Part I
New processes for Existing Active Compounds (APIs)

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Some Recent Examples in Developing Biocatalytic Pharmaceutical Processes

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1.1
Introduction

A confluence of factors is driving biocatalysis into a premier platform for the production of pharmaceuticals. First, the technology itself is more practical than ever for commercialization as a result of easy access of biocatalyst tool boxes from the GenBank, efficient expression systems for their production, and robust protein engineering techniques to improve their specificity, selectivity, and stability. Second, to improve the therapeutic index and absorption, desorption, metabolism, excretion, and toxicity (ADMET) profile, new chemical entities (NCEs) as pharmaceutical ingredients are structurally increasingly more complex, which conversely demand more selective transformations for bond connection and disconnection, manipulation of functional groups, and stereoselectivity. Third, catalytic process technology is posed to be the most crucial component in commercializing drug substances and even drug products as drug innovators or branded pharmaceutical companies are entering the generic business by launching generic versions of branded drugs. The premium paid to 'the first mover' by a generic company will be significantly decreased. Not only is biocatalysis intrinsically process efficient under the principles of green chemistry, it also provides a stronghold to generate novel routes with freedom to operate (FTO) and/or proprietary intellectual property (IP). This chapter focuses on the development of three chemoenzymatic routes to illustrate the dynamics of the field and the importance of strategic integration of chemical and biological transformation to shorten synthetic sequences, reduce energy input, and enhance process safety.

1.2
Levetiracetam (Keppra®)

Levetiracetam, (*S*)-α-ethyl-2-oxo-1-pyrrolidinacetamide, is the active pharmaceutical ingredient (API) of Keppra for the treatment of epilepsy, with sales of over US$1.5 billion in 2008. Most existing processes require either chromatographic separation

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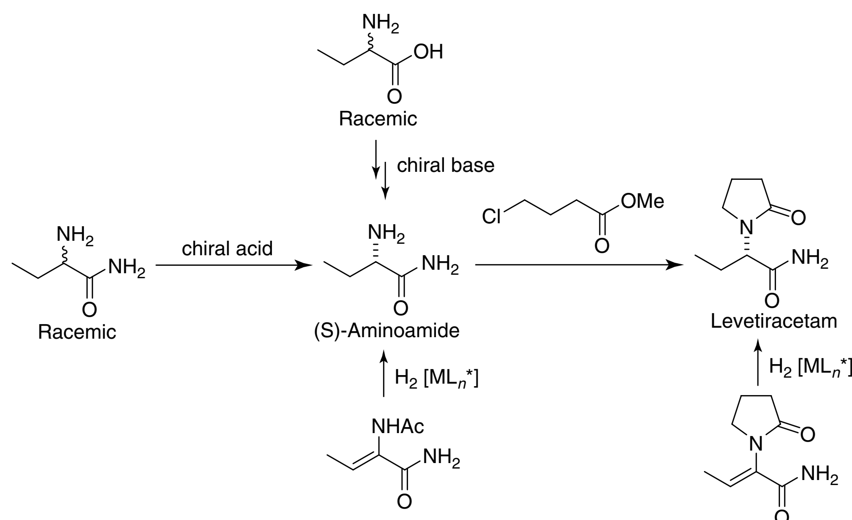
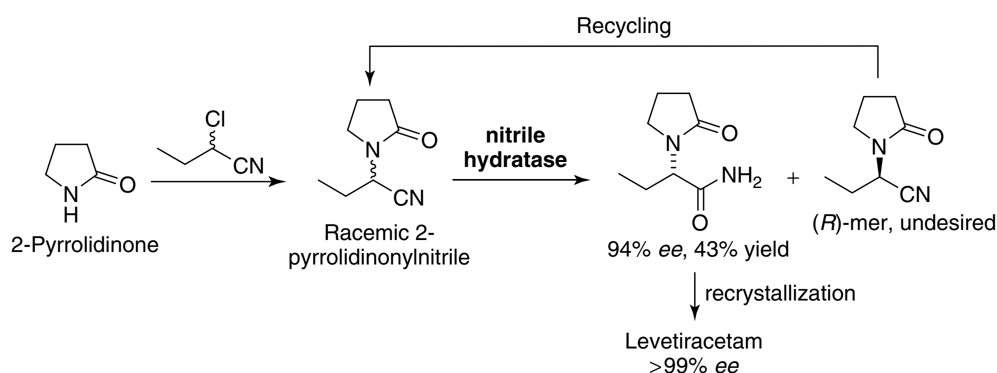


