

# Efficient Biocatalytic Synthesis of Chiral Chemicals

Zhi-Jun Zhang, Jiang Pan, Bao-Di Ma and Jian-He Xu

**Abstract** Chiral chemicals are a group of important chiral synthons for the synthesis of a series of pharmaceuticals, agrochemicals, and fine chemicals. In past decades, a number of biocatalytic approaches have been developed for the green and effective synthesis of various chiral chemicals. However, the practical application of these biocatalytic processes is still hindered by the lack of highly efficient and robust biocatalysts, which usually results in the low volumetric productivity and high cost of the bioprocesses. Further step forward of biocatalysis in industrial application strongly requires the development of versatile and highly efficient biocatalysts, aiming to increase the process efficiency and facilitate the downstream processing. Recently, the fast growth of genome sequences in the database in post-genomic era offers great opportunities for accessing numerous biocatalysts with practical application potential, and the so-called genome mining approach provides time-effective and highly specific strategy for the fast identification of target enzymes with desired properties and outperforms the traditional screening of soil samples for microbial enzyme producers of interest. A number of biocatalytic processes with industrial application potential were developed thereafter. Further development of protein engineering strategies, process optimization, and cooperative work between biologists, organic chemists, and engineers is expected to make biocatalysis technology the first choice approach for the eco-friendly, highly efficient, and cost-effective synthesis of chiral chemicals in the near future.

**Keywords** Biocatalysis · Data mining · Efficient biocatalytic synthesis · Chiral chemicals · High volumetric productivity

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## 1 Introduction

Chiral chemicals are usually required as key intermediates for the synthesis of a variety of pharmaceuticals, agrochemicals, food ingredients, flavors, and fine chemicals. Traditional chemical routes to chiral chemicals usually require harsh reaction conditions (e.g., elevated temperature, high pressure, strongly acidic, or basic condition etc.) and expensive metal-based catalysts, which have led to severe environmental problems. In the transformation of compounds bearing labile groups, tedious protection/deprotection steps are required, thereof leading to relatively low yields of the final products. Therefore, there is an urgent need for the development of green and cost-effective processes for the preparation of chiral chemicals. In past decades, there has been a significant rise in the application of biocatalysis for industrial settings since biocatalysts offer cheap, environmentally benign, excellently selective, and highly efficient alternatives to chemical routes [1–7].

However, the widespread industrial application of biocatalysis is still hindered by low volumetric productivity, unsatisfactory selectivity, and limited availability of robust biocatalyst [6]. The development of novel and robust biocatalysts still remains a great challenge in both academia and industry [8]. Great effort has been paid to develop powerful biocatalyst for biocatalytic processes with high substrate concentration in order to achieve cost-efficiency and competitiveness in practical biotransformation [6]. Traditional biocatalyst discovery is based on screening soil samples from various sources for microorganisms producing desired enzyme activity [9, 10]; however, this strategy is always time-consuming (typically 1–2 years), and the enzyme expression level in the original host strain is usually very low resulting in insufficient catalytic efficiency. Therefore, the cloning and over-expression of the target enzyme in a suitable host organism and even further protein engineering of the enzyme to meet specific application requirement is always necessary [11, 12]. Most importantly, only less than 1 % of microbes in the environment are culturable, limiting the versatility of biocatalysts explored. Due to the exponential growth of genome sequences in the database in post-genomic era, and most of them are uncharacterized for their definite biological functions, data mining offers an unprecedented opportunity for accessing novel and useful biocatalysts with industrial application potential [13, 14].

For data mining, the gene sequences of the already-known enzymes with best performance in the specific reaction are preferentially used as templates for BLAST in GenBank or SWISS-PROT database, then a series of gene sequences (preferentially uncharacterized before) with moderate sequence similarity to known enzymes are considered to be possible candidates. Multiple sequence alignment of the candidate sequences with known enzyme sequences with respect to key motifs, conserved regions, and catalytically important residues can further increase the successful rate of hits. If possible, three-dimensional structure analysis might be applied. Since the chosen candidate sequences only shows moderate similarity to known enzymes (usually lower than 80 %), they are considered to be relatively novel enzymes. The finally picked target genes are then obtained through PCR amplification from the genome DNA of the target strain or through gene synthesis, cloned into suitable expression vectors, and transformed into host strains. After protein expression and functional screening, one can get the suitable biocatalysts with desired properties.

In this chapter, efficient biocatalytic synthesis of a series of chiral pharmaceutical intermediates including carboxylic acids, epoxides, and alcohols will be discussed focusing on the newly developed biocatalytic processes using biocatalysts.

## 2 Bioresolution with Esterases and Lipases

Carboxylic ester-hydrolyzing enzymes, which are ubiquitous in animals, plants, and microorganisms, can catalyze the hydrolysis or formation of the ester bonds. Of them, lipases (EC 3.1.1.1, triacylglycerol hydrolases) and esterases (EC 3.1.1.3, carboxyl ester hydrolases) are two groups of biocatalysts that are widely used in

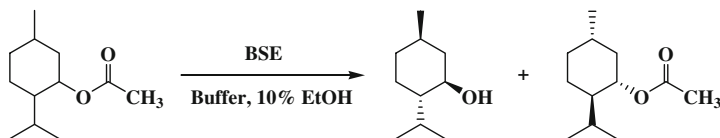
industry [15–18]. Esterase and lipase can be distinguished by substrate specificity and interfacial activation [19]. The esterases hydrolyze soluble substrates such as triglycerides with fatty acids shorter than C<sub>6</sub>, while the water-insoluble triglycerides with long-chain fatty acids are typical substrates for lipases. Furthermore, a minimum substrate concentration was needed for high activity of lipase because of the hydrophobic “lid” covering the active site of lipase.

The interests in esterase and lipase from academia and industry mainly reside in their desirable properties for practical application, such as high enantioselectivity toward a variety of substrates, robustness, and cofactor independence. They have been successfully applied in numerous industrial processes [20–23], including detergents, oils and fats, cheese making, and pharmaceutical industry. Enantioselective hydrolysis, transesterification, or synthesis of the single enantiomer ester from the racemic mixtures by esterase or lipase provides an attractive approach for the production of chiral chemicals. The intrinsic disadvantage of a maximum theoretical yield of 50 % in kinetic resolution can be overcome by the dynamic kinetic resolution (DKR) or stereoinversion of the unwanted enantiomer [24–27]. DKR combines the enzymatic resolution and racemization of the substrate in situ and has attracted great interest [28–30]. Therefore, the synthetic potential of esterase and lipase was pushed forward to a certain extent and the synthetic applications of these groups of biocatalyst are well reviewed [15, 18, 21–23]. Herein, we focus on the synthesis of important chiral chemicals by the recently exploited esterase or lipase.

## 2.1 Optically Pure Alcohols

### 2.1.1 *l*-Menthol

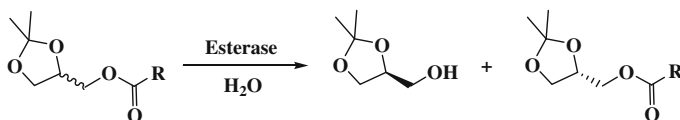
*l*-Menthol is one of the most important flavor components and widely used in the confectionary goods, pharmaceuticals, oral healthcare products, cosmetics, etc. Enzymatic resolution via enantioselective esterification/transesterification of racemic menthol in organic solvents [31, 32] or enantioselective hydrolysis of racemic menthol esters in aqueous medium [33, 34] is an extensively investigated approach to optically pure *l*-menthol. A prominent biocatalyst for the efficient preparation of *l*-menthol is the high substrate concentration-tolerable esterase from *Bacillus subtilis* ECU0554 (BSE), which was newly isolated from soil [35]. The low activity and poor stability of *B. subtilis* whole cells under operational conditions were overcome by overexpression of the *B. subtilis* esterase in *E. coli* BL21(DE3) [36] and immobilization by cross-linked enzyme aggregates [37], respectively. The cross-linked enzyme aggregates of BSE were recycled for the enantioselective hydrolysis of *dl*-menthol acetate with high substrate load (200 g/L, *ca.* 1.0 M) for 10 rounds, giving *l*-menthol with >94 % *ee* at conversion of >40 % (Fig. 1). In further work, the versatile BSE was also successfully employed for the production of (*S*)-1-phenyl-1,2-ethanediol [38] and *m*-substituted 1-phenylethanol acetates [39], which are valuable intermediates for pharmaceuticals and other fine chemicals.



**Fig. 1** Enzymatic resolution of *dl*-menthol acetate using BSE

### 2.1.2 (*S*)-1,2-*O*-Isopropylideneglycerol

(*S*)-1,2-*O*-Isopropylideneglycerol (IPG) is an important primary alcohol and serves as a starting material for the synthesis of  $\beta$ -adrenoceptor antagonists, prostaglandins, or leukotrienes. (*R,S*)-IPG could be selectively esterified with butyric acid in *n*-heptane by dry mycelia of *Rhizopus oryzae* and *Aspergillus oryzae*, with low enantiomeric ratio (*E*-value) of 3.4 and 8.0, respectively [40]. The yeast *Kluyveromyces marxianus* exhibited preference for the hydrolysis of (*S*)-IPG esters with moderate enantioselectivity (*E* = 28). The hydrolysis of (*R,S*)-IPG acetate by whole cells of the yeast was conducted in a membrane reactor, in which an ultrafiltration membrane (cut-off 10,000 Da) was used to recover the cells and released enzymes when the *ee<sub>s</sub>* reached 100 %. The repeated-batch operation in the membrane reactor was run for 20 cycles, and enantiomerically pure (*R*)-IPG acetate of 19.2 g/L was recovered from 60 g/L of racemic mixture [41]. However, most of the esterases or lipases involved in the resolution of IPG ester preferred to hydrolyze (*R*)-IPG esters leaving the (*S*)-IPG esters untouched and this biocatalytic process has been extensively studied for the preparation of (*S*)-IPG (Fig. 2) [42–44]. An interesting investigation was the enantioselective hydrolysis of benzoyl-1,2-*O*-isopropylideneglycerol by *Bacillus coagulans* NCIB 9365, 1.50 g of (*S*)-IPG with 88 % *ee* was obtained from 5 g/L of benzoyl-1,2-*O*-isopropylideneglycerol at 1-L scale under the optimized reaction conditions [45]. Enzyme purification study revealed that the insufficient enantioselectivity of *B. coagulans* resting cells was attributed to the existence of two different enzymes: The partially purified enzyme A is thermostable and enantioselective toward IPG ester, while the thermolabile enzyme B is not enantioselective [46]. A simple heat treatment of the whole cells at 65 °C for 1 h remarkably increased the enantioselectivity (*E* = 80–100 for (*R*)-benzoyl-1,2-*O*-IPG, 95–96 % *ee<sub>p</sub>*). Repeated-batch reaction was performed in the aforementioned membrane reactor for the preparation of enantiopure IPG by the heat-treated cells of *B. coagulans*, and no obvious activity loss was observed for 11 cycles. (*S*)-1,2-*O*-Isopropylideneglycerol benzoate of 9.55 g/L could be recovered from 24.0 g/L of

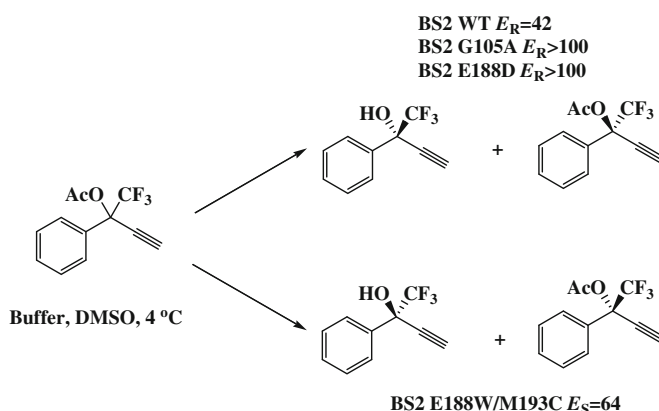


**Fig. 2** Esterase-catalyzed resolutions of IPG esters

racemic substrate [47]. Another promising candidate catalyst, *E. coli* esterase YbF, which shows 124/9.3 U/mg for (*R*)/(*S*)-IPG butyrate and 31/1.3 U/mg for (*R*)/(*S*)-IPG caprylate was reported in 2011 [48]. The moderate enantioselectivity of YbF was improved by site-directed saturation mutagenesis and resulted in a mutant, W235I, with *E*-values of 38 and 77 for IPG butyrate and IPG caprylate, respectively [49].

### 2.1.3 Optically Pure Tertiary Alcohols

Optically pure tertiary alcohols (TAs) represent a group of important building blocks for the synthesis of various chiral chemicals and valuable pharmaceuticals. Enzymatic preparation of the enantiopure TAs has gained great interest due to the harsh reaction conditions of the chemical methods [50, 51]. It is still a great challenge for the resolution of sterically hindered TAs [52–54] even though the esterase- or lipase-catalyzed kinetic resolution is the most practical approach and a standard procedure for the synthesis of enantiopure secondary and primary alcohols. Furthermore, there is no corresponding ketone to be reduced to a tertiary alcohol although asymmetric reduction represents a complementary route to enzymatic resolution for the preparation of optically active secondary alcohols [6]. Thus, enzymatic kinetic resolution of TAs is of particular importance and gains great attention. The observation that the GGGX motif in the oxyanion hole is crucial for enzyme activity toward TAs [53, 55] facilitated the discovery of several GGGX-type  $\alpha/\beta$ -hydrolases with hydrolytic activity toward esters of TAs [56–58]. However, the GGGX-type  $\alpha/\beta$ -hydrolases exhibited low-to-moderate enantioselectivity. Modern protein engineering strategies have substantially expanded the toolbox available for the preparation of enantiopure TAs. Rational design and site-directed saturation mutagenesis have yielded several mutants of the esterase BS2 from *B. subtilis* including the G105A [55] and E188D [59] with excellent



**Fig. 3** Kinetic resolution of 1,1,1-trifluoro-2-phenyl-but-3-yn-1-yl acetate using the wild-type esterase and its mutants

*R* enantioselectivity ( $E > 100$ ) toward 1,1,1-trifluoro-2-phenyl-but-3-yn-1-yl acetate (Fig. 3). A double-mutant, E188W/M193C, with inversed enantioselectivity ( $E_S = 64$ ) was created by focused directed evolution approach thereafter [60]. Another synthetically useful biocatalyst (mutant EstA-AGA) with excellent enantioselectivity was provided by engineering of the *Paenibacillus barcinonensis* esterase (EstA) based on the structure-guided alignment [61]. The EstA-AGA was used to resolve the racemic 1,1,1-trifluoro-2-phenyl-but-3-yn-1-yl acetate in preparative scale with 91 %  $ee_s$  and 99 %  $ee_p$ , demonstrating the feasibility of the EstA-AGA-catalyzed kinetic resolution for the synthesis of chiral TAs.

