catalysts, chemical catalysts are usually water sensitive and require moisture safeguards. Furthermore, the polymerization via lipase

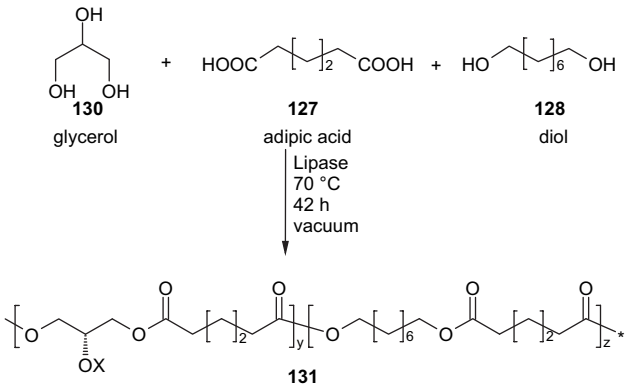

Figure 36. Lipase-catalyzed polymerization of glycerol **130**.[114]

reactions is metal free. The latter property is important in applications where metals can lead to problems in product use, such as toxicity and interference in electronic materials. [112]

3.1.1.1.3. Tertiary alcohols. Enzyme-catalyzed transesterification is an established strategy for the resolution of racemic secondary alcohols. The application of this method to tertiary alcohols is, however, less common. Towards a straightforward synthesis of the cyclopentenoid antibiotic, (+)-kjellmanianone, Christoffers et al. reported the enzymatic resolution of the kjellmanianone **135** using CAL-B

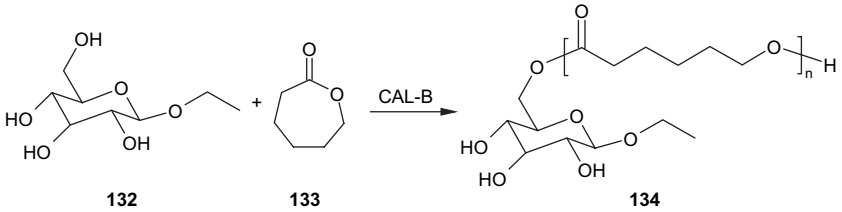

Figure 37. Lipase-catalyzed ring opening of lactone **133** with carbohydrate initiator.[112]

to afford (−)-kjellmanianone with high ee (>98%).[115] Attempts to resolve other tertiary alcohols[133–139] are documented in the literature[116–121] (Fig. 38).

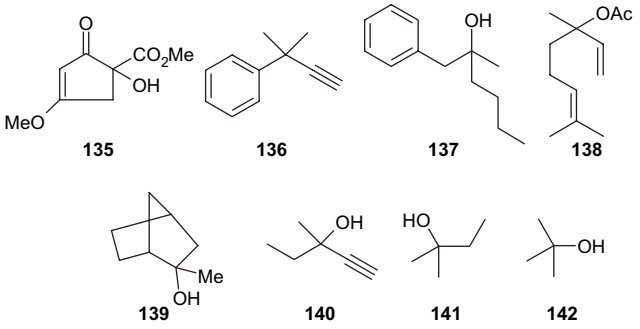

Figure 38. Lipase-catalyzed kinetic resolution of tertiary alcohols.

The lipase-catalyzed kinetic resolution of other chiral building blocks such as chiral carboxylic acids and diols has recently been reviewed.[85]

3.2. Dynamic and domino dynamic kinetic resolution

3.2.1. Dynamic kinetic resolution. As has been demonstrated above, kinetic resolution of racemic mixtures catalyzed by lipases is one of the most popular methods used to access enantiomerically pure/enriched compounds. Such conventional enzymatic kinetic resolution, however, has an intrinsic limitation. Thus, in the ideal case, the reaction is stopped after 50% conversion when all of the reactive enantiomer has been converted into the product and the resolution of the two enantiomers will provide a maximum 50% yield of the enantiomerically pure materials. In many cases, it is even less than 50%, as it is necessary to stop the reaction at a lower conversion than 50% when the differences in reaction rate between the two enantiomers are not large enough to achieve good enantioselectivity of the product. Furthermore, the product should be isolated from the unreacted pure/enriched enantiomer, which may cause separation problems. Such limitations can be overcome in several ways including the use of *meso* compounds or prochiral substrates,[14a] inversion of the stereochemistry (stereoinversion) of the undesired enantiomer (the remaining unreacted substrate),[122] racemization and recycling of the undesired enantiomer and dynamic kinetic resolution (DKR) by being coupled with a racemization reaction for the in situ conversion of undesired enantiomers to products.[123]

In both conventional and dynamic kinetic resolution (Fig. 39), the enantiomeric (*R*)-substrate is transformed into the (*R*)-product faster than the enantiomeric (*S*)-substrate ($k_R > k_S$). The only difference is that, in conventional kinetic resolution, the enantiomeric (*S*)-substrate is left behind as unreacted starting material, while, in the case of dynamic kinetic resolution, the substrate is continuously

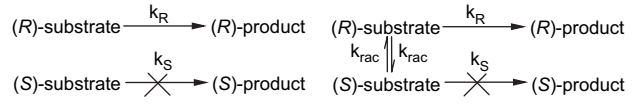

Figure 39. (a) Conventional kinetic resolution (max. 50% conv.). (b) Dynamic kinetic resolution with theoretical 100% yield.

enantiomerised during the resolution process, and thus the (*R*)- and (*S*)-substrates are in equilibrium, which allows for the possibility of converting all starting materials of (*R*)-substrate into (*R*)-product. Several conditions should be applied and these are reviewed in the literature.[123] Among these conditions, an efficient kinetic resolution has to be identified ($k_R \gg k_S$), an efficient racemization method should be established, and both the kinetic resolution and the racemization procedures should be compatible with one another.

Several groups have reported the use of a metal complex as the racemizing catalyst with an enzyme for the DKR. In one example, Williams and Allen reported a lipase–palladium combination for the DKR of allyl acetates,[124] and a lipase–rhodium combination for the DKR of secondary alcohols.[125] In the same year, Reetz et al. reported the DKR of 1-phenylethylamine by a lipase–palladium combination.[126] Soon after this work, the Bäckvall group reported substantially improved DKRs of secondary alcohols.[127,128]

Bäckvall et al.[128] used a combination of an enzyme and a transition-metal complex (Ru catalyst) to perform the DKR of a set of secondary alcohols (Fig. 40). Depending on the substrate, the chemical yield was ranged from 60 to 88%, with more than 99% ee.[129] The combination of ruthenium and enzyme catalysis was also applied to the DKR of secondary symmetrical diols (as *meso*/(±) mixtures) **143** to afford **144** in high yield and ee (Fig. 41).[130]

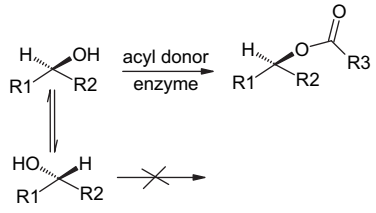

Figure 40. Dynamic kinetic resolution of secondary alcohols.

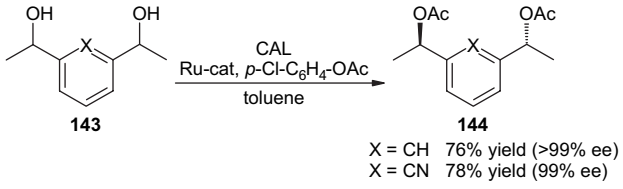

Figure 41. DKR of symmetrical secondary diols **143**.

Racemic α-hydroxy esters **145** were also subjected to the chemoenzymatic DKR methodology to afford **146**. Thus, transesterification in cyclohexane using immobilized PS-C as the biocatalyst with *p*-chlorophenyl acetate as the acyl donor was combined with ruthenium-catalyzed racemization (Fig. 42).[130] Under these conditions, various α-hydroxy

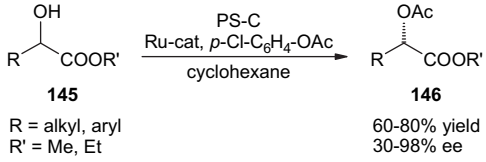

Figure 42. Chemoenzymatic DKR of racemic α-hydroxy esters.

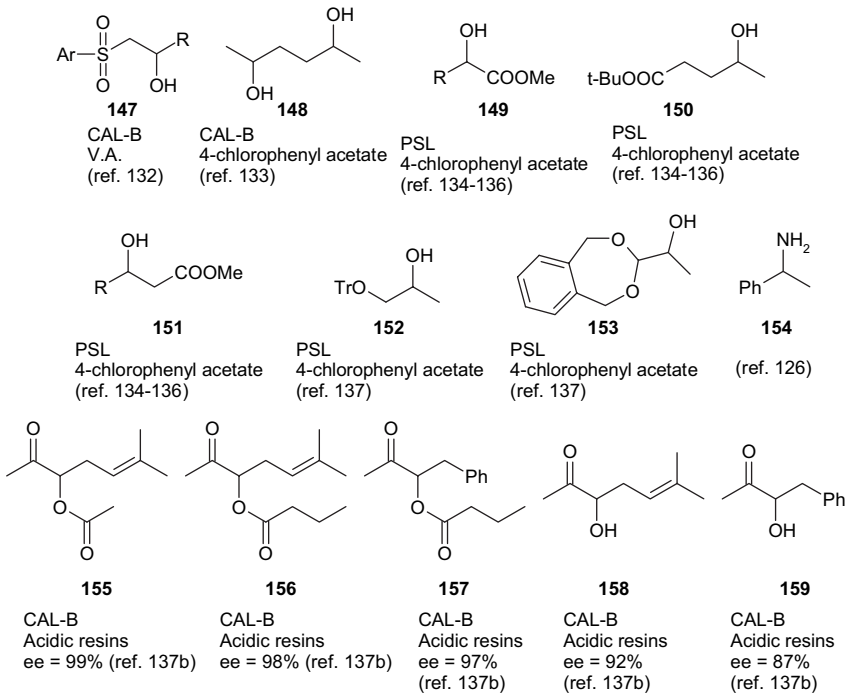

Figure 43. Selected examples of DKR (see Refs. 126, 132–137).

esters were deracemized in moderate to good yields and enantioselectivity. Other examples demonstrating the efficiency of DKR are reviewed in the literature,[130,131] some of which (**147**–**159**) are mentioned below (Fig. 43).