Scheme 1.1 Synthesis of levetiracetam by chemical resolution or asymmetric hydrogenation.

or chemical resolution using stoichiometric amounts of chiral acids or bases, and therefore excess amounts of chemical or solvents, resulting in high process mass intensity (PMI), whereas others use asymmetric hydrogenation or start from chiral pools (Scheme 1.1) [1, 2]. In addition, most reported syntheses employ a hazardous alkyl halide to install the pyrrolidinone ring in the final step. There is a need for efficient, cost-effective, and safer routes for the large-scale production of levetiracetam.

Recently, a biocatalytic process was disclosed in which the strategic step involves the kinetic resolution of a racemic 2-pyrrolidinonylnitrile catalyzed by nitrile hydratases (Scheme 1.2), which was prepared from 2-pyrrolidinone by *N*-alkylation



Scheme 1.2 Chemoenzymatic synthesis of levetiracetam by nitrile hydratases.

with racemic 2-chloro-*n*-butanonitrile [3]. It is difficult to execute such an approach using chemical transformations alone.

Nitrile hydratases are metalloenzymes that contain either iron or cobalt in their catalytic centers, and consist of α and β subunits with the active site being located at the interface of the two subunits. There is no homology between the two subunits. However, each subunit is highly homologous in amino acid sequences [4]. In this work, nearly 30 nitrile hydratases were discovered from the GenBank. Initial screening showed that all of them have poor selectivity though many with modest to good reactivity. The best nitrile hydratase, NH33 from *Bradyrhizobium japonicum*, has a poor *E*-value of less than 5.0 or approximately 60% enantiomeric excess (*ee*) at a conversion of 20%.

Subsequently, NH33 was engineered to improve its enantioselectivity through a combination of structure-based rational design and saturation mutagenesis [5, 6]. The three-dimensional structures of nitrile hydratases revealed almost superimposable metal coordination sites. Of the several residuals participating in recognition of substrates, several conserved aromatic residuals in the β-subunit form a hydrophobic pocket, which is thought to accommodate the side-chain of a nitrile substrate [4]. The three-dimensional structure of NH33 was constructed by homology modeling from the known crystal structure of a nitrile hydratase from *Bacillus smithii*, SC-J05-1 [7]. Computer modeling of the interaction between NH33 and the racemic 2-(2-oxopyrrolidin-1-yl)butanonitrile was performed to identify amino acid residues making up the substrate binding pocket and/or that reside within approximately 10–15 Å from the substrate binding pocket and catalytic center. These residues were then replaced by 19 other amino acids using saturation mutagenesis to screen up mutants with enhanced enantioselectivity. The results show that all positive mutation occurred in the β-unit and most mutation in the α-unit resulted in either deactivation or complete loss of enzymatic activity. The key mutation is βArg38Cys and additional scanning of double and triple mutation based on βArg38Cys led to nearly a ninefold increase in enantioselectivity with the *E*-value being improved to over 45 from less than 5 for the wild type (Table 1.1). It should be noted that although additional mutations around Ala42, Ala43, Leu76, and Val113 led to synergistic effects in improving enantioselectivity from the βArg38Cys template (Seq. ID 3–9, Table 1.1), further combinations of these upper mutation actually led to diminished *E*-values (Seq. ID 10–14, Table 1.1).

Medium engineering was then carried out for additional process optimization. For example, the optimal pH was found to be 6.5, and the enantioselectivity is higher in the Tris buffer than either acetate or phosphate buffers. Addition of $CoCl_2$ increased the reaction rate without adversely affecting the enantioselectivity, and its optimum concentration was set at 0.2 mM. At lower temperatures, higher enantioselectivity was obtained, as expected. To balance reactivity and enantioselectivity, the final process was conducted at 4 °C. Under these conditions, the enzymatic resolution of the racemic substrate proceeds with a high substrate loading of $100\,g\,l^{-1}$·per day, and good stereoselectivity of 94% *ee* for the amide product at a resolution yield of 43%, which was further enriched to over >99% *ee* upon recrystallization. Since the undesired *R*-enantiomer could be recycled by base-mediated

Table 1.1 Exemplary results from active site scanning of α-subunit and site saturation mutagenesis.