## 2.2 Optically Pure Carboxylic Acids

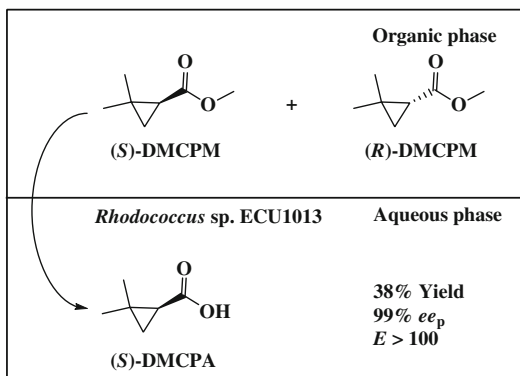
### 2.2.1 (*S*)-(+)-2,2-Dimethylcyclopropane Carboxylic Acid

(*S*)-(+)-2,2-Dimethylcyclopropane carboxylic acid [(*S*)-(+)-DMCPA] is a key precursor for cilastatin, an excellent renal dehydropeptidase-I inhibitor. Several approaches, including the chemical asymmetric synthesis, chemical, or enzymatic resolution, have been developed to prepare (*S*)-(+)-DMCPA [62–64]. Of these methods, the esterase- or lipase-catalyzed enantioselective hydrolysis of 2,2-dimethylcarboxylate ester represents an effective and environmentally benign approach. The commercially available Novozyme 435 was utilized for the enantioselective hydrolysis of ethyl-2,2-dimethylcyclopropanecarboxylate at substrate concentration of 60 mM. In repeated-batch operations, glutaraldehyde-modified Novozyme 435 retained 76 % of its original activity after 10 repeated cycles and the  $ee_p$  was kept above 98 % throughout the process [65]. Recently, a new bacterial strain *Rhodococcus* sp. ECU1013 [66] was isolated from soil for the enantioselective hydrolysis of (*S*)-DMCPA esters from their racemic counterparts, providing an alternative useful biocatalyst for the production of (*S*)-DMCPA. By using the resting cells of *Rhodococcus* sp. ECU1013 as catalyst, up to 400 mM ( $\pm$ )-DMCPM was enantioselectively hydrolyzed into (*S*)-(+)-DMCPA in an organic–aqueous biphasic system (Fig. 4), with an isolated yield of 38 and 99 %  $ee_p$ . Further, heterogeneous overexpression and activity improvement of this newly discovered esterase by protein engineering are under progress in our laboratory.

### 2.2.2 (2*S*,3*R*)-3-Phenylglycidate Methyl Ester

(2*S*,3*R*)-3-Phenylglycidate methyl ester (PGM) is a key intermediate for the synthesis of a potent anticancer drug Taxol<sup>®</sup>. Low-to-moderate enantioselectivity and the difficulty in separation of the desired product restricted the resolution of PGM via transesterification [67, 68]. The whole cells of *Pseudomonas putida* have been reported for the enantioselective hydrolysis of ( $\pm$ )-PGM at substrate concentration of 50–60 mM, furnishing (2*S*,3*R*)-PGM with 99 %  $ee$  [69]. Recently,

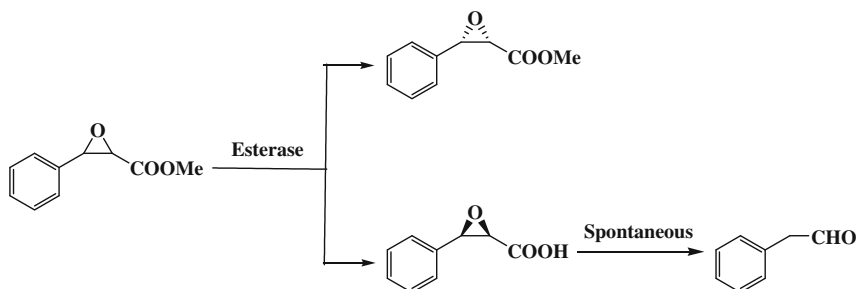
**Fig. 4** Enzymatic resolution of ( $\pm$ )-DMCPM in organic–aqueous biphasic system



Zhou et al. reported a newly isolated bacterial strain, *Enterobacter* sp. ECU1107 for the enantioselective hydrolysis of (2*R*,3*S*)-PGM with substrate concentration of 600 mM [70]. The recovery of the desired (2*S*,3*R*)-PGM in this process could be facilitated through the decomposition of the unstable product, (2*R*,3*S*)-3-phenylglycidic acid (Fig. 5). The great potential of *Enterobacter* sp. ECU1107 for industrial production of the key precursor of pharmaceutically important Taxol was further demonstrated by the reaction on a scale of 1.0 L, yielding 11.6 g (2*S*,3*R*)-PGM with >99 %  $ee$ .

### 2.2.3 Key Intermediates to Pregabalin

Pregabalin, a marketed GABA analog, is used for the treatment of neuropathic pain and partial seizures [169] and has been launched by Pfizer as Lyrica<sup>®</sup>. The chemoenzymatic process involving biocatalytic resolution of *rac*-2-carboxyethyl-3-cyano-5-methylhexanoic acid ethyl ester (CNDE) has been considered to be the most cost-effective and greenest route for pregabalin [71]. The enzymatic resolution process adopted a commercially available lipase from *Thermomyces lanuginosus*



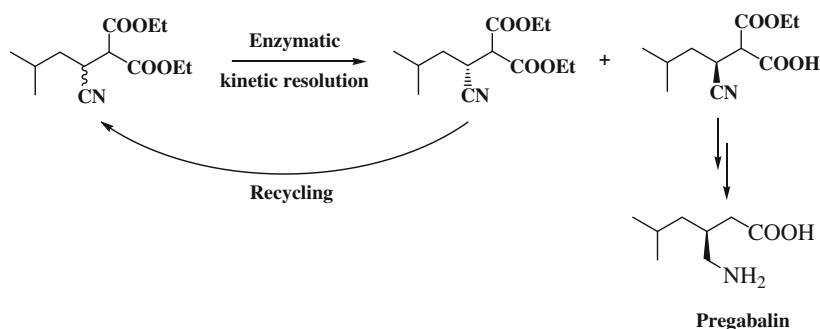
**Fig. 5** Enantioselective hydrolysis of racemic methyl trans-3-phenylglycidate [( $\pm$ )-PGM] using whole cells of *Enterobacter* sp. ECU1107 for the production of optically pure (2*S*,3*R*)-PGM



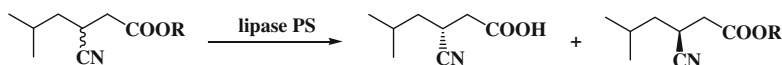
(Lipolase) to prepare the key enantiopure intermediate (*S*)-3-cyano-2-(ethoxycarbonyl)-5-methylhexanoic acid (*ee* > 98 %) with high yields and unprecedented high substrate load (765 g/L, 3.0 M) (Fig. 6). The undesired (*R*)-CNDE could readily be racemized using sodium ethoxide in ethanol at 80 °C. The enzymatic resolution was also tried for pilot runs at 900 kg scale and manufactured at 3.5 tons to demonstrate the scalability and consistency in performance. Besides the commercial lipase Lipolase, a newly isolated strain, *Morgarella morganii* ZJB-09203, could also be used for the resolution of CNDE [72]. Recently, an efficient route to pregabalin with higher atom economy was developed through the preparation of (*S*)-3-cyano-5-methylhexanoic acid ethyl ester [73]. The commercial lipase PS (Amano) from *Pseudomonas cepacia* was demonstrated to be the best enzyme for the hydrolytic resolution (Fig. 7). The substrate load was as high as 2.0 M (366 g/L), and (*S*)-3-cyano-5-methylhexanoic acid ethyl ester was produced in 99 % *ee* and 44.5 % yield.

### 2.3 Optically Pure Hydroxy Acids

Because of the dual functionality, optically pure hydroxy acids are versatile chiral synthons of particular interest in pharmaceutical industries. Optically active 2-hydroxy-phenyl acetic acid and its derivatives are the most important hydroxy acids. For example, (*R*)-2-hydroxy-2-(2'-chlorophenyl) acetic acid is the key chiral intermediate for the synthesis of (*S*)-clopidogrel, a platelet aggregation inhibitor. The enzymatic resolution catalyzed by esterase or lipase is a competitive method among the enzymatic routes to enantiomerically pure aromatic  $\alpha$ -hydroxy acids [74].

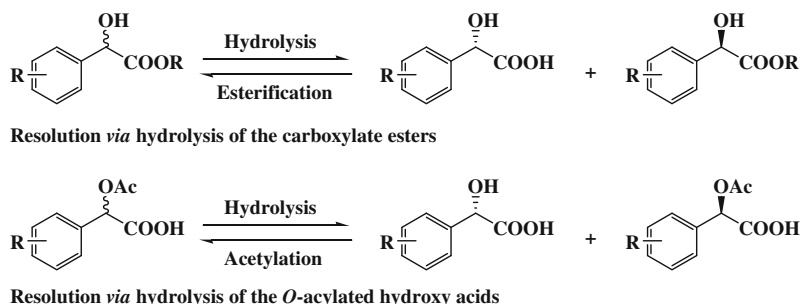


**Fig. 6** Lipolase-catalyzed resolution of *rac*-2-carboxyethyl-3-cyano-5-methylhexanoic acid ethyl ester

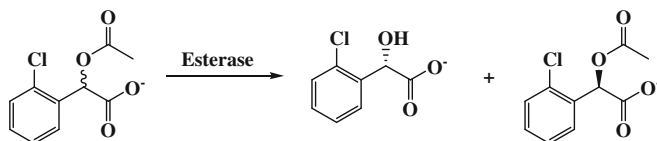


**Fig. 7** Lipase-mediated resolution of *rac*-3-cyano-5-methylhexanoic acid ester

Chiral 2-hydroxy-phenyl acetic acid and its derivatives can be prepared through the enantioselective synthesis or hydrolysis of hydroxy esters or *O*-acetylated hydroxy acids (Fig. 8). The ester group can be hydrolyzed directly from racemic hydroxy esters [75, 76] or the *O*-protected hydroxy esters [77]. The enantioselective synthesis or hydrolysis of *O*-acetylated hydroxy acids receives more interest and several promising catalysts including the *P. putida* esterase [78, 79], and commercially available lipases [80, 81] have been reported for this bioprocess in last decades. A series of substituted mandelic acids were enantioselectively acetylated by lipase PS (Amano) using vinyl acetate as acyl donor [81]. A thermostable *P. putida* esterase, rPPE01, which was screened and cloned for the resolution of 2-acetoxy-phenyl acetic acid and its derivatives, exhibited excellent enantioselectivity ( $E > 200$ ) to a series of acetylated aromatic  $\alpha$ -hydroxy acids at a substrate concentration of 100 mM [79]. In further work, the low activity was improved by semi-rational design, giving a single-point variant (rPPE01-W187H) with remarkably increased activity and excellent enantioselectivity [82]. Meanwhile, the biocatalyst deactivation was alleviated by carefully selection of suitable substrate counterion, among the five counterions tested,  $K^+$  showed the best stabilization effect. Finally, the resolution of 500 mM racemic potassium 2-acetoxy-2-(2'-chlorophenyl)acetic acid was successfully carried out with merely 0.5 g/L of lyophilized cells, and the conversion reached 49.9 % after 15 h with  $>99\%$   $ee_p$  and  $98.7\%$   $ee_s$  (Fig. 9).



**Fig. 8** Enzymatic resolution of 2-hydroxy-phenyl acetic acid and its derivatives



**Fig. 9** Esterase-catalyzed resolution of racemic *o*-Cl- $\alpha$ -acetoxyphenyl acetic acid

### 3 Chiral Synthesis with Epoxide Hydrolases

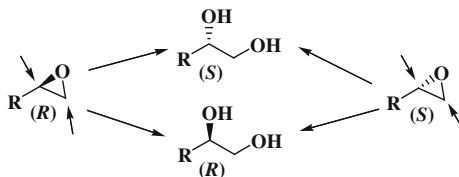
Enantiopure epoxides, as well as their corresponding vicinal diols are important chiral building blocks in organic synthesis. Epoxide hydrolase (EH)-catalyzed stereoselective hydrolysis of racemic or *meso*-epoxides are important methods for the preparation of enantiopure epoxides or corresponding vicinal diols.

Epoxides have two adjacent oxirane carbon atoms, and EH-catalyzed nucleophilic attack can occur on both carbon atoms, thereof EHs show not only enantioselectivity but also regioselectivity. According to the reaction mechanism, EHs catalyze the ring-opening via a nucleophilic  $S_N2$  attack by an aspartate residue that forms a transient covalent intermediate, and then, an activated water molecule attacks the carbonyl moiety of the ester-intermediate and releases the product diol. Therefore, inversion of configuration could happen when the attack was performed on a more substituted position.

Accordingly, besides the conventional kinetic resolution, EHs can also catalyze the enantioconvergent hydrolysis of racemic epoxides [83–85], once the two epoxide enantiomers are attacked at different positions as shown in Scheme 1. In contrast to traditional kinetic resolution, in which only 50 % theoretical yield is available, 100 % theoretical yield could be obtained in an enantioconvergent process.

#### 3.1 Styrene Oxide and Its Derivatives

Enantiopure styrene oxide and its derivatives are precursors of various pharmaceuticals and liquid crystal materials. They possess a benzylic carbon atom, which facilitates the formation of a carbo-cation stabilized by the adjacent aromatic ring. As a result, attack at the benzylic carbon is electronically, though sterically impeded. Thus, mixed regiochemical pathways (i.e., attack at both oxirane carbon atoms) are particularly easy within this group of substrates, and an enantioconvergent process could be expected.



**Scheme 1** Enantioconvergent hydrolysis of racemic epoxides

### 3.1.1 (*R*)-1-Phenyl-1,2-ethanediol

Recombinant *Pichia pastoris* expressing *Rhodotorula glutinis* EH shows high hydrolytic activity toward (*R*)-styrene oxide. Kinetic resolution of styrene oxide was conducted in a styrene oxide–aqueous biphasic system with a substrate load of 526 mM using whole cells of recombinant *P. pastoris*. Optically pure (*S*)-styrene oxide (>98 % *ee*) was obtained with 36 % yield [86]. After reaction optimization, (*S*)-styrene oxide of 98 % *ee* was formed with 41 % yield from 1.8 M *rac*-styrene oxide at pH 8.0, 4 °C in the presence of 40 % (v/v) Tween-20 and 5 % (v/v) glycerol [87].