Other examples of dynamic kinetic resolution of secondary alcohols using an air-stable racemization catalyst **160** are mentioned in the literature[138] (Fig. 44).

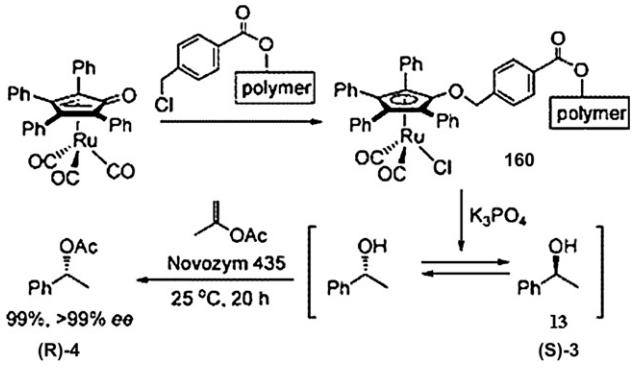

Figure 44. Air-stable racemization catalyst for dynamic kinetic resolution of secondary alcohols at room temperature.[138]

3.2.2. Domino dynamic kinetic resolution.

3.2.2.1. Definition. The usual way to synthesize an organic compound is the stepwise formation of the individual bonds in the target molecule. It would be advantageous, however, if several bonds could be formed in one sequence without isolating the intermediates, changing the reaction conditions, or adding reagents.[139] Such reactions would be ecologically and economically viable due to the minimization of waste, labour and energy necessary to conduct a stepwise reaction. This type of transformation is called a domino reaction. The term 'domino' was chosen from the game where several domino pieces are put up in one row and is in agreement with the time-resolved succession of reactions. If the first domino is knocked over, all others follow without altering the conditions. In some literature, the term 'cascade' has been used, but, it does not describe the real meaning and is used to describe other phenomena. Therefore, the term 'domino' is the best to describe a reaction correlated to the number of bonds formed in one sequence.[140]

3.2.2.2. Reactions. As has been noted by Brenna et al., in both DKR and KR, the installed acyl groups have seldom been utilized as part of the constituent structure for subsequent intramolecular reactions, and these groups are usually removed or replaced with other groups during subsequent transformations.[141] A realization of the effective use of the acyl moiety by a domino process has been highly desired, since it could minimize the use of chemicals, reduce the waste, and shorten the transformation steps.[142] Akai et al. reported the first example of a lipase-catalyzed domino process as an efficient approach towards the one-pot synthesis of optically active decalins.[143] Thus, three separate reactions are combined into one, where the DKR of racemic alcohols **161** (Fig. 45) was performed using lipase from *C. antarctica* B (CAL-B), functionalized ethoxyvinyl esters (racemic 3-vinylcyclohex-2-en-1-ols) **161** and a ruthenium catalyst in several organic solvents in the presence of molecular sieves (enantioselective KR), followed by the in situ racemization of the slow-reacting enantiomer (*S*) (DKR) and the Diels–Alder reaction of the intermediates (*R*). All proceeded under identical conditions to achieve the domino reaction with DKR. The fast racemization of the slow-reacting enantiomer (*S*) with reduced formation of the side product by the ruthenium catalysts used was the key to the success of this process. After five days, **162** (90–93% ee, 44–47% yield) was isolated along with (*S*)-**163** (99% ee, 46–50% yield).[143]

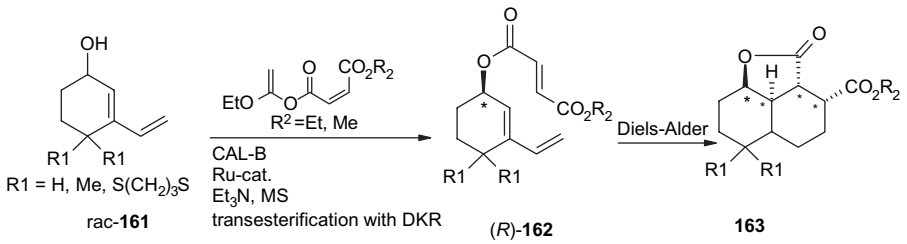

Figure 45. Lipase-catalyzed domino process as an efficient approach towards the one-pot synthesis of optically active decalins.

The domino kinetic resolution was extended to directly provide tetrahydrofuro[3,4-*c*]isoxazole derivatives (**5** and **9**) in 90% ee from racemic α-hydroxynitrones (**2** and **6**), which were used in the concise asymmetric total synthesis of (−)-rosmarinecine.[144] Thus, CAL-B effectively catalyzed the kinetic resolution of **161** in toluene at 20 °C. The subsequent intramolecular dipolar cycloaddition of the thus-generated ester (*R*)-**165** was so fast that the cycloadduct **166** (93% ee, 52% yield) was directly obtained as a single diastereomer along with the recovery of (*S*)-**164** (99% ee, 38% yield) (Fig. 46).

Based on these results, Akai et al. reported the domino reaction of the cyclic nitrone **167** with the acyl donor yielding **169**, which leads to (−)-rosmarinecine **170**, a typical necine-base portion of some natural alkaloids such as rosmarinine (Fig. 47). Although the reaction was problematic, due to some side reactions, the authors were able to isolate **168** with 94% ee and 49% yield.[144] Enzyme-initiated domino reactions have been reviewed.[140,142]

3.3. Lipase-catalyzed enantioselective synthesis of enantiomerically pure/enriched compounds

The use of lipases in organic synthesis is well established and has become an interesting area for organic and bioorganic chemists. For instance, some lipases catalyze the epoxidation of alkenes,[145] the conversion of sulfides into sulfoxides using hydrogen peroxide and a long-chain fatty acid,[146] ring opening,[147] and the Baeyer–Villiger reactions.[148] Lipases were also recently applied in aminolysis and ammonolysis reactions.[149] Conde et al. have recently described the regioselective amidation of *N*-blocked glutamic acid diesters **171** catalyzed by CAL-B. Depending on both the absolute configuration of the stereogenic centre and the *N*-protecting group of the aminodiester, different molecular ratios of γ:α monoamides are obtained.[150] As a conclusion, L-glutamic derivatives, L-**171**, produced α-regioisomers α-**172**, but D-glutamic derivatives, D-**171**, mainly led to γ-derivatives γ-**172** and **173** as a minor product with the regioselectivity being dependent on the nature of the *N*-protecting group of the diester (Fig. 48).

The regioselectivity of lipases in aminolysis reactions is also demonstrated in the case of the nucleophile. Thus, the regioselective acylation of pyrimidine-3,5-diamino-2,3,5-trideoxynucleosides **174** can be carried out by choosing different biocatalysts, e.g., lipase PCL-C preferentially catalyzes the acylation at the 3-position forming **176**, while CAL-B shows an excellent regioslectivity towards the 5-amino substituent giving **175** (Fig. 49). Other examples have also been reviewed.[149,151,152]

Lipases have recently been used in carbon–carbon bond-forming reactions. Thus, the mutants, Ser105Ala and Ser105Gly, which lack the nucleophilic serine residue in the catalytic triad, showed a clear aldolase activity and the aldol reaction was performed at room temperature with 0.3–0.5 M aldehyde (propanal or hexanal **177**) in cyclohexane to afford **178**, etc. in good yield (Fig. 50).[153]

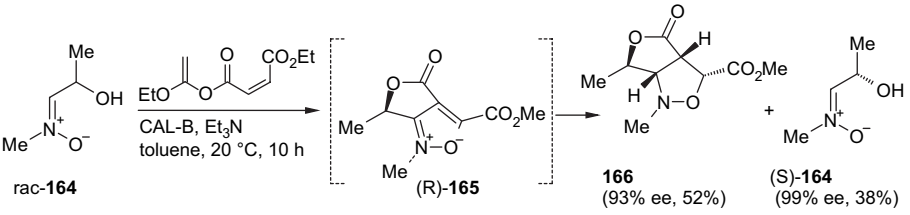

Figure 46. Lipase-catalyzed domino kinetic resolution of racemic α-hydroxynitrones **164**.

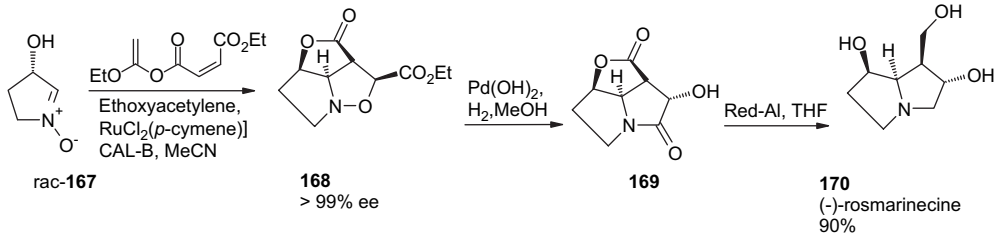

Figure 47. Lipase-catalyzed domino kinetic resolution of cyclic nitrone **167**.