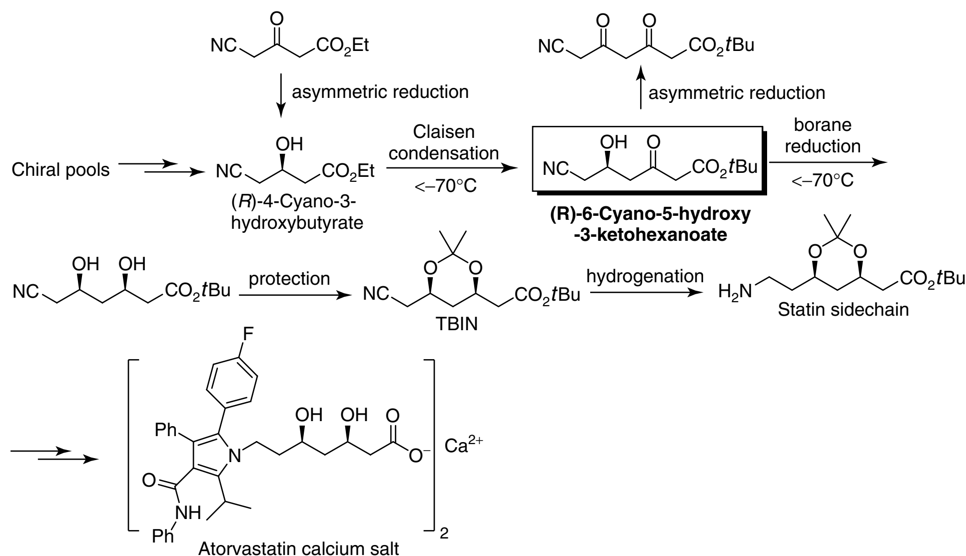
Seq. ID	Mutation site	*E*-value
1	Wild type	4.9
2	Arg38Cys	17.1
3	Arg38Cys, Ala42Val	54.6
4	Arg38Cys, Ala42Met	45.4
5	Arg38Cys, Ala43Gln	47.0
6	Arg38Cys, Ala43Gly	50.9
7	Arg38Cys, Ala43Ser	49.6
8	Arg38Cys, Leu76Phe	56.1
9	Arg38Cys, Val113Leu	53.9
10	Arg38Cys, Leu76Phe, Ala43Gln	42.7
11	Arg38Cys, Leu76Phe, Ala43Gly	29.4
12	Arg38Cys, Ala42Val, Ala43Ser, Val113Leu	42.4
13	Arg38Cys, Ala42Val, Ala43Met, Leu76Phe, Val113Leu	37.2
14	Arg38Cys, Ala42Val, Ala43Gln, Leu76Phe, Val113Leu	33.6

racemization, the biocatalytic process is more atom efficient than reported chemical routes by resolution or chromatographic separation. Furthermore, the hazardous alkylation chemistry was circumvented since this chemoenzymatic process starts with pyrrolidinone (Scheme 1.2).

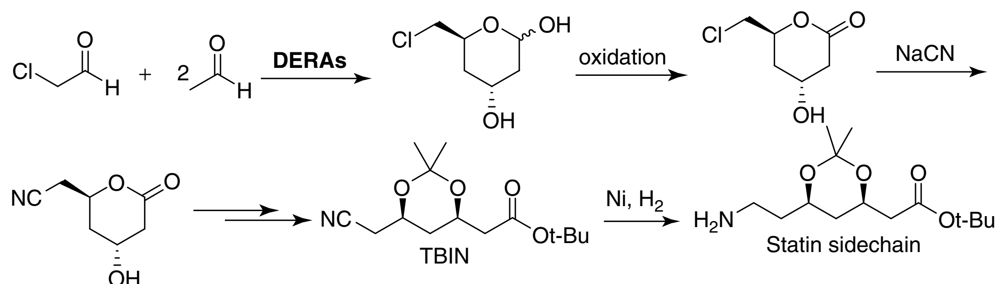
1.3
Atorvastatin (Lipitor®)