Several enantioconvergent processes have been developed for the preparation of (*R*)-phenyl-1,2-ethanediol, including the combination of EHs from *Aspergillus niger* and *Bacillus sulfurescens* [88]; *Agrobacterium radiobacter* and *Solanum tuberosum* [89]; *A. niger* or *R. glutinis* and *Caulobacter crescentus* [90]; *C. crescentus*, and marine fish *Mugil cephalus* [91]. In these processes, the EHs from *A. niger*, *A. radiobacter*, *R. glutinis*, and *M. cephalus* preferentially attack the terminal carbon of the oxirane ring with retention of the stereochemistry, and (*R*)-diol was formed. On the other hand, the EHs from *B. sulfurescens* and *C. crescentus* attack the benzylic carbon of the (*S*)-enantiomer, which also leads to the formation of (*R*)-diol.

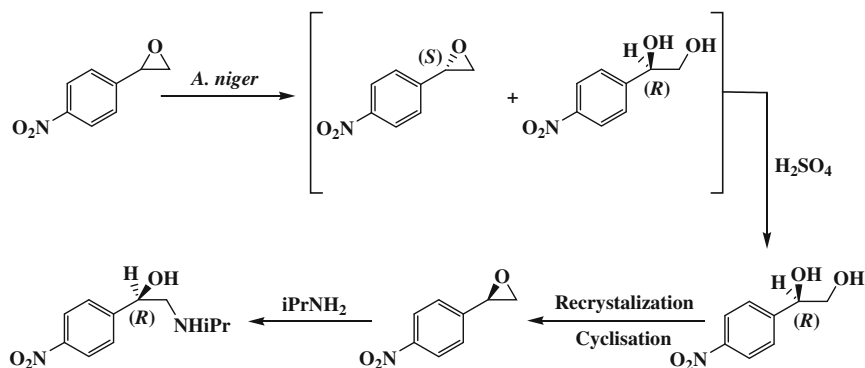
### 3.1.2 (*R*)-*p*-Nitro Styrene Oxide

(*R*)-*p*-Nitro styrene oxide [(*R*)-*p*NSO] and its corresponding diol are important precursors of (*R*)-Nifenalol, a  $\beta$ -adrenergic blocker with antianginal and antiarrhythmic properties.

*A. niger* EH (*AnEH*) can catalyze the kinetic resolution of *p*NSO, affording (*S*)-*p*NSO (96 % *ee*, 38 % yield) and (*R*)-diol (66 % *ee*, 49 % yield). After recrystallization, enantiopure (*R*)-diol was obtained with 32 % yield. In order to overcome the limitation of 50 % theoretical yield in the resolution process, acid hydrolysis of the remaining (*S*)-*p*NSO was investigated for the preparation of the corresponding (*R*)-diol with inversion of configuration at the stereogenic benzylic carbon atom and realized the desymmetric transformation of *rac-p*NSO into (*R*)-diol [92]. The produced (*R*)-diol was cyclized to (*R*)-*p*NSO and then condensed with isopropylamine, affording (*R*)-Nifenalol. The reaction details are shown in Fig. 10.

To obtain the (*R*)-diol with high optical purity, accurate tuning of these two consecutive reactions was necessary since the final *ee* of the diol was directly dependent upon the conversion ratio. According to calculation results based on the enantiomeric ratio, the acid hydrolysis of the remaining (*S*)-*p*NSO was initiated when *ee<sub>s</sub>* reached 95 %, and (*R*)-diol was formed in 90 % yield with 83 % *ee*, and after recrystallization, optically pure (*R*)-diol with 73 % yield was afforded.

Two EHs which could catalyze the enantioconvergent hydrolysis of *p*NSO were discovered from mung bean. Interestingly, these two EHs showed complementary enantioselectivities but with identical regioselectivity. Both EHs can catalyze the complete conversion of *p*NSO to (*R*)-diol. By using the mung bean crude powder as



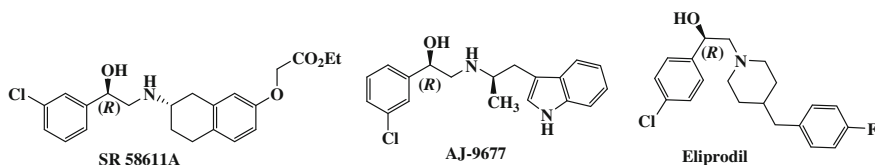
**Fig. 10** Chemoenzymatic synthesis of (R)-Nifenalol

biocatalyst, optically active (*R*)-diol was produced with 82.4 % *ee* and 83.5 % yield, and after recrystallization, enantiopure (*R*)-diol was obtained with an overall yield of 68.7 % [93]. The crude EH was then immobilized by diatomite adsorption, and Tween-80 was introduced for better substrate dispersion. After process optimization, the *ee* of (*R*)-diol was increased to 84.7 % [94]. An enantioconvergent EH (VrEH1) has been cloned from mung bean, which shows opposite regioselectivity toward (*S*)-*p*NSO (83 % to  $C_\alpha$ ) in contrast to (*R*)-*p*NSO (87 % to  $C_\beta$ ) [95].

### 3.1.3 Chlorostyrene Oxides

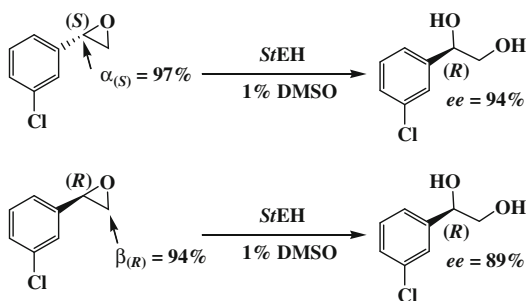
Enantiopure chlorostyrene oxides (CSOs) and their corresponding diols are important building blocks for the synthesis of a series of biologically active molecules, including  $\beta_3$ -adrenergic receptor agonists SR 58611A or AJ-9677, and an effective *N*-methyl-*D*-aspartic acid receptor antagonist eliprodil, as shown in Fig. 11.

Recombinant *S. tuberosum* EH (*St*EH) showed high enantioselectivity and complementary regioselectivity to the enantiomers of 3- and 4-CSO. For both epoxides, the (*S*)-enantiomer was preferentially attacked at the (benzylic) more



**Fig. 11** Pharmaceuticals synthesized from enantiopure chlorostyrene oxides

**Fig. 12** Enantioconvergent hydrolysis of *rac*-3-chlorostyrene oxide by *St*EH

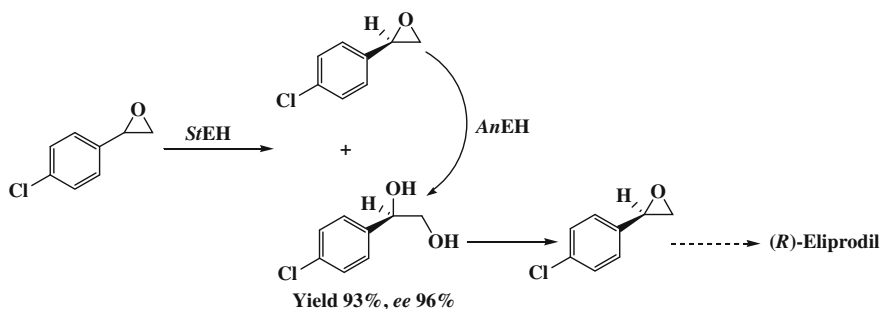


substituted carbon atom (97 %), whereas the *(R)*-antipode was attacked at the (terminal) less substituted carbon atom (94 %), as exemplified in Fig. 12. Enantioconvergent hydrolysis of 3- and 4-CSOs was performed, giving the corresponding *(R)*-diols with 91 and 74 % *ee*, respectively, after complete conversion of the substrates.

A preparative-scale biohydrolysis of *rac*-3-CSO was performed. For the purpose of minimizing the spontaneous hydrolysis of the 3-CSO and favor the stability of *St*EH, the reaction was performed at 20 °C. After complete conversion of the substrate, the homogeneous reaction mixture was filtered through an ultrafiltration membrane, and the recovered enzyme was reused. Totally, nine batches were performed at a substrate concentration of 10 g/L, the product was pooled, affording *(R)*-diol with 97 % *ee* and an isolated yield of 88 % [96].

*An*EH also shows high enantioselectivity to the 4-CSO and preferentially hydrolyzes the *(R)*-4-CSO, providing *(R)*-diols. Hydrolytic kinetic resolution of *rac*-4-chlorostyrene oxide was performed in heptane with an initial  $a_w$  of 0.9 [97]. Both the hydrolytic kinetic resolution efficiency and operational stability of *An*EH were found to be modest to excellent in various binary organic solvent mixtures of heptane and dioxane [98].

Since *St*EH and *An*EH showed complementary enantioselectivities, an enantioconvergent process for the enzymatic hydrolysis of 4-chlorostyrene oxide using a sequential bienzymatic strategy was adopted to realize the ideal 100 % yield of *(R)*-diol. In order to prevent the significant spontaneous hydrolysis, the enzymatic hydrolysis was conducted at 0 °C. *E*-value of about 100 was observed for both enzymes in a kinetic resolution process. As high as 2 M substrate could be efficiently resolved by *An*EH, while for *St*EH, substrate concentration above 200 mM was deleterious to the enzyme activity. Considering the *St*EH was an enantioconvergent EH and appeared to be more sensitive to inhibition by the *(R)*-diol formed, *St*EH was added first to completely transformed *(S)*-4-CSO before the addition of *An*EH. By this way, 0.2 M 4-chlorostyrene oxide was converted to *(R)*-diol with 96 % *ee* and 93 % yield [99]. The formed *(R)*-diol was a precursor for the preparation of *(R)*-eliprodil, as shown in Fig. 13.



**Fig. 13** Chemobioenzymatic preparation of (*R*)-eliprodil

### 3.2 Pyridyloxirane

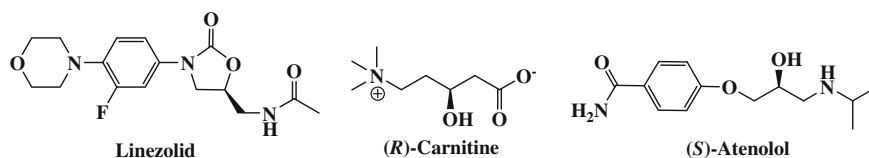
Enantiopure 2-, 3-, 4-pyridyloxirane are valuable chiral synthons, but they cannot be produced with high optical purity and yield using the conventional metal-based catalysts.

Among 14 EH collections, *AnEH* was found to be the best choice for the preparative-scale resolution of 2-pyridyloxirane [100]. Enantiopure (*S*)-2-pyridyloxirane (>99 % *ee*) was obtained in 43 % yield [101]. Gram-scale preparation of (*S*)-2-, 3-, and 4-pyridyloxirane was carried out by hydrolytic kinetic resolution with *AnEH* at a substrate concentration of 10 g/L (82 mM) in plain water, and (*S*)-pyridyloxiranes were afforded in a nearly enantiopure form (*ee* > 98 %) [102].

*A. radiobacter* EH (*ArEH*) can also catalyze the resolution of 2-, 3- and 4-pyridyloxiranes. An active-site mutation (Tyr215Phe) was introduced into the *ArEH* yielding a more suitable catalyst for kinetic resolution, and 127 mM (15.4 g/L) of (*S*)-2-pyridyloxirane was obtained in a preparative scale from 300 mM racemic substrate by the *ArEH* mutant [103].

### 3.3 Glycidyl Azide

Glycidyl azide is a key chiral C3 epoxide, wherein oxirane carbon atoms are highly active to many nucleophiles and azide is a precursor of amine. The optically pure glycidyl azides are important synthons for the synthesis of vicinal amino alcohols such as (*S*)-atenolol, (*R*)-carnitine, and synthetic antibiotic linezolid, as shown in Fig. 14. EH from *A. niger* showed high enantioselectivity toward *rac*-glycidyl azide and (*R*)-glycidyl azide was produced with 98 % *ee* and 40 % yield (*E* = 21) [104].



**Fig. 14** Pharmaceuticals prepared from optically pure glycidyl azides

### 3.4 *Epichlorohydrin and Aryl Glycidyl Ether*

#### 3.4.1 Epichlorohydrin

Enantiopure epichlorohydrins (ECHs) are important C3 chiral building blocks for the synthesis of pharmaceuticals, pesticides, and many other chemicals. For example, (*R*)-ECH serves as a key chiral intermediate for the synthesis of  $\beta$ -blocker drugs, such as metoprolol and alprenolol [105]. While (*S*)-ECH can be used as a precursor for atorvastatins, which is the top-selling cholesterol-lowering drug with global sales of 10 billion US dollars.

EHs with high enantioselectivity to epichlorohydrin are scarce. EHs from *A. niger* [106–108], *Rhodospiridium tortiloides* [109], and *Novosphingobium aromaticivorans* [110] prefer to hydrolyze the (*R*)-ECH, affording (*S*)-ECH, while EHs from *R. glutinis* [111, 112] and *A. radiobacter* [113, 114] prefer the (*S*)-ECH.

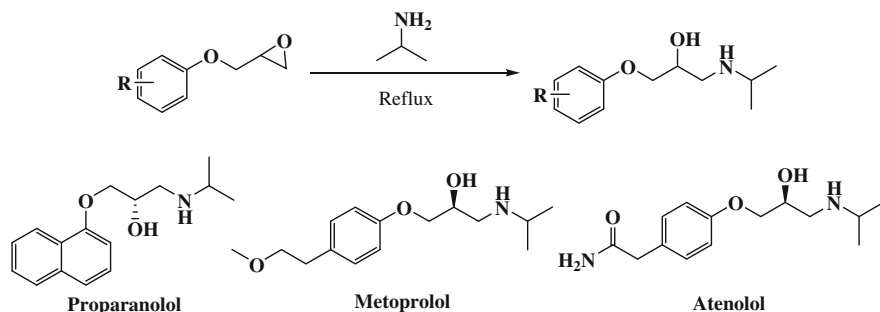
ECH is unstable in the aqueous medium. To overcome the spontaneous hydrolysis of ECH in aqueous buffer, organic solvents with little water [107, 108] and biphasic system [113, 114] were employed for the reaction. By using the recombinant *E. coli* whole cells expressing EH from *A. radiobacter* AD1 as biocatalyst, isooctane–aqueous (7:3) biphasic system was used for the reaction and 574 mM ECH was converted, producing (*R*)-ECH with 99.3 % *ee* and 37.5 % yield (analytical yield) [113].