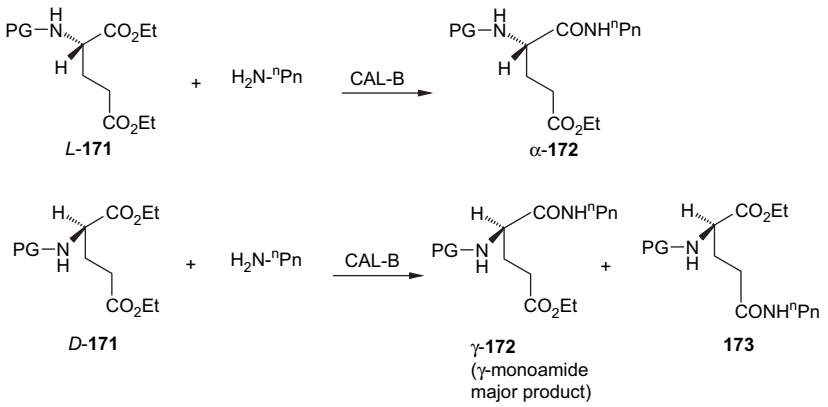

Figure 48. Lipase-catalyzed regioselective amidation of *N*-blocked glutamic acid diesters **171** (PG: protecting group).

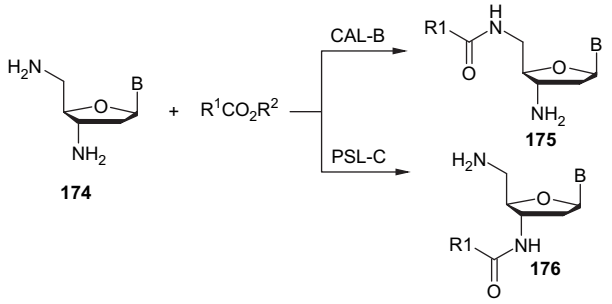

Figure 49. Lipase-catalyzed regioselective acylation of pyrimidine-3,5-diamino-2,3,5-trideoxynucleosides **174**.

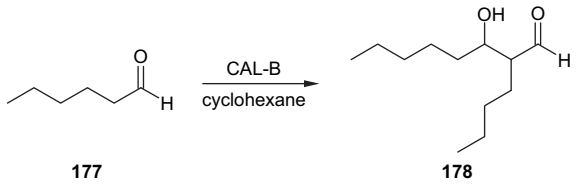

Figure 50. Lipase-catalyzed aldol reaction.

The wild-type enzyme was also able to promote this reaction, although to a lower extent. With the help of theoretical calculations, the authors proposed a mechanism in which the oxanion hole of the active site stabilizes the negative charge of the transition states and the His-Asp pair serves as a proton shuttle. Considering this mechanism, Gotor et al. realized that this enzyme could also catalyze the Michael addition.[154] Thus, in the presence of CAL-B, acrylonitrile reacted with a set of secondary amines **179** to afford the corresponding Michael adducts **180** up to 100-fold faster than the process in the absence of the biocatalyst (Fig. 51).

Other examples of enantioselective C–C bond synthesis catalyzed by enzymes have recently been reviewed by Hanefeld and Sukumaran.[155]

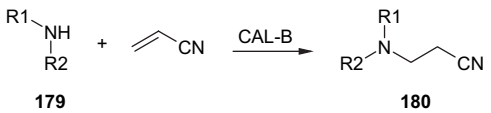

Figure 51. Lipase-catalyzed Michael addition of secondary amines to acrylonitrile.

4. Lipase mechanism, kinetics and enantioselectivity

4.1. Mechanism

Lipases differ widely in the number of amino acids in their primary sequences, e.g., *C. rugosa* lipase contains more than 500 amino acids, whereas *C. antarctica* lipase B contains 317 amino acids. The common feature of all lipases is that their active site is built up of three amino acids, serine, aspartate or glutamate and histidine, which are referred to as the catalytic triad. The 3D structure of all lipases reveals a feature known as an α/β-hydrolase fold. In essence, the α/β-hydrolase fold consists of a central β-sheet surrounded on both sides by a variable number of α-helices[1] (Fig. 52). The database, ESTHER (ESTerases, α/β-Hydrolase Enzymes and Relatives), lists the 3D structures of all known α/β-hydrolase-fold proteins (at http://www.ensam.inra.fr/cholinesterase/).

The catalytic triad and several oxanion-stabilizing residues are thought to compose the active centre of lipases. A lid or flap (a helical segment), which blocks the active centre, is responsible for the important characteristics of lipase interfacial activation. Based on the X-ray structure of lipase before and after binding to the substrate, researchers believe that lipases are activated before they take part in biochemical transformation reactions.[1] The water-soluble lipases take part in the catabolism of lipids. Thus, the lipase reaction takes place at a water–oil interface.

A freely dissolved lipase in the absence of an aqueous/lipid interface resides in its inactive state and a part of the enzyme molecule covers the active side. When the enzyme contacts the interface of a biphasic water–oil system, however, a short

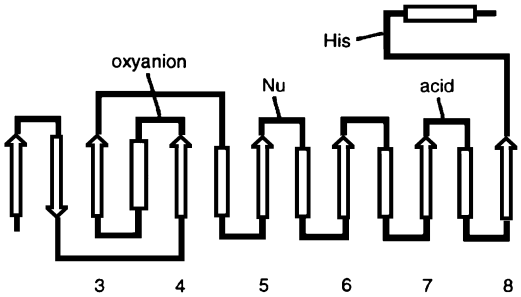

Figure 52. Schematic representation of α/β-fold of hydrolase.[1]

α-helix (the lid) is folded back, leading to activation of the lipase. In other words, with no substrate present, the lid is closed and the enzyme is inactive, whereas, in the presence of a substrate, the water–oil interface exists, the lid is opened therefore, the lipase is active.[13] In contrast to most other lipases, *C. antarctica* lipase B (CAL-B) does not display interfacial activation. This is considered to be due to the fact that this enzyme lacks the lids that regulate access to the active site.[14d] Another unusual feature of this lipase is that it does not have the consensus amino acid sequence, GXSXG, around the active-site serine that is common to the folding family of hydrolases. Instead, it has a threonine at the position of the first conserved glycine.[156]

The catalytic triad (Ser, His and Asp) exists in all hydrolases and the actual chemical operator at the active side is demonstrated in the mechanism of a serine-hydrolase-catalyzing hydrolysis of an ester (Fig. 53).

The special arrangement of these three groups causes a decrease of the p*K* value of the serine hydroxy group, thus enabling it to cause a nucleophilic attack on the carbonyl group of the substrate R_1COOR_2 (step 1). Thus, the acyl moiety of the substrate is covalently linked onto the enzyme, forming the acyl–enzyme intermediate and liberating the alcohol R_2OH. In the second step, water (regarded as the nucleophile, Nu) attacks the acyl–enzyme intermediate, regenerating the enzyme and releasing the carboxylic acid R_1COOH (step 2). Depending on the medium used (aqueous or organic), any other nucleophile can compete with water for the acyl–enzyme intermediate, thus leading to a number of synthetically useful transformations:

1. If the alcohol R_4OH attacks the acyl–enzyme intermediate, the ester R_1COOR_4 is formed (interesterification reaction).[18]
2. Attack of the amine R_3NH_2 results in the formation of an amide R_1CONHR_3 (enzymatic aminolysis of esters).[157]
3. Attack of hydrogen peroxide yields the peracid R_1COOOH.[146]
4. Hydrazinolysis yields hydrazides[158] and the action of hydroxylamine results in the formation of hydroxamic acid derivatives.[159]

4.2. Kinetics

Kinetic studies can reveal the enantiomer-discriminating step and the relative stability of the enantiomers in distinct

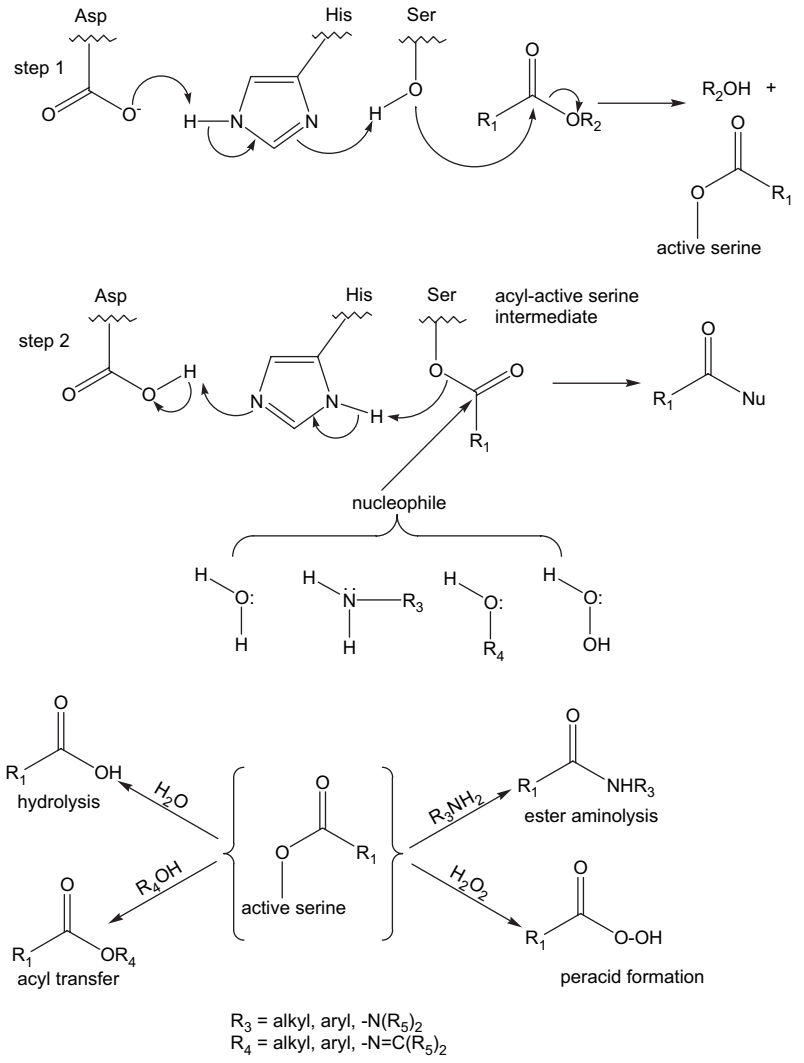

Figure 53. Serine hydrolase mechanism.