Atorvastatin is the API of Lipitor, a cholesterol-lowering drug with sales exceeding US$13 billion in 2008. A number of chemical processes have been reported and nearly all of them proceed through (*R*)-6-cyano-5-hydroxy-3-ketohexanoate (Scheme 1.3), which was obtained from either (*R*)-4-cyano-3-hydroxybutyrate by Claisen condensation or its ketone precursor under asymmetric hydrogenation conditions. This intermediate was then converted to the statin side-chain upon borane reduction at $-70\,^{\circ}$C, followed by protection of the two hydroxyl groups to give *tert*-butyl (3*R*, 5*S*)-3,5-*O*-iso-propylidene-3,5-dihydroxyhexanoate-6-nitrile (TBIN) and reduction of the nitrile group to finalize the statin side-chain [8–10] (Scheme 1.3). These approaches suffer from the lack of synthetic convergence and high energy input as a result of relying on cryogenic reactions.

The use of 2-deoxy-D-ribose-5-phosphate aldolase (DERA) was also reported for the synthesis of statin side-chain (Scheme 1.4). The chemoenzymatic synthesis is significantly shorter, more cost effective, and safer by avoiding cryogenic reactions and hazardous reduction. In Nature, DERAs catalyze reversible aldol reaction between a D-glyceraldehyde-3-phosphate (acceptor) and acetaldehyde (donor) to form 2-deoxy-D-ribose-5-phosphate. The discovery that the acceptors could also



Scheme 1.3 Chemical approaches for the syntheses of the atorvastatin side-chain.



Scheme 1.4 First-generation chemoenzymatic synthesis of the statin side-chain.

be non-phosphate substrates led to significantly expansion of DERA's synthetic applications [11]. The DERA-catalyzed synthesis of the statin side-chain starts with 1 equiv. of 2-chloroacetaldehyde and 2 equiv. of acetaldehyde to form a chiral lactol, which was subsequently oxidized to a lactone. The amino moiety in the statin side-chain was introduced through displacement of the chloro atom in the lactone with sodium cyanide followed by transesterification and Ni-catalyzed hydrogenation (Scheme 1.4). Almost all initial work focused on a DERA from *Escherichia coli* and limited success was achieved due to its strong substrate inhibition and relatively poor stability. Subsequently, the *E. coli* DERA was engineered by directed evolution which led to an almost 100-fold increase in volumetric activity [12]. Separately using a metagenomics approach by screening environmental DNAs, a DERA with high activity was discovered from environmental DNAs, where the substrate inhibition was partially overcome by a semi-continuous process [13]. However, the process

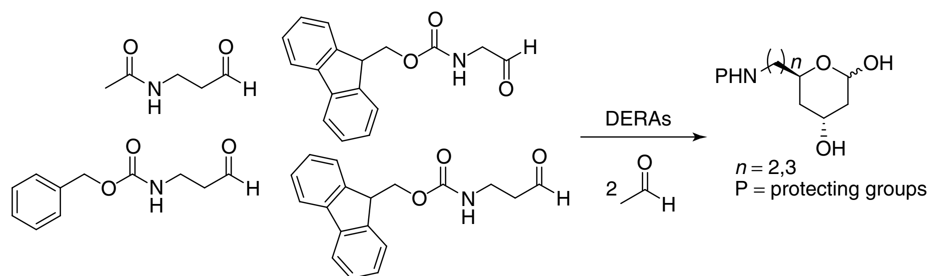
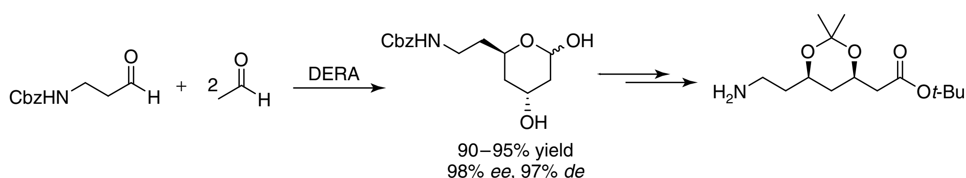


Figure 1.1 Some representative aminoaldehydes accepted by DERAs.