#### 3.4.2 Aryl Glycidyl Ether

Aryl glycidyl ethers are important precursors for the preparation of many  $\beta$ -blocker drugs with a suffix of “lol,” including propranolol, metoprolol, and atenolol, as shown in Fig. 15.

Due to the flexible property of the chiral center in aryl glycidyl ethers, few EHs show high enantioselectivity toward these substrates, and most of them are (*S*)-preferred, such as those from *A. niger* [115], *A. radiobacter* [116], and *Trichosporon loubierii* [117, 118]. Protein engineering was employed to increase the enantioselectivity of EH for the enantioselective hydrolysis of aryl glycidyl ethers. After one round of error-prone polymerase chain reaction (epPCR), the *E*-value of AnEH was increased from 4.6 to 10.8. The improved variant contained three amino acid substitutions, and two of them were spatially far away from the catalytically





**Fig. 15** Preparation of  $\beta$ -blocker drugs from (*R*)-aryl glycidyl ethers

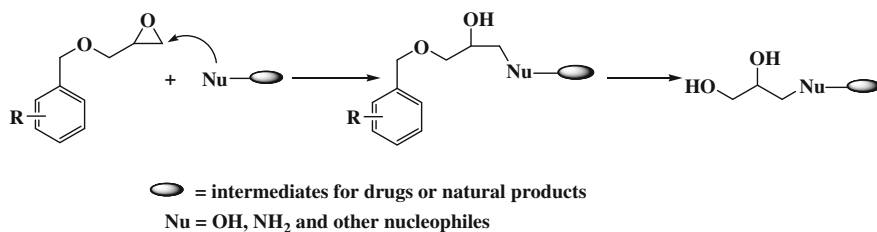
active center [119]. Moreover, the EH was evolved by iterative combinatorial active-site saturation test (CAST), leading to significantly improved enantioselectivity (enantiomeric ratio *E* from 4.6 to 115) for phenyl glycidyl ether (PGE) [120]. The enantioselectivity of EH from *A. radiobacter* was enhanced using epPCR and DNA shuffling, eight mutants showed significantly improved enantioselectivity (up to 13-fold) toward *p*-nitro-PGE and three other epoxides [121].

A bacterial strain, *Bacillus megaterium* ECU1001, was isolated from soil samples by using PGE as sole carbon and energy source [122], which preferentially hydrolyze the (*R*)-PGE, affording (*S*)-epoxide and (*R*)-diol with high enantioselectivity (*E* = 47.8) [123]. The *E*-value (enantiomeric ratio) was increased to 69.3 by using surfactant tween-80 as additive to help disperse the water-insoluble substrate [124]. Isooctane–aqueous biphasic system was employed to overcome the low solubility and instability of PGE in the aqueous phase, and *E*-value was further increased to 94. Resolution of 90.1 g/L PGE (based on isooctane phase) was carried out, affording enantiopure (*S*)-PGE with a yield (analytical yield) of 44.5 % [125, 126].

An (*R*)-enantioselective epoxide hydrolase (*BmEH*) was cloned from *B. megaterium* ECU1001 [127], high-to-excellent enantioselectivities (*E* > 200) were achieved in the bioresolution of PGE, *ortho*-substituted PGEs, and *meso*-nitro PGEs using the recombinant *BmEH*. The crystal structure of *BmEH* was resolved recently. By analyzing the active site of *BmEH*, two residues (Met145 and Phe128) were identified as potential hot spots for enhancing the *BmEH* activity toward the bulky substrates. After site-directed mutation of the two predicted hot spots, the activity of the *BmEH* was improved by 6–430-folds toward nine typical  $\beta$ -blocker precursors [128].

### 3.4.3 Benzyl Glycidyl Ether

Optically pure benzyl glycidyl ether (BGE) plays an important role in the synthesis of numerous drugs and natural products. For example, (*S*)-BGE is an intermediate of (+)-cryptocarya diacetate, a natural product used in the treatment of headaches, morning sickness, and cancer pulmonary diseases. (*R*)-BGE is an intermediate for



**Fig. 16** Application of optically active BGE and its derivatives in the synthesis of complex compounds

the synthesis of the anti-tumor and anti-leukemic drug, Synargentoide A. Optically active derivatives of BGE, such as methylbenzyl glycidyl ethers (MBGE) and dimethoxybenzyl glycidyl ethers (DMBGE), are important intermediates for the synthesis of more complex compounds [129] (Fig. 16). As a result, synthesis of optically pure BGE and its derivatives has received considerable interest.

Due to the linear structure adjacent to the chiral center, EHs with high enantioselectivity to these compounds are quite limited. EHs from *Rhodotorula* sp. and *A. niger* preferred (*S*)-BGE [130–132], while the EH from *Rhodococcus fascians* M022 preferred the (*R*)-enantiomer [131], the *E*-values of these EHs were lower than 10. *Talaromyces flavus* containing a constitutive EH showed relatively high enantioselectivity to BGE, with an *E*-value of 13. By using the whole cells as biocatalyst, (*R*)-BGE (96 % *ee*) was obtained [133].

The enantioselectivity of enzymatic resolution of BGE could be significantly enhanced with a methyl substitution at the 2-position of BGE. For example, whole cells of *Rhodococcus ruber* SM 1789 showed high enantioselectivity (*E* > 200) to *rac*-2-methyl-BGE, and (*R*)-2-methyl-BGE was obtained with 98 % *ee* and 43 % yield, and the (*R*)-diol was produced with 97 % *ee* and 44 % yield [134]. Furthermore, an enantioconvergent process was performed via a tandem reaction of *Rhodococcus* sp. CBS 717.73 EH-catalyzed kinetic resolution of 2-methyl-BGE and an acid-catalyzed hydrolysis of the remaining (*R*)-2-methyl-BGE with inversion of the configuration, furnishing (*R*)-diols as the sole product in 97 % *ee* and 78 % yield.

### 3.5 Cascade Reactions

Cascade reactions are green and promising process for organic synthesis since it can avoid the usually metal catalysts involved protecting and deprotecting steps and costly intermediate isolation process, thus making the process cost-effective for target molecule synthesis [135]. Recombinant *E. coli* cells coexpressing styrene monooxygenase and enantioconvergent EH have been constructed for efficient enantioselective dihydroxylation of various terminal aryl olefins [136]. Using styrene and its phenyl-substituted derivatives as substrates, (*S*)-vicinal diols were

produced with medium-to-excellent enantiopurities by the monooxygenase coexpressed with *Sphingomonas* sp. EH, while (*R*)-vicinal diols were formed with the monooxygenase coexpressed with *StEH*. This type of cascade biocatalysis provides an attractive alternative to Sharpless dihydroxylation, accepting *cis*-alkene and offering enantioselective *trans*-dihydroxylation.

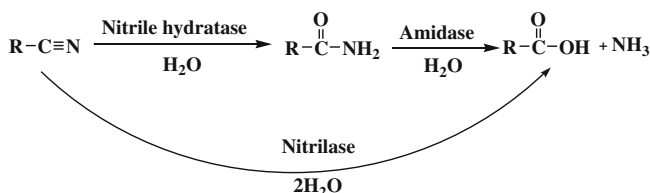
Recently, the recombinant *E. coli* cells expressing *A. radiobacter* halohydrin dehalogenase (HHDH) and *ArEH*, respectively, were immobilized by adsorption onto perlite and used for the preparation of (*R*)-epichlorohydrin from 1,3-dichloro-2-propanol in a cascade reaction. In the first step, racemic epichlorohydrin was produced with 73 % yield, and the final yield of enantiopure (*R*)-epichlorohydrin reached 25.1 % from 10 mM 1,3-dichloro-2-propanol [137].

## 4 Deracemization with Nitrilases

Nitrile compounds are ubiquitous in nature mainly in the form of cyanoglycosides, cyanolipids, ricinine, and phenylacetone nitrile, etc. [138]. They can be used for the manufacture of a series of polymers or as feedstock, solvents, and extractants in chemical industry or pesticides in agriculture. They are also very important intermediates for the synthesis of a variety of pharmaceuticals, agrochemicals, and fine chemicals because of their broad chemical versatility [139].

Nitrilases (EC 3.5.5.1) and nitrile hydratases (EC 4.2.1.84) are two classes of important nitrile-converting enzymes, the former directly hydrolyze nitriles into the corresponding carboxylic acids and  $\text{NH}_3$  in a single step, while the latter first convert nitriles into amides, which are then transformed into carboxylic acids and  $\text{NH}_3$  by amidases (EC 3.5.1.4) (Scheme 2) [140]. In recent years, nitrilase-mediated biocatalysis has attracted substantial interest from both academia and industry since it can be performed under mild reaction conditions combined with excellent selectivity (chemoselectivity, regioselectivity, and enantioselectivity). The enzymatic approach is significantly superior to traditional chemical methods that usually require harsh reaction conditions such as high temperatures, strongly acidic, or basic environment [141–143].

This section attempts to describe the use of nitrilase for the synthesis of pharmaceuticals, agrochemicals, fine chemicals, and their building blocks.



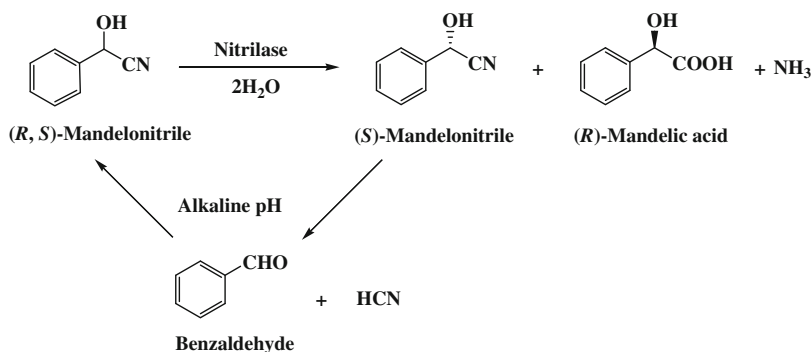
**Scheme 2** Pathways for nitrile hydrolysis

## 4.1 Optically Pure $\alpha$ -Hydroxy Carboxylic Acid and Its Derivatives

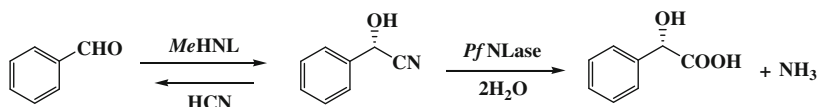
### 4.1.1 Optically Pure Mandelic Acid

Enantiopure  $\alpha$ -hydroxy acid and its derivatives serve as important chiral synthons for the synthesis of various pharmaceuticals, a chiral determination reagent, and a resolving reagent, for example, (*R*)-mandelic acid, is a key intermediate for the synthesis of semisynthetic penicillin, cephalosporin, antitumor agent, and antiobesity drugs [144], while (*S*)-mandelic acid can be used to synthesize the nonsteroidal anti-inflammatory drugs celecoxib and deracoxib [145]. Great effort has been paid on the development of nitrilase-catalyzed synthesis of optically pure mandelic acid from racemic mandelonitrile recently. The reaction is usually performed at slightly alkaline pH conditions, in which (*R*)-selective nitrilase preferentially hydrolyzes (*R*)-mandelonitrile to (*R*)-mandelic acid, whereas the unreacted (*S*)-mandelonitrile is spontaneously racemized in situ under the alkaline conditions and the newly formed (*R*)-mandelonitrile is used for the hydrolysis over again, thereby allowing the reaction to be proceeded in a DKR manner and affording 100 % theoretical yield (Fig. 17) [144].

Recently, a nitrilase producing strain *Alcaligenes* sp. ECU0401 was isolated from soil samples in our laboratory, which showed excellent enantioselectivity (>99.9 % *ee*) toward *R*-mandelonitrile [146]. The nitrilase gene was then cloned and over-expressed in *E. coli*, resulting in about 160-fold enhancement in nitrilase expression [147], and the enzyme production was further increased up to 19,000 U/L (50-fold improvement) by optimization of culture conditions and glycerol feeding [148]. Using the recombinant *E. coli* cells as catalyst, totally 520 mM (79 g/L) (*R*)-mandelic acid could be produced from 600 mM mandelonitrile in a fed-batch reaction and the space time yield (STY) reached 108 g (product) L<sup>-1</sup> d<sup>-1</sup> [149]. To relieve substrate inhibition, a toluene–water biphasic reaction system was adopted and the



**Fig. 17** Nitrilase-catalyzed synthesis of (*R*)-mandelic acid from racemic mandelonitrile in a dynamic kinetic resolution manner



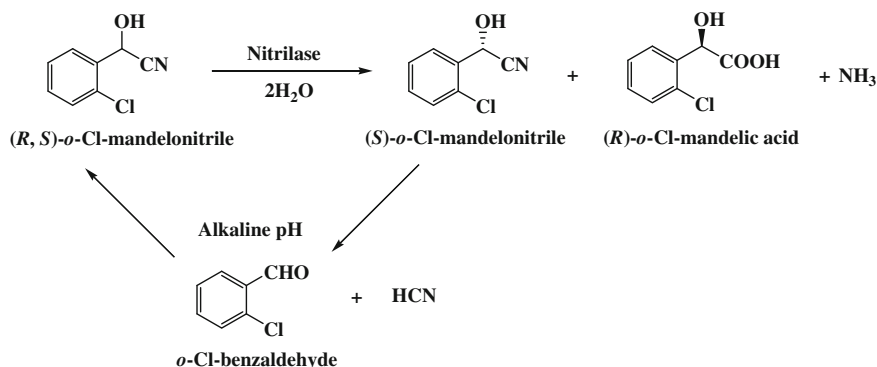
**Fig. 18** Enzymatic cascade procedure for the synthesis of (*S*)-mandelic acid using *S*-selective hydroxynitrile lyase (*MeHNL*) and non-selective nitrilase in tandem

STY was increased to 352 g (product) L<sup>-1</sup> d<sup>-1</sup>. Furthermore, the recombinant *E. coli* cells were immobilized in calcium alginate to facilitate product isolation and biocatalyst recovery; finally, 110.7 g (*R*)-mandelic acid was obtained by recycling the immobilized biocatalyst in a 2-L-stirred tank reactor, giving a catalyst productivity of 13.8 g (product) g<sup>-1</sup> (cells) [150]. Xue et al. developed an integrated bioprocess for the efficient production of (*R*)-mandelic acid with the immobilized *Alcaligenes faecalis* ZJUTB10 in a packed bed bioreactor which was incorporated with an in situ product recovery system to overcome product inhibition. This reaction system was very stable and gave a productivity of 8.87 mM/h in 16 h of reaction; totally, 550 mmol of (*R*)-mandelic acid with excellent enantiomeric excess (>99 %) was accumulated after 80 h of reaction [151]. Currently, BASF and Mitsubishi are producing (*R*)-mandelic acid from racemic mandelonitrile at several tons per year [74].