steps. Despite the usefulness of the kinetic data, only a limited number of kinetic studies have been performed on the enantioselective hydrolase-catalyzed reactions.[160] Kinetic studies have demonstrated that lipases do not follow the Michaelis–Menten kinetics, but rather show a sigmoidal increase in the reaction velocity with increasing substrate concentration. Many enzymatic reactions, however, follow a pattern of the basic model presented by Michaelis–Menten (Eq. 1). From the steady state assumption, they postulated the existence of an enzyme–substrate complex (ES), which is formed by the reversible reaction of substrate S and enzyme E and the rate of formation of ES from the free enzyme and substrate is exactly balanced by the rate of conversion of ES into to P. Thus, for a relatively short time during the experimental measurement of the velocity, the concentration of ES remains essentially constant. The latter is considered to be an oversimplified statement. Jencks has pointed out that the steady state criterion is met if the absolute rate of change of concentration of a transient intermediate is very small, compared to that of the reactants and products.[161]

$$\mathrm{E}+\mathrm{S} \underset{K_2}{\overset{K_1}{\rightleftharpoons}} \mathrm{ES} \overset{K_3}{\rightleftharpoons} \mathrm{E}+\mathrm{P} \tag{1}$$

The dissociation of the complex (ES) to E and P (product) is assumed to be the rate-determining step (Eq. 1).

K_1 is the rate constant for the association constant of the ES complex; K_2 is dissociation rate constant of the ES complex into E and S; K_3 is turnover number (also referred to as K_{cat}).

The Michaelis–Menten equation for the initial rate of reaction of a single substrate with an enzyme is given by Eq. 2.

$$v=\frac{v_{max}[\mathrm{S}]}{K_m+[\mathrm{S}]} \quad \text{where } K_M=\frac{K_2+K_3}{K_1} \tag{2}$$

K_M is Michaelis constant.

This equation provides a relationship between the velocity observed at any particular substrate concentration and the maximum velocity that would be achieved at infinite substrate concentration. The two quantities, v_{max} and K_M, are often referred to as the kinetic parameters of an enzyme and their determination is an important part of the characterization of any enzyme.

Quite often, enzymatic reactions involve two or more competing substrates, e.g., lipase-catalyzed esterification (Fig. 54). In this case, the standard state assumption is also valid. Two substrate enzymatic reactions often follow three mechanisms, i.e., rapid equilibrium random *bi bi*, ordered *bi bi* and Ping-Pong *bi bi* mechanisms.[162]

The lipase-catalyzed esterification follows the Ping-Pong *bi bi* kinetics. If a fatty acid (A) is bound to the lipase in the first step, followed by the release of water (P), then the alcohol (B) is bound to the acyl enzyme (F). Finally, the new ester (Q) is formed, regenerating the enzyme. The lipase oscillates between the free enzyme form (E) and the acyl-enzyme intermediate (F) (Fig. 55).

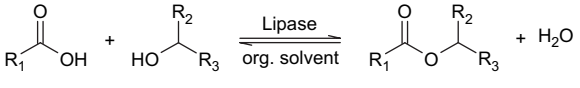

Figure 54. Lipase-catalyzed esterification reaction.

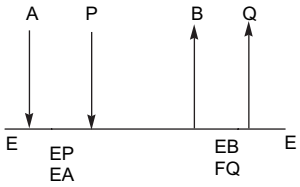

Figure 55. Ping-Pong *bi bi* mechanism.

The reaction equation corresponds to the following expression (Eq. 3):

$$v=\frac{v_{max}[\mathrm{A}][\mathrm{B}]}{K_m\mathrm{B}[\mathrm{A}]+K_m\mathrm{A}[\mathrm{B}]+[\mathrm{A}][\mathrm{B}]} \tag{3}$$

In the case of competitive substrate inhibition, the reaction rate (Eq. 4) can be expressed as

$$v=\frac{v_{max}[\mathrm{A}][\mathrm{B}]}{K_m\mathrm{B}[\mathrm{A}]+K_m\mathrm{A}[\mathrm{B}](1+[\mathrm{B}]/K_i+[\mathrm{A}][\mathrm{B}])} \tag{4}$$

If the concentration of alcohol B ([B]) in the reaction phase is constant, this equation can be written as the Michaelis–Menten equation (Eq. 5):

$$v=\frac{v_{max}[\mathrm{A}]}{K_m+[\mathrm{A}]} \tag{5}$$

Despite the differences in enzymes, substrates and reaction conditions, the following trend can be seen when searching in the literature: the difference in the v_{max} (or K_{cat}) value between the two enantiomers is much larger than that of the K_M value between the enantiomers. Therefore, it seems generally true that chiral recognition in the binding step (Michaelis complex) is unimportant and that the enantioselectivity results from the subsequent transition state.

Ema indicated, after several studies on lipases, that the enantioselectivity results from the greatly reduced activity of the enzymes for the slower-reacting enantiomers, but not from the enhanced activity for the faster-reacting enantiomers.[160] Comparison of his data revealed that the larger substituents of both enantiomers interact repulsively with the protein in the transition state. The author concluded that binding interactions are not at all important for enantioselectivity and the principle of enantioselectivity of lipases towards unnatural secondary alcohols cannot be classified into the traditional lock-and-key paradigm. Therefore, the 'non-lock-and-key concept' that differs from the traditional lock-and-key concept has been proposed.[160]

4.3. Enantioselectivity and chiral recognition

At present, when designing a biocatalytic process, much time and resources are spent on optimization and screening.

Therefore, a knowledge that would enable predictions of the outcome of the reaction would be most beneficial. A large amount of such information and knowledge has been acquired since the breakthrough of biocatalysis in the 1980s and several empirical and modelling-based rules and criteria are now available.[162] An enzyme model always describes the mechanism of the enantioselectivity in enzymatic reactions. The simplest models, more accurately referred to as rules, do not attempt to predict the degree of enantioselectivity, but only predict which enantiomer reacts faster. The earliest example of such a model is Prelog's rule,[163] which predicts the enantioselectivity of the reduction of ketones by yeast, based on the size of the two substituents on the carbonyl group. Other models based on pockets, which give an indication of the size and shape of the molecules tolerated in the active site, have been reviewed by Ema.[160] Examples of such models are the model of Jones et al. for pig liver esterase (PLE), subtilisin[164] and several lipases.[165–167] A more recent example is the empirical rule of Kazlauskas for chiral recognition by lipases.[168] This rule states that most lipases have the same enantiopreference towards a certain substrate, but only show a different enantioselectivity. It is translated into an active-site model for lipases consisting of two pockets of different size, a large and a small pocket (Fig. 56).

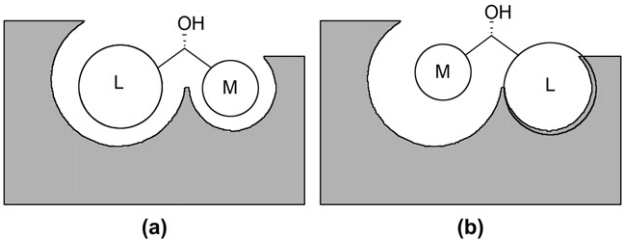

Figure 56. (a) Fast-reacting enantiomer (left) and (b) slow-reacting enantiomer (right) in the active-site model for lipases derived from Kazlauskas' rule.

The enantioselectivity for substrates bearing a small and a large substituent (e.g., a secondary alcohol, as shown in Fig. 56) is explained by assuming that, upon subjecting the secondary alcohol to resolution by a lipase, the fast-reacting enantiomer binds to the active side in the manner shown in Figure 56a. When the other enantiomer reacts with the lipase, however, it is forced to accommodate its large substituent into the smallest pocket (Fig. 56b). Thus, the steric repulsions between this substituent and the enantioselectivity pocket disrupt the catalytic triad and account for the lower reaction rate for this enantiomer. Regardless of the direction of the reaction (hydrolysis/esterification), (*R*)-enantiomers react faster.[160] Empirical rules for primary alcohols and carboxylic acid esters have been reported, although their reliability is lower than those for secondary alcohols.[160]

The enantiomeric purity of a chiral compound is expressed in terms of its enantiomeric excess (ee) value (Eq. 6) defined as

$$\%\mathrm{ee}_R = \frac{R-S}{R+S}\times 100 \quad \text{for } R>S \tag{6}$$

where R is the concentration of the (*R*)-enantiomer and S is the concentration of the (*S*)-enantiomer. Thus, for a racemic compound, the ee value is zero, whereas, for an enantiomerically pure compound, the ee value is 1 (or 100%).