Since most mandelonitrile hydrolases are *R*-selective, it is not applicable to synthesize (*S*)-mandelic acid directly from racemic mandelonitrile. To overcome this limitation, Baum et al. developed a bienzymatic cascade reaction system including an (*S*)-hydroxynitrile lyase from *Manihot esculenta* and a non-selective arylacetone nitrilase from *Pseudomonas fluorescens* EBC191 for the synthesis of (*S*)-mandelic acid from benzaldehyde and cyanide (Fig. 18). An aqueous–ionic liquid biphasic system was adopted to alleviate the inhibitory effect of benzaldehyde on nitrilase activity; this system allowed to convert up to 700 mM benzaldehyde in the ionic liquid phase with a product yield of 87–100 %. Unfortunately, the nitrilase also showed nitrile hydratase activity; therefore, (*S*)-mandeloamide was formed as a by-product in about 50 % of the total product [152]. A third enzyme, an amidase from *Rhodococcus erythropolis*, was then incorporated to the bienzymatic cascade system, and all three enzymes were co-immobilized in cross-linked enzyme aggregates, allowing the production of (*S*)-mandelic acid in 90 % yield and >99 % enantiomeric purity without any by-product [153].

#### 4.1.2 Optically Pure *o*-Chloromandelic Acid

(*R*)-*o*-Chloromandelic acid is a key chiral precursor for the synthesis of the platelet aggregation inhibitor, (*S*)-clopidogrel, which is sold under the commercial name of Plavix<sup>®</sup>. Plavix<sup>®</sup> is a very important drug in reducing the risk of stroke, heart attack, and death in patients with a previous stroke, unstable angina, heart attack, or peripheral arterial disease caused by blood clots. In 2009, Plavix<sup>®</sup> has become the



**Fig. 19** Synthesis of (*R*)-*o*-chloromandelic acid from *o*-chloromandelonitrile by nitrilase-mediated hydrolysis

second top-selling drug in the world with global sales of over 10 billion US dollars per year. Several enzymatic methods have been developed for the synthesis of (*R*)-*o*-chloromandelic acid; of them, the nitrilase-catalyzed DKE of *o*-chloromandelonitrile represents one of the most promising approaches since under slightly alkaline conditions the unreacted (*S*)-*o*-chloromandelonitrile is spontaneously racemized to its racemate similar to nitrilase-mediated mandelonitrile hydrolysis, thereby affording 100 % theoretical yield (Fig. 19).

In order to explore new nitrilase for *o*-chloromandelonitrile hydrolysis, the data mining strategy based on BLAST was employed using the nitrilase sequence of *Alcaligenes* sp. ECU0401 as the template. Totally, seven nitrilases showing 40–60 % amino acid identities with the template were cloned and expressed in *E. coli*, after screening based on activity and enantioselectivity, a new nitrilase from *Labrenzia aggregata* (LaN) was discovered, which could catalyze the enantioselective hydrolysis of *o*-chloromandelonitrile to (*R*)-*o*-chloromandelic acid with 96.5 % *ee*. To enhance the process efficiency, a toluene–water biphasic reaction system was used to relieve substrate inhibition, in which up to 300 mM *o*-chloromandelonitrile could be completely transformed, giving an isolated yield of 94.5 %, and a space time yield of 154 g (product) L<sup>-1</sup> d<sup>-1</sup>, respectively [154].

## 4.2 Enantiomerically Pure $\beta$ -Hydroxy Carboxylic Acids

Optically pure  $\beta$ -hydroxy carboxylic acids are key building blocks for the synthesis of natural products, antibiotics, and chiral auxiliaries. Meanwhile, they can also be used for the manufacture of copolyesters in the film, fiber, molding, and coating industry [155]. The versatile application of chiral  $\beta$ -hydroxy carboxylic acids as important synthons has triggered the development of efficient and eco-friendly methodologies for the preparation of enantiomerically pure  $\beta$ -hydroxy carboxylic

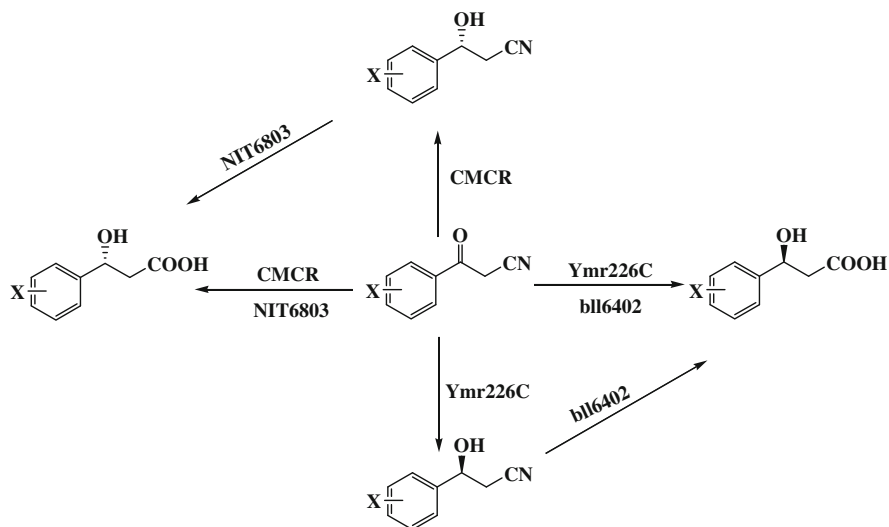
acids, including enzymatic reduction of  $\beta$ -ketoesters [156], enzymatic resolution of racemic acylated  $\beta$ -hydroxyesters [157], or kinetic resolution of racemic  $\beta$ -hydroxy carboxylic acid esters [158]. Optically pure  $\beta$ -hydroxy carboxylic acids can also be accessed by enantioselective hydrolysis of the easily available  $\beta$ -hydroxy nitriles. However, conventional chemical hydrolysis of nitriles usually requires harsh reaction conditions and elevated temperatures, which in turn result in the undesirable elimination of the functional group carried by the nitriles and lead to by-product formation. Nitrilase-mediated hydrolysis of nitriles represents an attracting alternative since the reaction can be carried out at environmental benign conditions, avoiding the protection and deprotection of functional groups, and most importantly, nitrilases are always highly selective.

#### 4.2.1 Optically Pure 3-Hydroxy-3-phenylpropionate and Its Derivatives

In an attempt to explore the synthetic applicability of nitrilases obtained by genome mining,  $\beta$ -hydroxy nitriles were subjected to hydrolysis by these nitrilases. Both nitrilases (NIT6803 from cyanobacterium *Synechocystis* sp. strain PCC 6803 and bll6402 from *Bradyrhizobium japonicum* strain USDA110) could catalyze the enantioselective hydrolysis of  $\beta$ -hydroxy nitriles to give enantioenriched  $\beta$ -hydroxy carboxylic acids, NIT6803 produced (*S*)-enriched  $\beta$ -hydroxy carboxylic acids, while bll6402 produced (*R*)-enriched  $\beta$ -hydroxy carboxylic acids, but both with low-to-moderate enantioselectivity [159, 160]. To address the enantioselectivity issue, a two-step one-pot process involving carbonyl reductase and nitrilase was developed, in which  $\beta$ -ketonitriles were stereoselectively reduced by carbonyl reductase to afford (*R*)- or (*S*)- $\beta$ -hydroxy nitriles, which were then hydrolyzed by nitrilase to produce optically active  $\beta$ -hydroxy carboxylic acids in high yields (Fig. 20). Another advantage of this bienzymatic cascade reaction process is that the isolation of the intermediates  $\beta$ -hydroxy nitriles could be avoided, thereby lowering the process cost and minimizing the environmental impact [161].

#### 4.2.2 (*R*)-4-Cyano-3-hydroxybutyric Acid

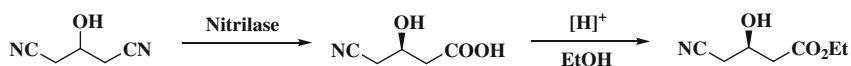
Lipitor<sup>®</sup>, the world's top-selling drug, is a cholesterol-lowering drug, and a member of the statin family of HMG-CoA (HMG = 3-hydroxy-3-methylglutaryl) reductase inhibitors. It contains a (3*R*,5*S*)-dihydroxyhexanoate side chain with two chiral centers. The huge market requirement of cholesterol-lowering drugs has stimulated great efforts invested on the efficient and economic synthesis of the chiral side chain, including ketoreductase-catalyzed asymmetric reduction of carbonyl precursors, lipase-promoted kinetic resolution of respective esters, aldolase-mediated carbon-carbon bond-forming reaction of aldehydes, and nitrilase-based desymmetrization of prochiral 3-hydroxyglutaronitrile. Among these methods reported, the nitrilase-catalyzed desymmetrization of 3-hydroxyglutaronitrile to afford (*R*)-4-cyano-3-hydroxybutyric acid shows great advantage since it can be carried



**Fig. 20** Two-step one-pot synthesis of optically pure  $\beta$ -hydroxy carboxylic acids

out in 100 % theoretical yield; furthermore, nitrilase is a cofactor-free enzyme, thereby avoiding the supplement of expensive cofactor in the bioreaction process. DeSantis et al. created a nitrilase tool box containing more than 200 new nitrilases by extracting DNA directly from environmental samples collected from different locations of the world [162]. By screening the nitrilase tool box, four enzymes were found to be able to produce (*R*)-4-cyano-3-hydroxybutyric acid from 3-hydroxyglutaronitrile with high conversion (>95 %) and *ee* (>90 %). One of them was then chosen to perform gram-scale (1 g, 240 mM substrate) preparation, affording (*R*)-4-cyano-3-hydroxybutyric acid in 98 % yield and 95 % *ee*, the ethyl ester of which is an important intermediate for the manufacture of cholesterol-lowering drug Lipitor<sup>®</sup> (Fig. 21).

Unfortunately, the enantioselectivity of the nitrilase dramatically decreased as the substrate concentration increased for the cost-effective production of (*R*)-4-cyano-3-hydroxybutyric acid. To address this problem, a novel directed evolution technology named as the gene site saturation mutagenesis (GSSM) was employed aiming to obtain a nitrilase variant that could convert 3-hydroxyglutaronitrile to (*R*)-4-cyano-3-hydroxybutyric acid with high enantioselectivity at high substrate concentration (3 M). Combined with a high-throughput screening method, they were able to identify a best variant (A190H) which could efficiently transformed



**Fig. 21** Nitrilase-catalyzed desymmetrization of 3-hydroxyglutaronitrile to produce (*R*)-4-cyano-3-hydroxybutyric acid



3 M 3-hydroxyglutaronitrile to (*R*)-4-cyano-3-hydroxybutyric acid (96 % isolated yield, 98.5 % *ee*) in 15 h with a space time yield of 619 g (product) L<sup>-1</sup> d<sup>-1</sup> [163].

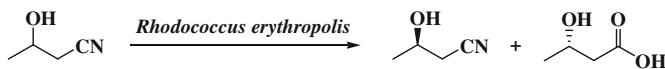
Based on the above results, Bergeron et al. developed a three-step process for the synthesis of (*R*)-4-cyano-3-hydroxybutyric acid starting from the low-cost epichlorohydrin, in which the epichlorohydrin was first cyanided by sodium cyanide to give 3-hydroxyglutaronitrile, which was then subjected to hydrolysis by nitrilase. After 16-h reaction, (*R*)-4-cyano-3-hydroxybutyric acid was produced with 100 % conversion and 99 % *ee* [164].

#### 4.2.3 (*S*)-3-Hydroxybutyric Acid

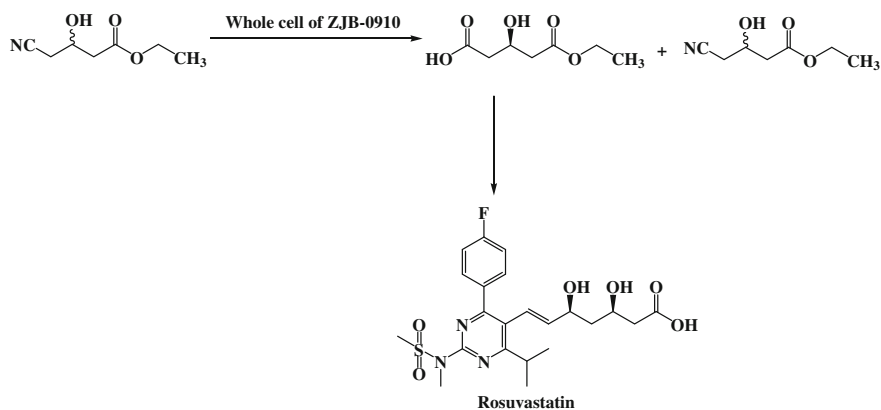
Lennon et al. devised a strategy that combines toxicity, starvation, and induction studies together with subsequent high-throughput screening method based on a 96-well plate system to rapid identification of bacterial isolates showing nitrilase activity [165]. This strategy enabled the fast screening of 256 novel nitrilase producing bacterial strains toward  $\beta$ -hydroxy nitriles. One of the bacterial strains, which was identified as *Rhodococcus erythropolis* SET1, was found to catalyze deracemization of 3-hydroxybutyronitrile with excellent enantioselectivity. In a preparative-scale reaction, optically pure (*S*)-3-hydroxybutyric acid was successfully produced from 3-hydroxybutyronitrile with 42 % yield and >99.9 % *ee* (Fig. 22).

#### 4.2.4 Ethyl (*R*)-3-Hydroxyglutarate

Optically active (*R*)-ethyl-3-hydroxyglutarate is a key precursor for the synthesis of a potent statin drug Rosuvastatin, which has received great interest in the therapy of patients with coronary artery disease because of its great potential in lowering the level of low-density lipoprotein cholesterol and medical security as compared to other statins. In order to develop a highly efficient and cost-effective method for the synthesis of enantiopure (*R*)-ethyl-3-hydroxyglutarate, a  $\beta$ -hydroxy aliphatic nitriles hydrolase-producing strain, identified as *R. erythropolis* ZJB-0910, was isolated by a colorimetric screening method [166]. Under the optimal reaction conditions using



**Fig. 22** Enantioselective hydrolysis of 3-hydroxybutyronitrile for the synthesis of optically pure (*S*)-3-hydroxybutyric acid

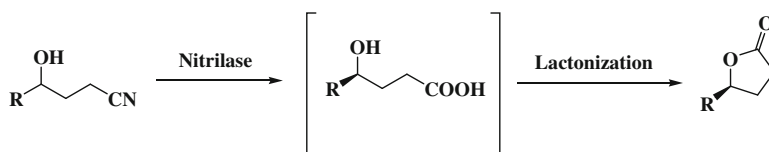


**Fig. 23** Enantioselective hydrolysis of racemic ethyl 4-cyano-3-hydroxybutyrate for the synthesis of optically active (*R*)-ethyl-3-hydroxyglutarate

the whole cells of *R. erythropolis* ZJB-0910 as biocatalyst, enantiomerically pure (*R*)-ethyl-3-hydroxyglutarate was produced from racemic ethyl 4-cyano-3-hydroxybutyrate with 46.2 % yield and >99 % *ee* (Fig. 23).