Since lipases are chiral, they possess the ability to distinguish between the two enantiomers of a racemic mixture. The progress of the ee of the substrate ($\mathrm{ee_s}$) and product ($\mathrm{ee_p}$) versus the degree of conversion for a kinetic resolution is displayed in Fig. 57. At low conversion, the enzyme experiences a nearly

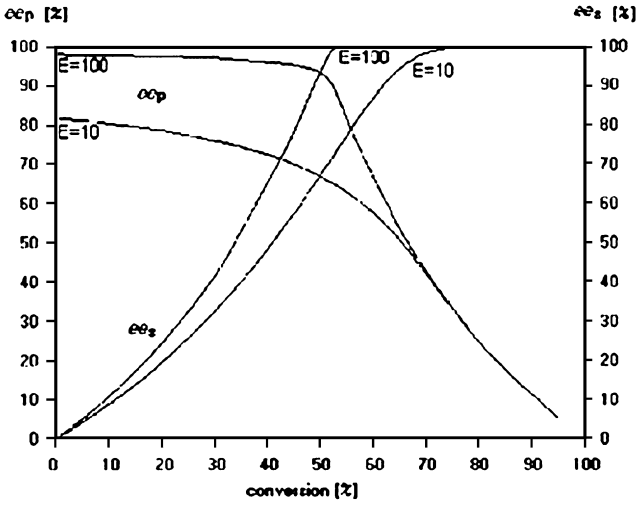

Figure 57. Plot of enantiomeric excess of substrate, $\mathrm{ee_s}$, and product, $\mathrm{ee_p}$, as a function of conversion of an enzyme-catalyzed irreversible kinetic resolution reaction. The plots were made with the computer program 'Selectivity' developed by the group of Faber, University of Graz (http://borgc185.kfuni-graz.act.at).

racemic concentration of the substrate (no or low $\mathrm{ee_s}$) and practically only catalyzes the conversion of the preferred enantiomer, yielding a high $\mathrm{ee_p}$. As the reaction proceeds further, the concentration of the preferred enantiomer decreases and the enzyme now experiences a relatively higher concentration of the other enantiomer, raising its probability to be converted, i.e., $\mathrm{ee_s}$ increases and $\mathrm{ee_p}$ decreases. The parameter of choice to describe the enantioselectivity (or stereoselectivity) of a lipase-catalyzed reaction is the enantioselectivity, which is also called the enantiomeric ratio E.

The E value is defined as the ratio of specificity constants for the two enantiomers (Eq. 7).

$$E_{RS} = \frac{(K_{\mathrm{cat}}/K_{\mathrm{M}})_R}{(K_{\mathrm{cat}}/K_{\mathrm{M}})_S} \tag{7}$$

where K_{cat} is the rate constant or the turnover number and K_{M} is the Michaelis–Menten constant.[169] Sih et al.[170,171] developed this equation in terms of the ee of the product ($\mathrm{ee_p}$), the unreacted substrate ($\mathrm{ee_s}$) and the conversion (c). Thus, for a reversible enzymatic reaction, the E value is expressed by Eq. 8:

$$E = \frac{\ln\left[1-(1+K)c\left(1+\mathrm{ee_p}\right)\right]}{\ln\left[1-(1+K)c\left(1-\mathrm{ee_p}\right)\right]} = \frac{\ln[1-(1+K)(c+\mathrm{ee_s})\{1-c\}]}{\ln[1-(1+K)(c-\mathrm{ee_s})\{1-c\}]} \tag{8}$$

where K is the equilibrium constant. When the reaction is irreversible or the reverse reaction is negligible (K=0), this equation is reduced to Eq. 9:

$$E = \frac{\ln\left[1 - c\left(1 + \mathrm{ee_p}\right)\right]}{\ln\left[1 - c\left(1 - \mathrm{ee_p}\right)\right]} = \frac{\ln[1 - c(1 - \mathrm{ee_s})]}{\ln[1 - c(1 + \mathrm{ee_s})]} \tag{9}$$

where c is expressed by Eq. 10:

$$c = \frac{\mathrm{ee_s}}{\mathrm{ee_s} + \mathrm{ee_p}} \tag{10}$$

Holmberg and Karlsson used Eqs. 8 and 9 of Sih et al. to distinguish between the enantiomeric ratio of the substrate (E_s) and that of the product (E_p),[172] where E_s and E_p are calculated according to Eqs. 11 and 12:

$$E_s = \frac{\ln[(1 - c)(1 - \mathrm{ee_s})]}{\ln[(1 - c)(1 + \mathrm{ee_s})]} \tag{11}$$

$$E_p = \frac{\ln\left[1 - c\left(1 + \mathrm{ee_p}\right)\right]}{\ln\left[1 - c\left(1 - \mathrm{ee_p}\right)\right]} \tag{12}$$

According to Eqs. 11 and 12, for the substrate, a high enantiomeric purity ($\mathrm{ee_s}$>98%) can be obtained, even with an E_s value as low as 5. The course of the kinetic resolution is, however, different for the product. Initially, $\mathrm{ee_p}=(E-1)/(E+1)$, then, at $c\sim$50%, the $\mathrm{ee_p}$ value rapidly diminishes, ending up as the racemic product at c=100%. Only systems with an E_p>100 can be used to obtain an $\mathrm{ee_p}$>98%. This kind of enantioselectivity is often not obtained and other strategies such as recycling of the product, coupled enantioselective action and sequential biocatalytical resolution have to be considered in order to obtain the desired $\mathrm{ee_p}$.[172]

It should be noted that the estimation of E by single-point measurements of $\mathrm{ee_s}$ or $\mathrm{ee_p}$ at one conversion is extremely unreliable as the determination of the conversion often lacks a high degree of accuracy. This may be overcome by the use of algorithms that calculate E by nonlinear regression of data obtained at several degrees of conversion.[173,174] The determination of conversion can be avoided if E can be calculated by another equation presented by Rakels et al.[175] (Eq. 13), where E is expressed only in terms of the $\mathrm{ee_s}$ and $\mathrm{ee_p}$ as follows:

$$E = \frac{\ln\left[\dfrac{1 - \mathrm{ee_s}}{1 + \left(\mathrm{ee_s}/\mathrm{ee_p}\right)}\right]}{\ln\left[\dfrac{1 + \mathrm{ee_s}}{1 + \left(\mathrm{ee_s}/\mathrm{ee_p}\right)}\right]} \tag{13}$$

A non-selective reaction has an E value of 1, while resolution with an E value above 20 is necessary to conduct a synthetic reaction.[1] These and other methods for the evaluation of E and their advantages and disadvantages are reviewed by Straathof and Jongejan.[175b]

4.3.1. Theory. Orrenius et al.[176,177] have developed a model to show how *C. antarctica* lipase B discriminates between the enantiomers of *sec*-alcohols. This model involves the binding of the two enantiomers in two different productive binding modes in transition states to allow catalysis and the conversion of the substrate enantiomers. Experimental and molecular modelling research showed that the two modes were necessary to allow both enantiomers to develop the hydrogen-bonding pattern within the active site required for catalysis. In Figure 58, a schematic presentation of the alcohol-binding site and the two productive binding modes are presented. As shown, the active site of CAL-B is deep and narrow. In the innermost part, there is a free volume designated as an enantioselectivity pocket. The fast-reacting enantiomer, generally (*R*), positions its medium-sized substituent of the alcohol moiety into the enantioselectivity pocket and its large substituent towards the active-site entrance. To keep the hydrogen-bonding pattern, the slow-reacting enantiomer, usually (*S*), is sterically more hindered and, hence, has to place its large substituent into this enantioselectivity pocket. Although the large substituent is not easily fitted into the enantioselectivity pocket, this is the catalytically active binding mode for the slow-reacting enantiomer. A steric difference between the transition states of the enantiomers can therefore explain the CAL-B ability to resolve the enantiomers. The model only considers differences in activation enthalpy, however, and not activation entropy, both of which are important for enantioselectivity.[178]

Taking into consideration that entropy is contributing to the enantioselectivity and enzyme catalysis in general, Hult

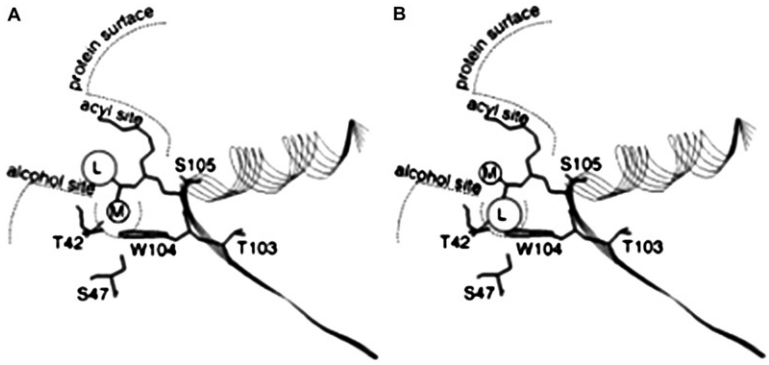

Figure 58. Simplified scheme of the two productive binding modes in the model proposed by Orrenius et al.[176,177] that rationalizes the enantioselectivity of *Candida antarctica* lipase B. (A) Fast-reacting enantiomer. (B) Slow-reacting enantiomer. (M) medium-sized substituent; (L) large-sized substituent.

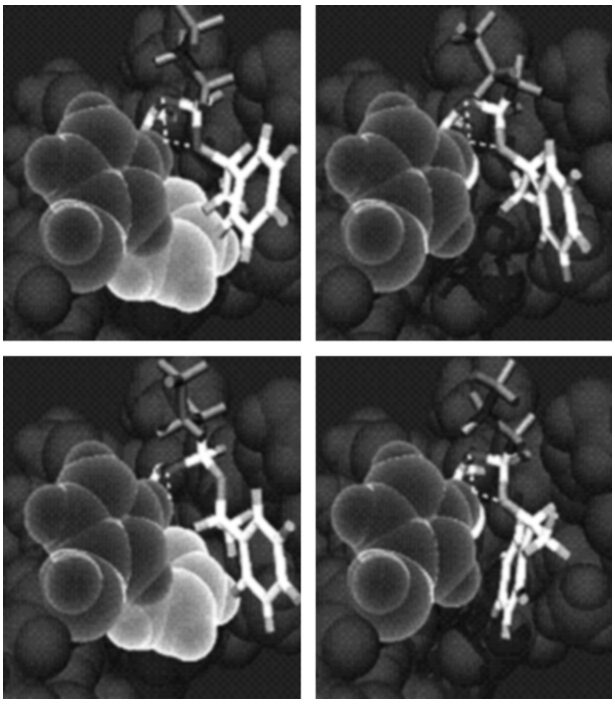

Figure 59. Active site of wild-type CAL-B (left) and Trp 104 Ala mutant (right) with the butanoate ester of (*R*)-1-phenylethanol (top) and (*S*)-1-phenylethanol (bottom) covalently bound to the catalytic serine in the tetrahedral reaction intermediate. The substrate is presented with a stick model and amino acid 104 with a space-filling model in white. The (*R*)-enantiomer has a similar configuration in the wild-type CAL-B and Trp 104 Ala mutant: the large substituent (phenyl) points towards the active-site entrance and the medium-sized substituent (methyl) is positioned in the enantioselectivity pocket. In the wild-type CAL-B, the (*S*)-enantiomer cannot position its phenyl group in the enantioselectivity pocket, and not all of the hydrogen bonds required for catalysis can be formed. In the Trp 104 Ala mutant, the phenyl group is comfortably accommodated in the space liberated by the mutation in the enantioselectivity pocket.[179]

et al. redesigned the enantioselectivity pocket of *C. antarctica* lipase B (CAL-B) to accommodate much larger groups than the wild-type lipase (Fig. 59). This change transformed the strongly *R*-selective wild-type CAL-B into an *S*-selective mutant. The *S*-selectivity increased with temperature and was dominated by the activation entropy. Furthermore, the altered enantioselectivity of CAL-B is a demonstration of the possibilities offered by protein redesign and shows the importance of the entropy contribution.[179]

4.3.2. Thermodynamic analysis. The enantioselectivity or enantiomeric ratio E is the result of a difference in activation free energy between enantiomers, $\Delta_{R-S}\Delta G^{\ddagger}$, which is related to the enantiomeric ratio E as $-RT\ln E$. Thus, the relation between the difference in Gibbs free energy of the transition states of the enantiomers (Fig. 60) and the enantiomeric ratio E can be shown with transition state theory (Eq. 14) by

$$\Delta_{R-S}\Delta G^{\ddagger} = \Delta G_R^{\ddagger} - \Delta G_S^{\ddagger} = -RT\ln E \tag{14}$$

Furthermore, $\Delta_{R-S}\Delta G^{\ddagger}$ is related to the difference in activation enthalpy and entropy as shown in Eq. 15.

$$\Delta_{R-S}\Delta G^{\ddagger} = \Delta_{R-S}\Delta H^{\ddagger} - T\Delta_{R-S}\Delta S^{\ddagger} = -RT\ln E \tag{15}$$

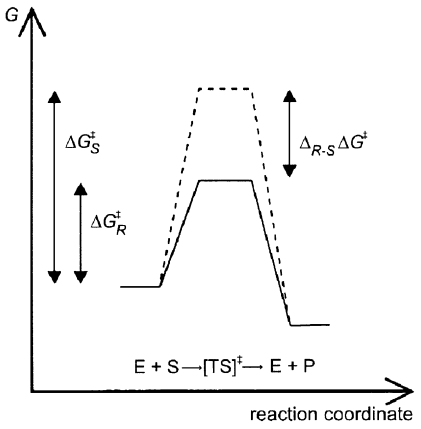

Figure 60. Schematic free energy profile of an enantioselective lipase-catalyzed reaction favouring the *R* enantiomer.[178]

Therefore, the enthalpic and entropic components of the enantiomeric ratio E have been determined by temperature studies by Phillips and E is determined according to Eq. 16.[180]

$$\ln E = -\frac{\Delta_{R-S}\Delta H^{\ddagger}}{R}\times\frac{1}{T} + \frac{\Delta_{R-S}\Delta S^{\ddagger}}{R} \tag{16}$$

According to Eq. 16, the thermodynamic parameters $\Delta\Delta H^{\ddagger}$ and $\Delta\Delta S^{\ddagger}$ can be easily estimated. The enantiomeric ratio E or, rather, $\ln E$ will vary with the reciprocal of the temperature to an extent determined by the enthalpic term (the slope of Eq. 16, $\Delta_{R-S}\Delta H^{\ddagger}/R$), and at a level determined by the entropic term (the intercept of Eq. 16, $\Delta_{R-S}\Delta S^{\ddagger}/R$). The differential activation enthalpy is related to the differences in the complementary of each enantiomer in the transition state and comprises steric and electrostatic interactions between the reaction components, the enzyme, its substrate and the solvent.[178] The differential activation entropy is, however, is more difficult to visualize and comprises differences between the enantiomers in conformational degrees of freedom of the protein, losses in conformational entropy of the substrates or solvation. Figure 61 presents an overview of the theoretically possible relations of $\ln E$ or $\Delta_{R-S}\Delta G^{\ddagger}$ with temperature according to Eqs. 15 and 16. The differential activation parameters can be either positive or negative, depending on which enantiomer is favoured by enthalpy and entropy. A temperature at which there is no enantioselectivity, E=1 and $\Delta_{R-S}\Delta G^{\ddagger}$=0, the racemic temperature T_R, can be determined as the ratio of the differential activation enthalpy and entropy, according to Eq. 17:[180]

$$T_R = \Delta_{R-S}\Delta H^{\ddagger}/\Delta_{R-S}\Delta S^{\ddagger} \tag{17}$$

In general, the enthalpic and entropic contributions counteract each other, i.e., they are either both positive or both negative. This case has been confirmed by Hult et al., who showed that, in the resolution of most *sec*-alcohols by CAL-B, $\Delta_{R-S}\Delta H^{\ddagger}$ and $\Delta_{R-S}\Delta S^{\ddagger}$ were of the same sign, resulting in a real positive T_R.[178,181]

This is in agreement with the statement of 'better transition-state binding, low enthalpy, comes with a more rigid transition state'. In other words, the enantiomer favoured by enthalpy is disfavoured by entropy and vice versa. As shown

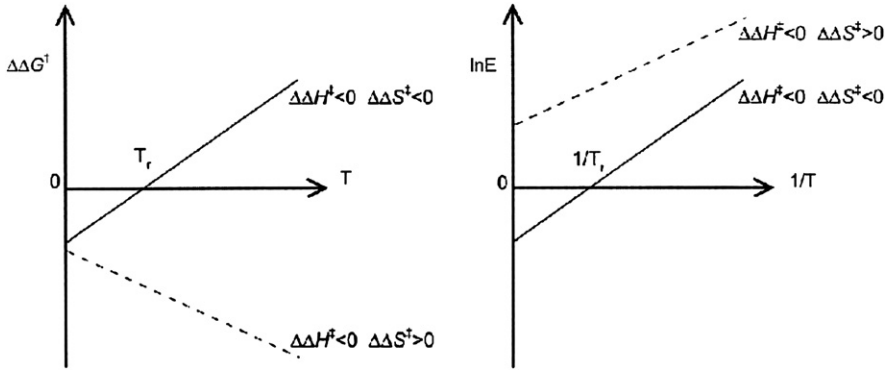

Figure 61. Schematic overview of the theoretically possible relationships between differential activation free energy, $\Delta\Delta G^{\ddagger}$, and temperature (Eq. 15) or LnE and reciprocal temperature (Eq. 16). In the opposite enantiopreference, both $\Delta\Delta H^{\ddagger}$ and $\Delta\Delta S^{\ddagger}$ change sign.

in Figure 61 (theoretically possible), below T_R, the major term in the differential free energy is the enthalpic term (Eq. 15) and the enzyme will preferentially catalyze the enantiomer favoured by enthalpy and, thus, the enantioselectivity E will decrease with temperature as the entropic term ($T\Delta_{R-S}\Delta S^{\ddagger}$) of the equation increases.

On the other hand, above T_R, the major term is the entropic term and the enzyme's enantiopreference is changed to the enantiomer favoured by entropy and the enantioselectivity E will increase with temperature as $T\Delta_{R-S}\Delta S^{\ddagger}$ increases. Theoretically, the differential activation enthalpy and entropy may be of different signs, i.e., the favoured enantiomer is favoured both by enthalpy and entropy. The latter produces a negative T_R with no physical meaning.[178]

As has been confirmed by Hult et al., the T_R for CAL-B-catalyzed resolutions of *sec*-alcohols is above (>100 K) the experimental temperature.[178] Several researchers have shown that the differential activation entropy significantly affects the enzyme-catalyzed resolution of enantiomers[180,182,185] and, therefore, in order to create a complete model of the mechanism of enantioselectivity, entropy considerations must be included.[178,181–184]

The dependence of enantioselectivity on temperature is well documented in the literature[54,55,185] and it has become clear that the entropic term is by no means negligible, but actually has a strong influence on the enantioselectivity. Generally, enzyme-catalyzed reactions have racemic temperatures above the experimental temperature, and the enantioselectivity decreases with increasing temperature. Several examples are, however, known of reactions above the racemic temperature with the somewhat counterintuitive property of increasing enantioselectivity with increasing temperature. The latter is considered a fascinating topic in lipase catalysis as both the enantioselectivity and the activity can be increased by simply increasing the reaction temperature. In addition, the variation of temperature might result in switching the enantiopreference of the lipase. Hult et al. reported an efficient example of an *S*-selective lipase created by rational redesign and the enantioselectivity was strongly increased with temperature. The thermodynamic analysis showed that the altered enantioselectivity was dominated by entropy.[179]