### 4.3 Enantiomerically Pure $\gamma$ -Hydroxy Carboxylic Acids

$\gamma$ -Butyrolactones serve as important intermediates for the synthesis of natural products and pharmaceuticals, and they are also main components of flavors, fragrance, and insect pheromones [167].  $\gamma$ -Butyrolactones can be produced directly from  $\gamma$ -hydroxy carboxylic acids via lactonization, while  $\gamma$ -hydroxy carboxylic acids can be accessed by nitrilase-catalyzed hydrolysis of  $\gamma$ -hydroxynitriles under mild reaction conditions (Fig. 24). A series of optically active  $\gamma$ -hydroxy carboxylic acids with different side-chain lengths and structures were prepared from their respective  $\gamma$ -hydroxynitriles by commercial nitrilases with moderate-to-high enantioselectivity. The formed  $\gamma$ -hydroxy carboxylic acids can then be transformed into optically pure lactones through lactonization [168].



**Fig. 24** Nitrilase-mediated hydrolysis of hydroxynitriles to hydroxyacids and subsequent lactonization of hydroxyacids to form lactones

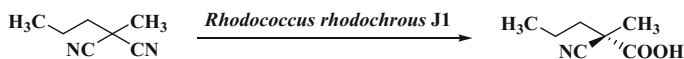
## 4.4 Important Enantioenriched Cyano Acids

### 4.4.1 (S)-2-Cyano-2-methylpentanoic Acid

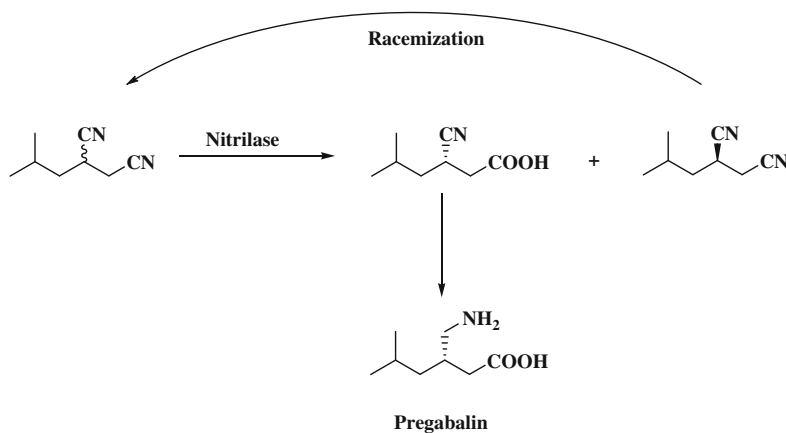
(S)-2-Cyano-2-methylpentanoic acid can be converted to  $\beta$ -amino acids by chemical reduction of the cyano group, while  $\beta$ -amino acids are key building blocks for the synthesis of a series of biologically active compounds. Therefore, the prerequisite to access the biologically active compounds is the development of an efficient and green synthetic route to produce (S)-2-cyano-2-methylpentanoic acid. The well-studied nitriles converting bacterium *Rhodococcus rhodochrous* J1, which produces both nitrilase and nitrile hydratase activity, were chosen as the potential biocatalyst. To prevent potential influence of nitrile hydratase activity on the reaction, the nitrile hydratase activity was removed by cloning the nitrilase gene responsible for the target reaction alone from *R. rhodochrous* J1 and expressing it in *E. coli* JM109. The recombinant *E. coli* cells could enantioselectively hydrolyze 2-methyl-2-propylmalononitrile to form (S)-2-cyano-2-methylpentanoic acid (Fig. 25). In a scale-up reaction, 80 g (S)-2-cyano-2-methylpentanoic acid was successfully produced from 2-methyl-2-propylmalononitrile with 97 % molar yield and 96 % *ee* after 24-h transformation without any by-product [169].

### 4.4.2 (3S)-3-Cyano-5-methyl Hexanoic Acid

(3S)-3-Cyano-5-methyl hexanoic acid is a key precursor for the preparation of a marketed GABA analog, pregabalin (Lyrica<sup>®</sup> API), which is used for the treatment of neuropathic pain and partial seizures [170]. One approach to synthesize (3S)-3-cyano-5-methyl hexanoic acid is the (S)-selective nitrilase-mediated regio- and enantioselective hydrolysis of racemic isobutylsuccinonitrile, and the untouched enantiomer (3R)-isobutylsuccinonitrile could readily be racemized under basic conditions, allowing to afford (3S)-3-cyano-5-methyl hexanoic acid in 100 % theoretical yield (Fig. 26). Ten plant and bacterial nitrilases were cloned from GenBank and their ability to catalyze the target reaction was preliminarily examined by a fluorescent assay based on the  $\text{NH}_3$  product and further verified by chiral GC analysis. Five nitrilases showed activity toward isobutylsuccinonitrile with the best one AtNit 1 giving 45 % conversion and 98 % *ee*, while the others are very poor nitrilase toward the target substrate. However, from the view point of synthetic application, the specific activity of AtNit 1 was still too low to serve as an efficient catalyst. Therefore, protein engineering based on error-prone PCR was then



**Fig. 25** Enantioselective hydrolysis of 2-methyl-2-propylmalononitrile to produce (S)-2-cyano-2-methylpentanoic acid by recombinant *Rhodococcus rhodochrous* J1 nitrilase



**Fig. 26** Nitrilase-catalyzed synthesis of (3*S*)-3-cyano-5-methyl hexanoic acid from racemic isobutylsuccinonitrile

performed to engineer the AtNit 1 aiming to improve its catalytic efficiency. After screening of about 9,962 mutant clones (1 % of the mutant library), a single-mutant C236S was found to show about threefold increase in the activity for the hydrolysis of isobutylsuccinonitrile without affecting its enantioselectivity.

## 5 Asymmetric Synthesis with Keto Reductases

Chiral alcohols are key building blocks for the synthesis of a variety of biologically active molecules and active pharmaceutical ingredients. Numerous biocatalytic processes, including ketoreductase-catalyzed asymmetric reduction of prochiral carbonyl compounds, EH-mediated hydrolysis of epoxides, lipase-promoted kinetic resolution of esters, HHDH-catalyzed ring-opening of epoxides, and aldolase-based aldehyde condensation, have been developed for the efficient and eco-friendly synthesis of optically pure alcohols [6, 171, 172]. Of these methods, the ketoreductase-catalyzed asymmetric reduction of prochiral ketones represents a promising approach for the synthesis of chiral alcohols since it can be performed under mild reaction conditions with a theoretical yield of 100 % and excellent stereoselectivity. From the view point of industrial application, a promising biocatalytic process is expected to meet the criteria of high substrate concentration ( $\geq 100$  g/L), low catalyst usage ( $\leq 5$  g/L), excellent enantioselectivity ( $>99.5$  %), and high volumetric productivity ( $\geq 100$  g L<sup>-1</sup> d<sup>-1</sup>) combined with no or little amount of cofactor consumption ( $<0.5$  g/L) [173].

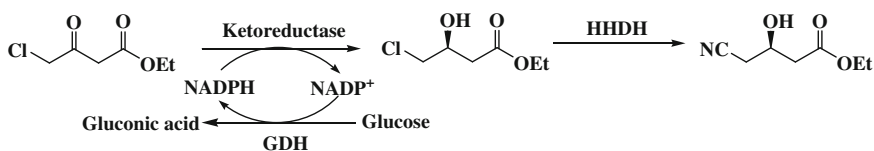
In this section, the efficient and cost-effective synthesis of chiral alcohols by ketoreductase-catalyzed asymmetric reduction will be discussed.

## 5.1 Optically Active Ethyl 4-chloro-3-hydroxybutyrate

### 5.1.1 Ethyl (S)-4-chloro-3-hydroxybutyrate

Ethyl (S)-4-chloro-3-hydroxybutyrate is an important chiral building block for the synthesis of HMG-CoA reductase inhibitor, which is the active component of the cholesterol-lowering drug Lipitor. An impressive contribution for the synthesis of ethyl (R)-4-cyano-3-hydroxybutyrate is presented by Codexis, which involves a two-step process: At first, a ketoreductase coupled with a glucose dehydrogenase (GDH) for cofactor regeneration was employed to asymmetrically reduce ethyl 4-chloroacetoacetate resulting in the formation of ethyl (S)-4-chloro-3-hydroxybutyrate, which was then transformed into ethyl (R)-4-cyano-3-hydroxybutyrate by HHDH via the formation of an epoxide intermediate (Fig. 27). Protein engineering was adopted to improve the catalyst efficiency and the process is now running at 2,000 L scale [174]. This pioneer work was assigned the Presidential Green Chemistry Challenge Award in 2006.

Besides the work of Codexis, great effort has also been paid on the development of an efficient process to produce ethyl (S)-4-chloro-3-hydroxybutyrate based on ketoreductase-catalyzed asymmetric reductions by several different research groups. An acetoacetyl-CoA reductase was discovered from *Ralstonia eutropha* by a bioinformatic-based enzyme-screening method, which could catalyze the highly stereoselective reduction of ethyl 4-chloroacetoacetate to ethyl (S)-4-chloro-3-hydroxybutyrate. By using the recombinant *E. coli* cells coexpressing the reductase from *R. eutropha* and a glucose dehydrogenase from *B. subtilis* for the regeneration of NADPH as catalyst, 48.7 g/L ethyl (S)-4-chloro-3-hydroxybutyrate was successfully produced from ethyl 4-chloroacetoacetate with an optical purity of 99.8 % *ee* [175]. Recently, Wang et al. reported the cloning and expression of a highly active and stereoselective NADH-dependent reductase from *Streptomyces coelicolor* by genome mining [176]. In a water–toluene biphasic reaction system, up to 600 g/L (3.6 M) ethyl 4-chloro-3-oxobutyrates was asymmetrically reduced to ethyl (S)-4-chloro-3-hydroxybutyrate by the recombinant *E. coli* cells using isopropanol as co-substrate for cofactor regeneration, giving a product yield of 93 and >99 % *ee*. The volumetric productivity of the process reached as high as 609 g L<sup>-1</sup> d<sup>-1</sup> and a total turnover number of more than 12,000, indicating the great potential of this reductase for industrial application.



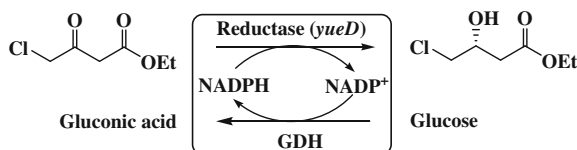
**Fig. 27** A two-step biocatalytic process for the synthesis of ethyl (R)-4-cyano-3-hydroxybutyrate

### 5.1.2 Ethyl (*R*)-4-chloro-3-hydroxybutyrate

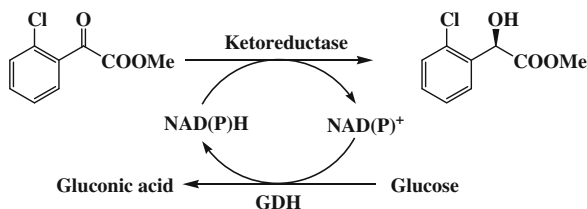
Optically pure ethyl (*R*)-4-chloro-3-hydroxybutyrate is an intermediate for L-carnitine, and several biocatalytic methods have been developed to produce ethyl (*R*)-4-chloro-3-hydroxybutyrate [177–180]. Nevertheless, the substrate loading used in these processes was relatively low, and the enantioselectivity was unsatisfactory, thereby hindering their practical application. Therefore, there is still an urgent need for novel and highly efficient biocatalyst for the cost-effective production of ethyl (*R*)-4-chloro-3-hydroxybutyrate. A carbonyl reductase gene (*yueD*) was identified from the genome sequence of *Bacillus* sp. ECU0013 and overexpressed in *E. coli*. The recombinant reductase showed activity toward a series of substrates including aromatic ketones,  $\alpha$ - and  $\beta$ -keto esters, especially ethyl 4-chloro-3-oxobutyrate. In an aqueous–toluene biphasic system using the recombinant *E. coli* cells coexpressing carbonyl reductase (*yueD*) and glucose dehydrogenase as catalyst, 215 g/L (1.3 M) ethyl 4-chloro-3-oxobutyrate was transformed into ethyl (*R*)-4-chloro-3-hydroxybutyrate by a fed-batch strategy with a product yield of 91.7 and 99.6 % *ee* (Fig. 28) [181].

## 5.2 Methyl (*R*)-*o*-Chloromandelate

As the key intermediate for the synthesis of the top-second selling drug Plavix<sup>®</sup> (clopidogrel bisulfate), methyl (*R*)-*o*-chloromandelate has attracted great interest from both academy and industry. A variety of synthetic methods have been reported for the synthesis of this chiral intermediate, including ketoreductase-catalyzed asymmetric reduction of methyl *o*-chlorobenzoylformate, nitrilase-mediated DKR of *o*-chloromandelonitrile, hydroxynitrile lyase-based hydrocyanation of *o*-chlorobenzaldehyde followed by hydrolysis with nitrilase, lipase/esterase-promoted kinetic resolution of *o*-chloromandelic acid esters, or  $\alpha$ -acetoxy-*o*-chlorophenyl acetic acid. Among these methods, the ketoreductase-based asymmetric reduction of methyl *o*-chlorobenzoylformate represents an attractive approach, since the reaction can be carried out at 100 % theoretical yield, and most importantly, the expensive cofactor can readily be regenerated in situ by coupling a glucose dehydrogenase (Fig. 29).



**Fig. 28** Ketoreductase-catalyzed asymmetric synthesis of (*R*)-4-chloro-3-hydroxybutyrate



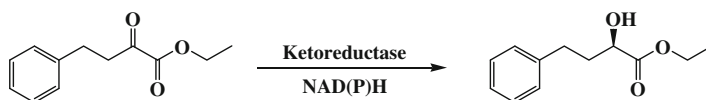
**Fig. 29** Stereoselective reduction of methyl *o*-chlorobenzoylformate to methyl (*R*)-*o*-chloromandelate by ketoreductase coupled with glucose dehydrogenase

An efficient biocatalytic reduction process for the synthesis of methyl (*R*)-*o*-chloromandelate was developed by Ema et al., in which a recombinant *E. coli* cell coexpressing a carbonyl reductase (Gre2p) from *Saccharomyces cerevisiae* and a glucose dehydrogenase from *B. megaterium* was utilized for the reduction of methyl *o*-chlorobenzoylformate with externally added  $\text{NADP}^+$ , affording methyl (*R*)-*o*-chloromandelate at 198 g/L [182]. A new carbonyl reductase, CgKR1, which shows high activity and excellent stereoselectivity toward methyl *o*-chlorobenzoylformate, was discovered from *Candida glabrata* by in silico data mining based on sequence homology using Gre2p as the template. Using the crude enzyme of CgKR1 together with glucose dehydrogenase as catalyst, as much as 300 g/L of methyl *o*-chlorobenzoylformate could be stoichiometrically reduced to methyl (*R*)-*o*-chloromandelate with a product yield of 87 and 98.7 % *ee*. The volumetric productivity of this process reached as high as  $700 \text{ g L}^{-1} \text{ d}^{-1}$ , suggesting a great potential for practical application [183]. Recently, a more efficient carbonyl reductase, YtbE, was identified from a tool box of carbonyl reductases cloned from *Bacillus* sp. ECU0013 and coexpressed with a glucose dehydrogenase in *E. coli*. Up to 500 g/L of methyl *o*-chlorobenzoylformate could be completely converted to optically pure methyl (*R*)-*o*-chloromandelate in an aqueous–ethyl caprylate biphasic reaction system by the recombinant *E. coli* cells with an isolated yield of 88 and >99 % *ee*, affording a volumetric productivity of  $812 \text{ g L}^{-1} \text{ d}^{-1}$ . It is noteworthy that no any external cofactor was added during the biocatalytic process, which will significantly reduce the production cost [184].

### 5.3 Ethyl 2-hydroxy-4-phenylbutyrate

#### 5.3.1 Ethyl (*R*)-2-hydroxy-4-phenylbutyrate

Ethyl (*R*)-2-hydroxy-4-phenylbutyrate is a key chiral synthon for the preparation of a variety of angiotensin-converting enzyme (ACE) inhibitors, while the latter is widely used for the treatment of hypertension and congestive heart failure. Although a number of methods for the preparation of ethyl (*R*)-2-hydroxy-4-phenylbutyrate have been investigated [185–187], the ketoreductase-catalyzed



**Fig. 30** Synthesis of ethyl (*R*)-2-hydroxy-4-phenylbutyrate by asymmetric reduction of ethyl 2-oxo-4-phenylbutyrate with reductase

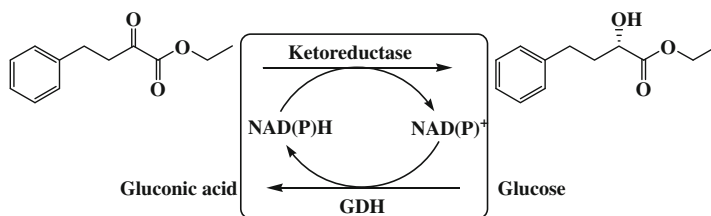
asymmetric synthesis from ethyl 2-oxo-4-phenylbutyrate represents an attractive alternative due to its high theoretical product yield, excellent stereoselectivity, and environmental friendliness (Fig. 30). Therefore, several microorganisms including *Candida boidinii* CIOC21, *Candida krusei* SW2026, *Pichia angusta*, and *S. cerevisiae*, have been employed for the synthesis of ethyl (*R*)-2-hydroxy-4-phenylbutyrate [188–191]. However, they share some common limitations such as low substrate loading, the requirement of external cofactor addition, and/or inadequate stereoselectivity that hinder their industrial application.

To discover more efficient catalyst for the practical synthesis of ethyl (*R*)-2-hydroxy-4-phenylbutyrate, a new reductase, CgKR2, was identified as the most promising catalyst candidate from 13 recombinant reductases obtained by genome mining due to its highest activity and stereoselectivity. Using the recombinant *E. coli* cells expressing CgKR2 and glucose dehydrogenase as catalyst, as much as 206 g/L (1 M) of ethyl 2-oxo-4-phenylbutyrate was completely reduced to ethyl (*R*)-2-hydroxy-4-phenylbutyrate with 84 % isolated yield and >99 % *ee*, the volumetric productivity reached 700 g L<sup>-1</sup> d<sup>-1</sup>, which is significantly higher than the highest value reported so far in literature. It should be noted that during the biocatalytic process, no cofactor was externally added, thereby greatly lowering the production cost [192]. The reductase, CgKR1, discovered by Ma et al. also showed very high activity in the reduction of ethyl 2-oxo-4-phenylbutyrate, and up to 412 g/L (2 M) of substrate could be stoichiometrically converted to the target product but with a little lower enantioselectivity (98.1 % *ee*) [183]. Recently, an aqueous–octanol biphasic reaction system combined with fed-batch strategy was developed by Ni et al., in which 330 g/L (1.6 M) ethyl 2-oxo-4-phenylbutyrate was successfully reduced to ethyl (*R*)-2-hydroxy-4-phenylbutyrate with 99.5 % *ee* using the recombinant *E. coli* cells coexpressing a reductase gene and a glucose dehydrogenase gene from *B. subtilis* as catalyst [193].

### 5.3.2 Ethyl (*S*)-2-hydroxy-4-phenylbutyrate

Similar to the synthesis of ethyl (*R*)-2-hydroxy-4-phenylbutyrate, its (*S*)-enantiomer can also be accessed through asymmetric reduction of ethyl 2-oxo-4-phenylbutyrate by reductases with reverse enantioselectivity. An impressive work on the efficient production of ethyl (*S*)-2-hydroxy-4-phenylbutyrate was presented by Ni et al., in which an extremely high substrate concentration (620 g L<sup>-1</sup>, equal to 3 M) could be completely transformed by the recombinant *E. coli* cells coexpressing a reductase



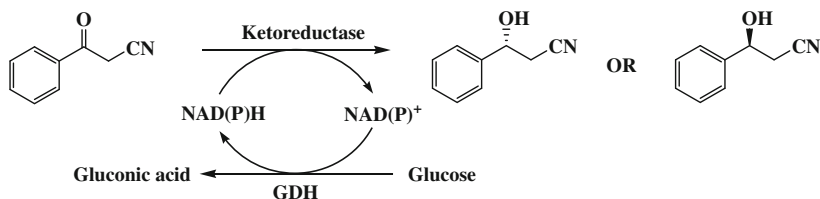


**Fig. 31** Preparation of ethyl (*R*)-2-hydroxy-4-phenylbutyrate by asymmetric reduction of ethyl 2-oxo-4-phenylbutyrate by reductase coupled with glucose dehydrogenase for cofactor regeneration

(FabG) identified from *Bacillus* sp. by genome mining and a glucose dehydrogenase in the absence of expensive cofactor, affording ethyl (*S*)-2-hydroxy-4-phenylbutyrate with 91 % isolated yield and >99 % *ee* (Fig. 31) [194].

#### 5.4 Optically Active $\beta$ -Hydroxynitriles

Optically pure  $\beta$ -hydroxynitriles find widespread application in the synthesis of various biologically active compounds and pharmaceuticals. For example, they are important precursors of the popular serotonin/norepinephrine reuptake inhibitors under the commercial name of fluoxetine, atomoxetine, and nisoxetine, which are widely used for the treatment of inception and disorders, including anxiety, alcoholism, bulimia, chronic pain, migraine headaches, sleep and memory disorders, and urinary incontinence [195]. A number of methods have been utilized for the synthesis of chiral  $\beta$ -hydroxynitriles including chemical routes and enzymatic approaches; however, the chemical methods usually require expensive heavy metals as catalyst, which will result in environmental pollution and toxicity issues, and the enantioselectivity is also unsatisfactory [196, 197]. While the enzymatic kinetic resolution approaches are limited by the low theoretical yield of 50 % [198, 199]. Therefore, the ketoreductase-catalyzed asymmetric reduction of  $\beta$ -ketonitriles offers an interesting alternative, in which both enantiomers are accessible in 100 % theoretical yield using biocatalysts with reverse enantioselectivity (Fig. 32).

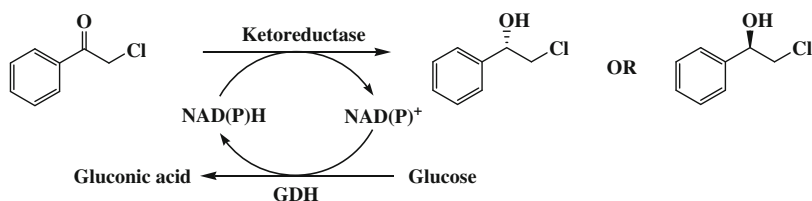


**Fig. 32** Synthesis of (*R*)- or (*S*)- $\beta$ -hydroxynitriles by asymmetric reduction of benzoylacetonitrile using reductase with reverse enantioselectivity

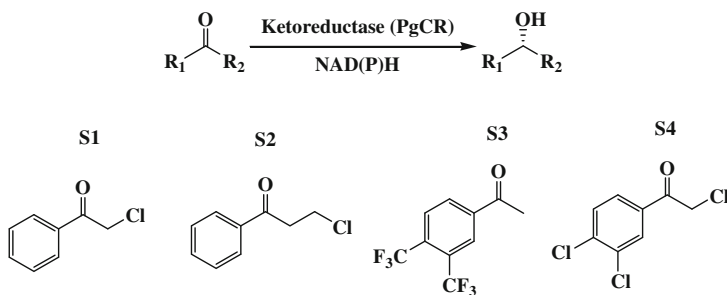
Optically active (*R*)- or (*S*)- $\beta$ -hydroxynitriles were produced with excellent enantioselectivity and yield from  $\beta$ -ketonitriles by using a carbonyl reductase (CMCR) from *Candida magnoliae* or an alcohol dehydrogenase (Ymr226c) from *S. cerevisiae* in its isolated form, respectively, without any  $\alpha$ -ethylated by-product, which is often observed in the whole cell biocatalysis [161]. Recently, Xu et al. reported the development of a biphasic system, in which recombinant *E. coli* cells coexpressing a data-mined carbonyl reductase *DhCR* from *Debaryomyces hansenii* or *CgCR* from *C. glabrata*, and a glucose dehydrogenase for in situ cofactor regeneration were employed for the asymmetric reduction of benzoylacetone, as much as 145 g/L substrate based on the organic phase was completely transformed into (*R*)- or (*S*)- $\beta$ -hydroxynitriles with >99 % *ee*, and no any  $\alpha$ -ethylated by-product was detected [200].

### 5.5 Optically Active Aryl Halohydrins

Optically pure aryl halohydrins play an important role in the synthesis of a variety of pharmaceutical relevant compounds, such as  $\beta$ -blockers,  $\beta$ -lactam antibiotics, and chiral biphosphines. [201]. To date, numerous work on the synthesis of enantioenriched aryl halohydrins especially those with biocatalyst have been extensively investigated (Fig. 33). For example, Xie et al. demonstrated the preparation of (*R*)-aryl halohydrin in >99 % *ee* from  $\alpha$ -chloroacetophenone using crude enzyme of Adzuki bean with a space time yield of 61.6 g L<sup>-1</sup> d<sup>-1</sup> [202], while the (*S*)-aryl halohydrin was produced from  $\alpha$ -chloroacetophenone at a substrate concentration of 144 g/L using the alcohol dehydrogenase (LsADH) from *Leifsonia* sp. strain S749 with a space time yield of 104 g L<sup>-1</sup> d<sup>-1</sup> and a total turn over number of 935 [203]. Recently, a carbonyl reductase tool box was developed by genome data mining, after screening based on specific activity and substrate tolerance using  $\alpha$ -chloroacetophenone as a model substrate, a robust carbonyl reductase (KtCR) from *Kluyveromyces thermotolerans* was discovered. In a biocatalytic reduction process using the recombinant *E. coli* cells expressing KtCR as biocatalyst, up to 154 g/L (1 M) of  $\alpha$ -chloroacetophenone was asymmetrically reduced to (*S*)-aryl halohydrin, giving an isolated yield of 92 and >99 % *ee* [204].



**Fig. 33** Synthesis of (*R*)- or (*S*)-2-chloro-1-phenylethanol via asymmetric reduction of  $\alpha$ -chloroacetophenone using reductase with reverse enantioselectivity

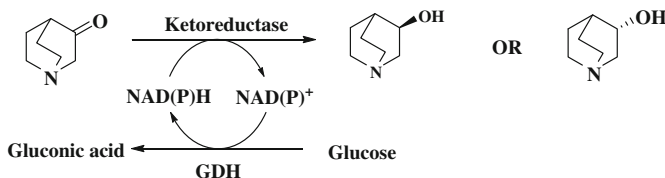


**Fig. 34** Asymmetric reduction of aryl-halogenated ketones with ketoreductase (PgCR)

To further explore the synthetic potential of this reductase tool box, a series of aryl-halogenated ketones were subjected to reduction using the reductases from this tool box. Another reductase, PgCR from *Pichia guilliermondii* NRRL Y-324, was found to show broad substrate spectrum, including aryl ketones, aliphatic ketones, and ketoesters. Four aryl-halogenated ketones were then asymmetrically reduced by the isolated reductase coupled with a NADPH regeneration system in a semi-preparative scale (Fig. 34). All of the four aryl halohydrins were produced with excellent enantioselectivity (>99 %) and isolated yields (>80 %) [205].

## 5.6 (R)-3-Quinuclidinol

Enantiomerically pure 3-quinuclidinol is an important chiral intermediate with widespread applications, for example, (R)-3-quinuclidinol is a precursor for the synthesis of talsacidine, revatropate, and cevimeline [206], while (S)-3-quinuclidinol is a very promising chiral synthon for serotonin receptor antagonist drugs and new anticholinergic drugs [207]. Therefore, a number of methods including chemical routes and enzymatic approaches have been developed for the preparation of optically pure 3-quinuclidinol. Of these methods reported, the ketoreductase-catalyzed asymmetric reduction of 3-quinuclidinone has attracted most attention since it can be carried out in 100 % theoretical yield, and by coupling a glucose dehydrogenase for in situ cofactor regeneration, the process cost could be significantly reduced (Fig. 35).