Shortly, enzyme enantioselectivity will provide an excellent opportunity for fundamental studies of enzyme catalysis, due to the identical ground-state energies of enantiomers, which reduces a comparison of their reaction-path energies to the difference in the two enzyme–substrate transition states. Measurements of the absolute rates are often problematic, e.g., for instance under denaturing conditions, where the amount of active enzyme is difficult to determine. In enantioselective systems, determinations of the relative reaction rates are sufficient. It is important to note that the accuracy of the thermodynamic data obtained by kinetic resolution experiments is relatively high, since the E values are determined under competitive reaction conditions. Additionally, the determination of E itself is reliable when comparing and studying the performance of enzymes in enantioselective catalysis. Any conclusions on enzyme mechanism and performance drawn from changes in E may otherwise be faulty. Although a comparison of the ee of the reaction products is a very good measure of product quality, it cannot be used to make any conclusions about enzyme enantioselectivity unless the enantiomeric excesses are determined at identical degrees of conversion. In studies on enzyme enantioselectivity, the enantiomeric ratio E should be the parameter compared.[186] Particular precautions should be taken when determining high E values, as these are difficult to measure with a high accuracy, because analysis of very high ee values is required. Enzyme enantioselectivity is, apart from the reaction conditions, influenced by the substrate structure, the variation on the catalyst itself and by the reaction medium. Several efforts have been made to enhance the enantioselectivity of enzyme-catalyzed reactions with substrates, and protein and solvent engineering.[6]

As previously shown for substrate effects on CAL-B enantioselectivity, modifications of the protein affect both the enthalpic and the entropic components of the enantiomeric ratio E.[181] This fact stresses the importance of entropy in enzyme catalysis, in general, and enantioselectivity, in particular. The observed changes in enthalpy and entropy displayed a compensatory character in that, if $\Delta_{R-S}\Delta H^{\ddagger}$ was large, so was $\Delta_{R-S}\Delta S^{\ddagger}$, and vice versa. They were not perfectly compensatory, however, allowing for modifications of the enzyme to improve E. Figure 62 shows the general energy profile diagrams of enthalpy and entropy for an enzyme-catalyzed kinetic resolution. Changes in the components of enantioselectivity, $\Delta_{R-S}\Delta H^{\ddagger}$ and $\Delta_{R-S}\Delta S^{\ddagger}$, caused by mutations on the enzyme must be related to one or both enantiomers shifting their individual activation energy, $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$, up or down relative to each other.[178]

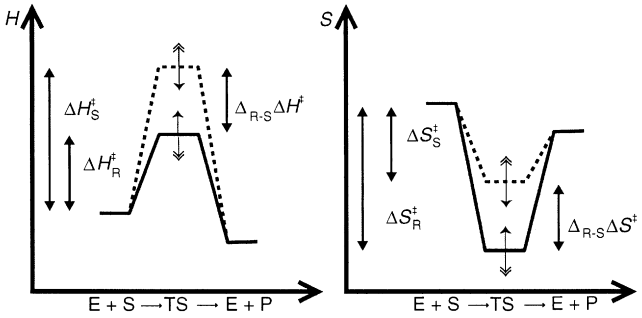

Figure 62. Enthalpy and entropy profile diagrams of the enantioselective reaction catalyzed by lipase.[178]

5. Novel biocatalysts

One of the most important obstacles faced by researchers working in the field of enzyme technology is to identify or design an optimal enzyme capable of catalyzing a specific reaction that they need. This enzyme can be found among natural variants or constructed by gene technological methods. Natural evolutions of enzymes are excluded in this context. Only the improvement of enzyme properties by in vitro evolution or rational design and the creation of new enzymes by biosynthesis (catalytic antibodies) are considered.

5.1. In vitro evolution

The conventional way to identify new enzymes is based on the screening of, e.g., soil samples or strain collections by enrichment cultures. Upon identification of a suitable biocatalyst for a specific reaction, strain improvement as well as cloning and expression of the encoding gene(s) enable production on a large scale. Not all microorganisms are culturable, however, by using common fermentation methods and, additionally, not all enzymes found in nature are capable of catalyzing a specific reaction in organic synthesis. If the gene encoding the enzyme and its 3D structure were available, these limitations can be overcome by rational protein design, where mutation sites can be identified followed by the introduction of appropriate amino acids by site-directed mutagenesis[1] (Fig. 63).

Another approach, which has emerged during the mid-1990s, is the strategy of in vitro evolution (also called directed or molecular evolution). This evolution in a test tube comprises essentially two steps: (1) random mutagenesis of the gene encoding the enzyme; and (2) identification of the desired biocatalyst variants within these mutant libraries by screening or selection (Fig. 63). A prerequisite of the in vitro evolution is the availability of gene(s) encoding the enzyme(s) of interest, a suitable (usually microbial) expression system, an effective method to create mutant libraries, and a suitable screening or selection system. These methods have been extensively discussed and reviewed in the literature.[187–191]

5.2. Rational design of enzymes

With the advances in protein engineering during the early 1990s, where the dominant method used to improve enzyme properties was site-directed mutagenesis (the genetic exchange of one amino acid residue with another to alter the enzyme properties), the vision of tailor-made proteins is becoming a reality.[1] Finding the optimal biocatalyst and reaction conditions for a specific reaction, however, still required a great deal of experimental screening. Enabling a rational design of enzymes would relieve this need for screening large numbers of biocatalysts to suit to a target reaction and allow the introduction of new enzymatic activities.[192,193] The rational design of enzymes enables the identification of the amino acid residues that might be function modulating or the cause of the limited stability of the enzyme in a given enzymatic reaction.[178] These residues can then be mutated using site-directed mutagenesis to select the mutant enzymes having improved properties.[1] The selection of the amino acid residues is based, in most cases, on the 3D structure of the enzyme itself.

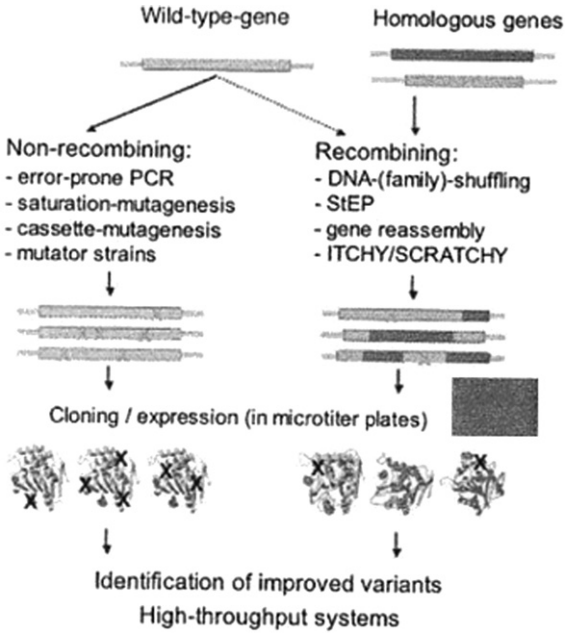

Figure 63. Schematic presentation of the principle of directed evolution. The gene(s) encoding the wild-type or homologous enzyme(s) are subjected to random mutagenesis using non-recombining or recombining methods. The resulting mutant libraries are then cloned and expressed (often in microtiter plates). The desired improved variants are identified by high-throughput screening systems, usually using microtiter plate-based assays or selection.[1]

5.3. Enzymes by biosynthesis: catalytic antibodies

By raising antibodies to the appropriate hapten, a variety of catalytic activities may be generated. Typically, this method is used to create monoclonal antibodies. These catalytic antibodies could be used as biocatalysts for the conversion of substrates, and can be selected from different monoclonal antibodies using a special screening technique.[192]

Catalytic antibodies have been under development for about 20 years and many examples are mentioned in the literature, e.g., a 10-fold enhancement in the rate of hydrolysis of *p*-nitrophenyl-β-lactam was observed for antibodies raised against a *p*-nitrophenyl carbonate hapten. Although their specificity constants have increased, they remain many orders of magnitude below those of the enzymes able to convert the same substrates.[194]

5.4. Enzymes by synthesis (synzymes)

A number of possibilities now exist for the construction of artificial enzymes. These are generally synthetic polymers or oligomers with enzyme-like activities, often known as synzymes, and have been used by polymer chemists to mimic the sites in chemically synthesized polymers. They must possess two structural entities, a substrate-binding site and a catalytically effective site. It has been found that producing the facility for substrate binding is relatively straightforward, but catalytic sites are somewhat more difficult. Both sites may be designed separately, but it appears that, if the synzyme has a binding site for the reaction transition state, this often achieves both functions. Synzymes generally obey the saturation Michaelis–Menten kinetics. Although studies on synzymes have been conducted for over 30 years, the best specificity constants obtained remain many orders of magnitude lower than those of the enzyme for which the active site is being mimicked.[1]

6. Lipase catalysis on an industrial scale

As the use of lipases for industrial chemical synthesis becomes easier, several chemical companies have begun to significantly increase their biocatalytic process activities used in synthetic applications.[85] Among these companies is BASF, which produces enantiomerically pure alcohols, e.g., (*S*)-**3** and unreacted (*R*)-**181** and amines (*S*)-**182** and unreacted (*R*)-**183** on an industrial scale (Fig. 64).[195]

The (*R*)-amide (*R*)-**183** is separated from the (*S*)-amine (*S*)-**182** by distillation or extraction, and the free (*R*)-amine is released through basic hydrolysis. In addition, the undesired enantiomers can be racemized and the acylating agent (e.g., methoxyacetic acid ethyl ester) can be recovered. BASF produces more than 2500 tonnes of amines annually using this process.