**Fig. 35** Synthesis of optically pure 3-quinuclidinol via asymmetric reduction of 3-quinuclidinone with ketoreductase

A recombinant *E. coli* cells coexpressing the reductase from *Rhodotorula rubra* and a glucose dehydrogenase were employed by Uzura et al. for the conversion of 100 g/L (618 mM) of 3-quinuclidinone to (*R*)-3-quinuclidinol in 98.6 % yield and >99.9 % *ee* [208]. (*S*)-3-Quinuclidinol in 92 % yield and >99 % *ee* was formed by asymmetric reduction of 3-quinuclidinone using an *S*-selective reductase producing strain *R. erythropolis* WY1406 [209]. A promising process for the efficient preparation of (*R*)-3-quinuclidinol was demonstrated by Zhang et al. recently [210]. In this process, a new reductase (*ArQR*) was identified from *A. radiobacter* ECU2556 by screening the laboratory stock microorganisms, which showed high activity and excellent stereoselectivity in the asymmetric reduction of 3-quinuclidinone. For the cofactor regeneration, the reductase and a glucose dehydrogenase from *B. megaterium* was then coexpressed in *E. coli*, and the resultant recombinant *E. coli* cells were utilized for the synthesis of (*R*)-3-quinuclidinol from 3-quinuclidinone. Up to 242 g/L of substrate could be stoichiometrically reduced to the target product with >99 % *ee* and a space–time yield of 916 g L<sup>−1</sup> d<sup>−1</sup>, indicating its great potential for practical application.

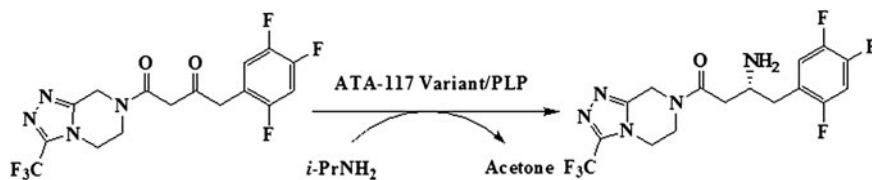
## 6 Chiral Amine Synthesis with Amine Transaminases

Optically pure amines and amino acids are usually used as active pharmaceutical ingredients, and also as resolving agents to obtain enantiomerically pure carboxylic acids. Traditional chemical methods to prepare chiral amines or amino acids usually require the involvement of expensive transition metal catalysts and unavoidably result in environmental pollutions, while the eco-friendly enzymatic routes represent a promising alternative [211–213]. Till date, several enzymatic approaches for the preparation of optically active amines and amino acids including lipase, amidase, monoamine oxidase, amine dehydrogenase, and amine transaminase have been developed; among these methods reported, the amine transaminase-catalyzed kinetic resolution of racemic amines or asymmetric synthesis from the corresponding prochiral ketones represents one of the most promising approaches. Especially, the amine transaminase-mediated asymmetric synthesis seems more advantageous, since it can afford 100 % theoretical yield.

In this section, the asymmetric synthesis of chiral amines catalyzed by amine transaminases will be discussed.

### 6.1 Sitagliptin

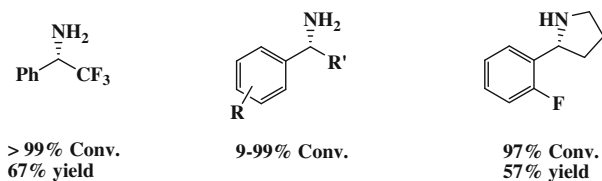
So far the most successful application of amine transaminase for organic synthesis is the large-scale manufacture of antidiabetic compound sitagliptin developed by Codexis and Merck & Co together (Fig. 36) [214]. A substrate walking, modeling, site saturation mutagenesis, and directed evolution strategy were adopted to tailor



**Fig. 36** Biocatalytic synthesis of sitagliptin mediated by an amine transaminase (ATA-117) variant

an amine transaminase (ATA-117) originally inactive toward prositagliptin ketone for practical application in a manufacturing setting. Structural homology model analysis and docking studies revealed that the enzyme could not bind the target substrate due to steric hindrance in the small binding pocket and potentially unfavorable interactions in the large binding pocket. Therefore, a truncated methyl ketone analog of prositagliptin ketone was applied to the screening of the site saturation libraries of residues lining the large pocket of the active site, resulting in a variant (S223P) with 11-fold activity improvement toward the methyl ketone analog. In the second library construction, residues potentially interact with the trifluorophenyl group and those selected from structural considerations were subjected to saturation mutagenesis based on S223P, which gave the first detectable transaminase activity toward the target substrate. The variant active toward the target substrate was then used for the second round of evolution, in which beneficial mutations from both small and large binding pockets were combined. A variant with 75-fold increased activity toward prositagliptin ketone was found after screening of the library. In order to tolerate the harsh reaction conditions such as high substrate/co-substrate loading, high organic solvent concentration, and high reaction temperatures, the best variant from the second round was subjected to another 9 rounds of evolution, and process-like conditions were applied to the screening steps; specifically, the substrate concentration was increased from 2 to 100 g/L, the  $i\text{PrNH}_2$  concentration from 0.5 to 1 M, DMSO from 5 to 50 %, the pH from 7.5 to 8.5, and the temperature from 22 to 45 °C. Finally, a best variant containing 27 mutations was obtained, which met the required process target and could convert 200 g/L prositagliptin ketone to sitagliptin with >99.95 % *ee* and 92 % yield.

Interestingly, the engineered amine transaminase also showed broad substrate specificity, and some chiral amines including various trifluoromethyl-substituted amines as well as phenylethylamines with electron-rich substituents and pyrrolidines that cannot be prepared via traditional reduction amination previously can now be produced by the amine transaminase variant with excellent stereoselectivity (Fig. 37).



**Fig. 37** Chiral amines and pyrrolidine prepared by ATA-117 variants

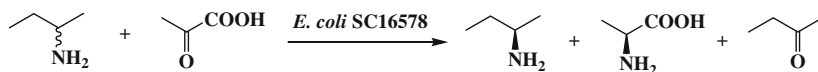
## 6.2 *sec*-Butylamine

To obtain optically active (*R*)-*sec*-butylamine, a biocatalytic resolution process was developed, in which whole cells of recombinant *E. coli* expressing an (*S*)-selective amine transaminase from *B. megaterium* SC6394 were employed for the resolution of racemic *sec*-butylamine using pyruvate as amino acceptor and pyridoxal phosphate as a cofactor (Fig. 38) [215]. This process was performed in 15-L scale, in which 750 g of racemic substrate was resolved to afford 585 g (*R*)-*sec*-butylamine·1/2H<sub>2</sub>SO<sub>4</sub> salt (46.6 % isolated yield, 99.2 % *ee*).

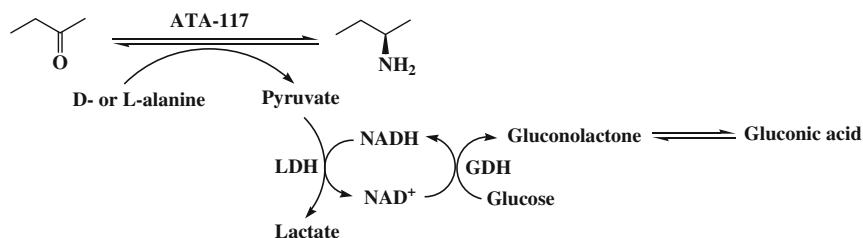
The bottleneck of the above-mentioned resolution process is the theoretical yield of 50 %; thereof, an asymmetric synthesis process would be a useful alternative for practical application. The ω-transaminase (ATA-117)-catalyzed asymmetric synthesis of optically pure (*R*)-*sec*-butylamine from 2-butanone using D- or L-alanine as amino donor (ATA-117) was developed by Kroutil et al. [216]. In order to shift the reaction equilibrium to full conversion, lactate dehydrogenase was applied to remove the by-product pyruvate. Under the optimized reaction conditions, 50 mM 2-butanone could be converted with 98 % conversion and >99 % *ee* (Fig. 39).

## 6.3 Other Chiral Amines

Although amine transaminase-catalyzed asymmetric synthesis theoretically provides a 100 % yield of the product, the existence of the reaction equilibrium that favors the substrate over the product will result in incomplete conversion. One solution for the problem is the use of excess amino donor; the other strategy is the introduction of a second reaction to further transform the by-product. For example, Hohne et al. reported the use of lactate dehydrogenase and pyruvate decarboxylase for the removal of the by-product pyruvate in the asymmetric synthesis of several



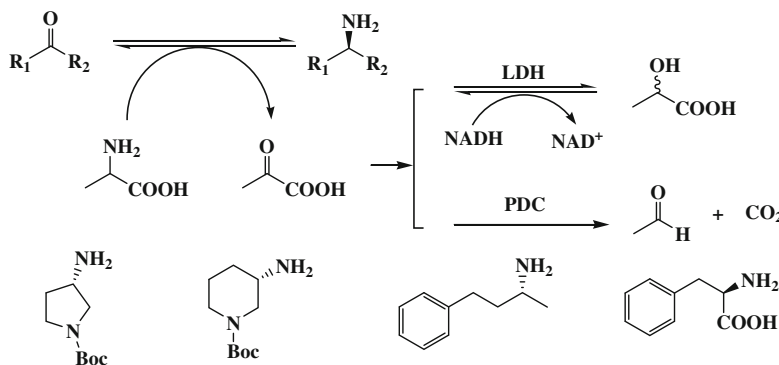
**Fig. 38** Resolution of *sec*-butylamine by transaminase-catalyzed transamination



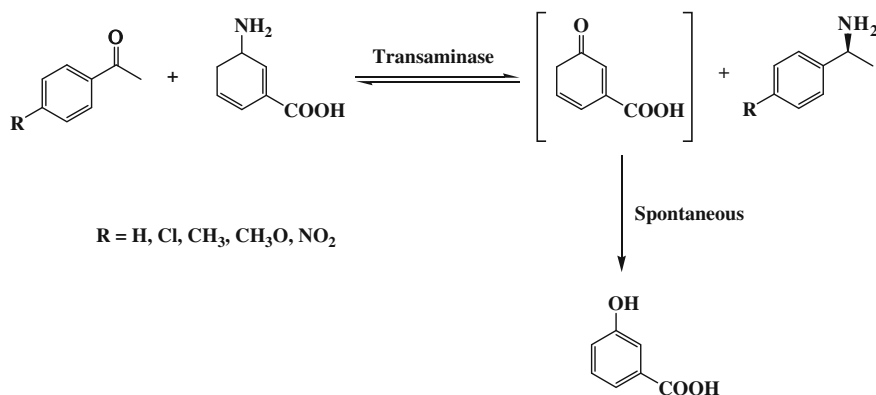
**Fig. 39** Asymmetric synthesis of sec-butylamine with  $\omega$ -transaminase. Lactate dehydrogenase was employed to remove the by-product pyruvate and shifted the equilibrium to the product direction

optically active amines, both resulted in significantly higher conversion rate and pyruvate decarboxylase showed slightly better results (Fig. 40) [217]. Recently, an efficient single-enzymatic cascade for the asymmetric synthesis of chiral 1-phenylethylamine and its derivatives employing 3-aminocyclohexa-1,5-dienecarboxylic acid as the amino donor was developed by Berglund et al. [218], in which the by-product ketone was spontaneously transformed into 3-hydroxybenzoic acid, pushing the equilibrium to the desired direction, allowing a theoretical yield of 100 % (Fig. 41).

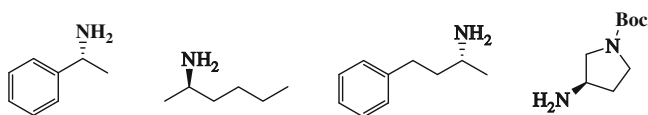
Unlike various (*S*)-selective amine transaminases reported, the number of (*R*)-selective enzymes are relatively scarce. To find more (*R*)-selective amine transaminases for (*R*)-amines synthesis, an *in silico* strategy for enzyme identification was developed [13]. They first analyzed the structural information of respective enzymes to assess the possibility for the evolution of an (*R*)-selective amine transaminases; then, a prediction of key amino acids need to be changed was made; an annotation algorithm based on key motifs was adopted to exclude unwanted enzyme activities; after database search, protein sequences fulfilled the predicted criteria were identified and cloned from synthetic genes; at the last step,



**Fig. 40** Asymmetric synthesis of chiral amines by combining transaminase and lactate dehydrogenase or pyruvate decarboxylase



**Fig. 41** Asymmetric synthesis of chiral 1-phenylethylamines and its derivatives by  $\omega$ -transaminase. The employment of 3-aminocyclohexa-1,5-dienecarboxylic acid drove the reaction to full conversion via spontaneous transformation of the product ketone to 3-hydroxybenzoic acid



**Fig. 42** Chiral amines synthesized by the data-mined (*R*)-selective amine transaminases

after protein expression of respective genes, the resultant enzymes were subjected to activity and selectivity investigation. Finally, up to 17 (*R*)-selective amine transaminases that could catalyze the synthesis of several (*R*)-amines with excellent optical purity were discovered by this in silico approach (Fig. 42). This is also a nice example for the data mining of novel enzymes.

## 7 Perspectives

In the past years, we have seen a big step forward in the application of biocatalysis for the synthesis of a series of optically pure chiral chemicals both in laboratory and industry with the fast growth of various powerful biocatalysts discovered. Although the genomic database offers a large pool of potential biocatalyst resources and provides great opportunities for the discovery of novel and robust biocatalyst, protein engineering including rational design, semi-rational design, random mutagenesis, and de novo enzyme design combined with reliable high-throughput screening strategy is required to further tailor the enzyme to meet specific requirement for industrial application. The development of suitable expression vectors and host strains is an important factor influencing the availability of robust



biocatalyst in large quantity with acceptable cost. Furthermore, immobilization of biocatalyst on appropriate carrier using proper methods or supplement of suitable additives is crucial for enzyme storage, transportation, and application in large-scale transformation. Additionally, process engineering such as the use of aqueous–organic biphasic reaction system, designing of suitable bioreactors, in situ product recovery, and continuous operations is beneficial for process efficiency and volumetric productivity.

It is also noteworthy that cascade reactions involving two or more enzymatic reaction steps or chemoenzymatic process in one pot have emerged as a promising strategy for the preparation of chiral pharmaceutical building blocks without the need for intermediate isolation, thus making the process cost-effective for target molecular synthesis. Finally, collaboration between biologists, organic chemists, and engineers is expected to promote the biocatalysis technology to be a first choice approach for the eco-friendly, highly efficient and cost-effective synthesis of various chiral chemicals in large scale in the near future.

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