The enantioselective hydrolysis of the racemic acetamide **184** was developed at Bayer AG in the mid-1990s. The reaction was performed using *C. antarctica* lipase B (CAL-B) to afford the free amine (*R*)-**185** in high ee (>99.5%) and the unreacted amide (*S*)-**184** (Fig. 65).[196] The requirement of a high concentration of the catalyst, however, limits the exploitation of the process on an industrial scale.[197]

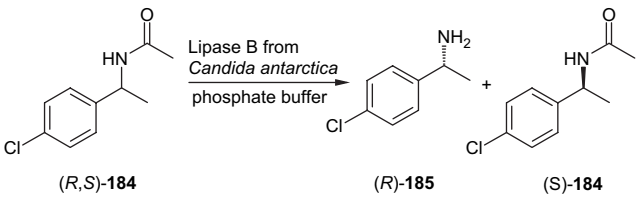

Figure 65. Enantioselective hydrolysis of racemic acetamide (*R*,*S*)-**184** developed at Bayer AG.

Apart from amines and secondary alcohols, Ladner and Whitesides have developed a procedure to resolve racemic glycidyl butyrate *rac*-**186** (Fig. 66) with porcine pancreatic lipase (PPL) to afford the enantiomer (*R*)-186 in 89% chemical yield and with 92% ee.[198] This process was further developed and used by Andeno-DSM to produce the epoxy alcohol (*R*)-glycidol (*R*)-**187** on a multitonne scale.

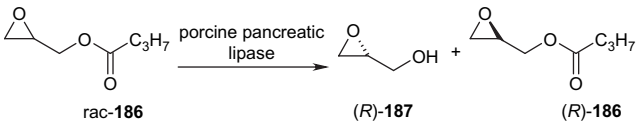

Figure 66. Enantioselective hydrolysis of racemic glycidyl butyrate **186** developed at Andeno-DSM.

In addition, DSM has developed a lipase-catalyzed process for the production of captopril **190** intermediates starting from (*R*)-3-chloro-2-methyl propionate (*R*)-**188** obtained from racemic **188** (Fig. 67). The intermediate **189** can be easily transferred to captopril **190**. All lipases preferentially hydrolysed the (*S*)-enantiomer, and up to 98% ee at 64% conversion was observed with a lipase from *Candida cylindracea*.[1]

In the pharmaceutical industry, salt-activated biocatalysts have been used to synthesize a library of paclitaxel (taxol) derivatives. CAL was used in the hydrolysis of the terminal vinyl ester in taxol 2-vinyladipate **191** (Fig. 68). The resulting taxol 2-adipic acid derivative **192** was nearly 1700-fold more soluble in water than the native taxol, an important

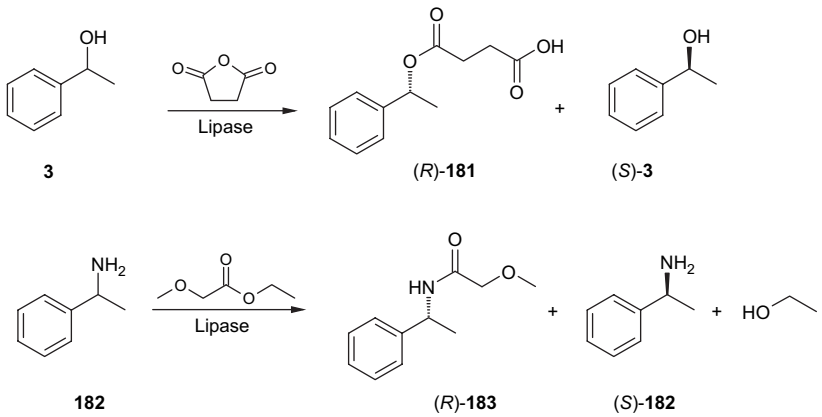

Figure 64. Some of the biocatalytic steps using lipase developed at BASF: lipase-catalyzed kinetic resolution of (a) phenylethanol **13** using succinic anhydride, (b) secondary amine **182** using ethyl methoxyacetate as acyl donor.[195]

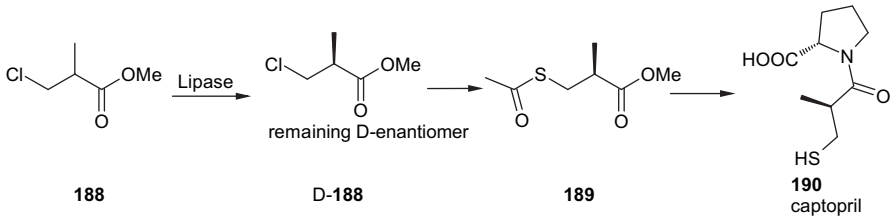

Figure 67. Lipase-catalyzed kinetic resolution of a building block for the synthesis of captopril.

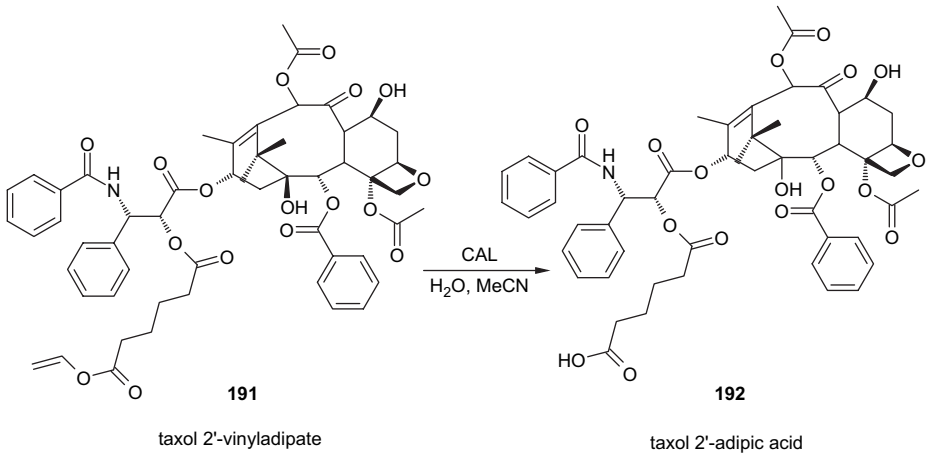

Figure 68. Lipase-catalyzed hydrolysis of terminal vinyl ester in taxol 2-vinyladipate **191**.

result in the design of taxol prodrugs with increased bioavailability.[199]

A broader overview of the industrial methods used for the production of optically active intermediates is available from Breuer et al.[197] Other examples of the use of lipases in pharmaceutical preparations have been reviewed.[200–202]

7. Concluding remarks and perspectives

Lipases continue to serve as viable biocatalysts with extraordinary enantioselectivity for synthetic purposes. These enzymes have competed well with chemical methods used for resolution. A maturing understanding of these enzymes allows scientists to use them predictably on more sophisticated chemical structures and to attempt to resolve more 'difficult to resolve' substrates. The selected examples shown in this survey demonstrate the broad applicability of lipases in terms of substrate structures and enantioselectivity. More recently, modern molecular biology methods such as rational protein design and, especially, directed evolution[193] will further boost the development of tailor-made lipases for future applications in the synthesis of enantiomerically pure compounds. It has already been shown that a virtually non-enantioselective lipase (E=1.1 in the resolution of 2-methyldecanoate) could be evolved to become an effective enantioselective biocatalyst (E>50). Furthermore, variants were identified, which showed opposite enantiopreference. Moreover, extremophiles (a group of microorganisms that can thrive under, and actually prefer, conditions previously thought to be too harsh for sustaining life) offer useful enzymes that should enable the range of reaction conditions suitable for biocatalysis to be expanded. This has indeed proved to be the case, and various promising applications in detergent production, sugar chemistry, lipid and oil chemistry, and food processing have been initiated or are being explored.[203]

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Biographical sketch

Ashraf Ghanem was born in 1974 in Cairo, Egypt. He studied chemistry at the University of Stuttgart, Germany, where he conducted his master degree research work on the synthesis of optically active cyanohydrins at the Institute of Organic Chemistry under the supervision of Professor Franz Effenberger and in collaboration with Prof. G. H. El-Gemeie, University of Helwan, Egypt. In 1999, he received his master degree and joined the group of Professor Rolf D. Schmid at the Institute for Technical Biochemistry, University of Stuttgart and Professor Uwe Bornscheuer currently at the University of Greifswald, Germany, where he worked on the industrial project of lipase-catalyzed kinetic resolution of DL-menthol. In 2000, he received a scholarship from the graduate college of the German Research Council (DFG) to conduct his Ph.D. at the University of Tuebingen, Germany under the supervision of Prof. Volker Schurig working on the lipase-catalyzed kinetic resolution of racemates. In 2002, he received his Ph.D. and joined the group of Prof. Paul Muller as a postdoctoral fellow at the institute of Organic Chemistry, University of Geneva, Switzerland, where he worked on the asymmetric cyclopropanation of olefins using rhodium catalysts. In 2004, he got a scientist position at the Biomedicinal Chemistry Unit, Biological and Medical Research Department, King Faisal Specialist Hospital & Research Centre, Riyadh, Saudi Arabia. In 2005, he was selected among the hundreds of promising scientists from all over the world to participate in the 2005 class fellow at the Biovision NXT 2005, Lyon, France. In 2006, He received a grant award from the King Abdul-Aziz City for Science and Technology (KACST) for his project 'From enzymes to chiral metal catalysts: asymmetric access to enantiomerically pure pharmaceuticals and related compounds'. Dr. Ghanem is the author of more than 40 international scientific publications covering subjects ranging from lipase and rhodium catalysis to chiral separation of biologically active compounds.