Scheme 1.5 Second-generation chemoenzymatic synthesis of the statin side-chain by DERAs.

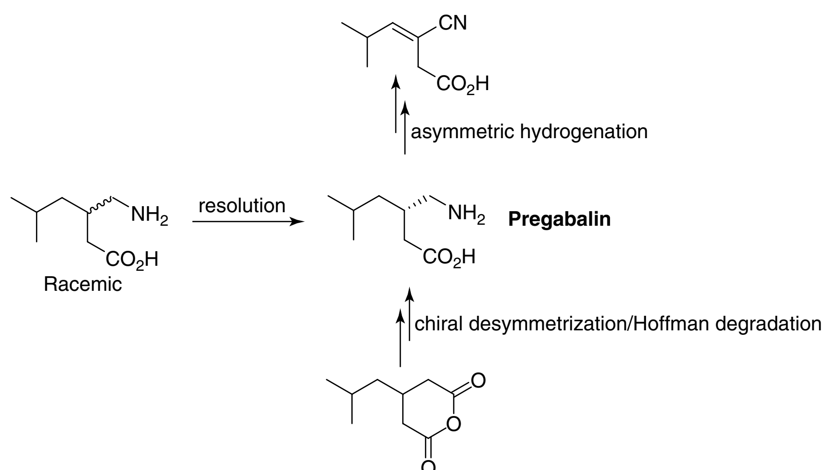
is still linear where the cyano group has to be introduced using NaCN and then hydrogenated to the desired amino group of the statin side-chain.

More recently, a more convergent DERA-catalyzed synthesis of the statin side-chain was reported using aminoaldehydes as the acceptors [14]. In this work, a representative library of DERAs were cloned from GenBank to study their activity in catalyzing sequential aldol condensation between 1 equiv. of an aminoaldehyde and 2 equiv. of acetaldehyde to form an amino lactol. It is striking that some DERAs are fairly promiscuous and active towards even bulky and lipophilic aminoaldehydes considering that the native substrate is a glyceraldehyde-3-phosphate, which is hydrophilic and anionic at pH 7 (Figure 1.1).

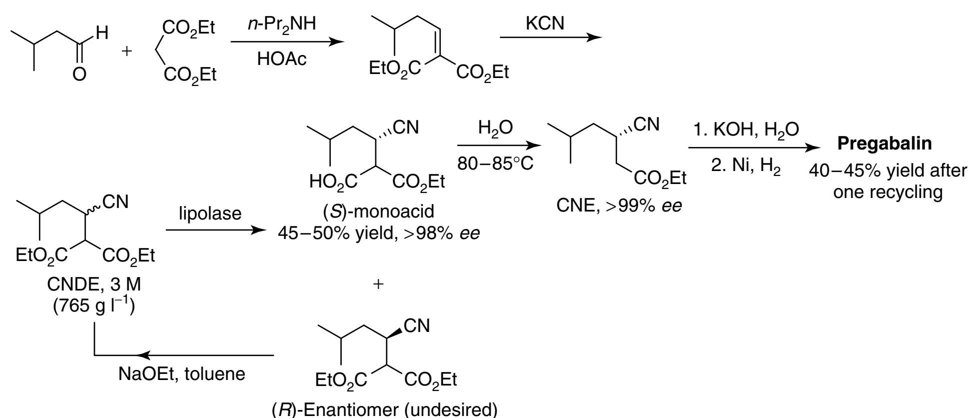
The *O*-benzyloxycarbonyl (Cbz)-3-aminopropionaldehyde was selected for further process development due to commercial availability. The biotransformation proceeds with high throughput (200 g l^{-1}·per day), high yields (90–95%), excellent stereocontrol (98% *ee*), and 97% diastereomeric excess (*de*), which could subsequently be converted to the statin side-chain upon oxidation, protection, and esterification (Scheme 1.5). This new DERA process is two steps shorter than the first-generation DERA route and the hazardous cyanation chemistry was circumvented.

1.4
Pregabalin (Lyrica®)

Pregabalin is the API of Lyrica for the treatment of neuropathic pain and epilepsy with sales of over US$2.5 billion in 2008. A number of syntheses have been



Scheme 1.6 Chemical syntheses of pregabalin.

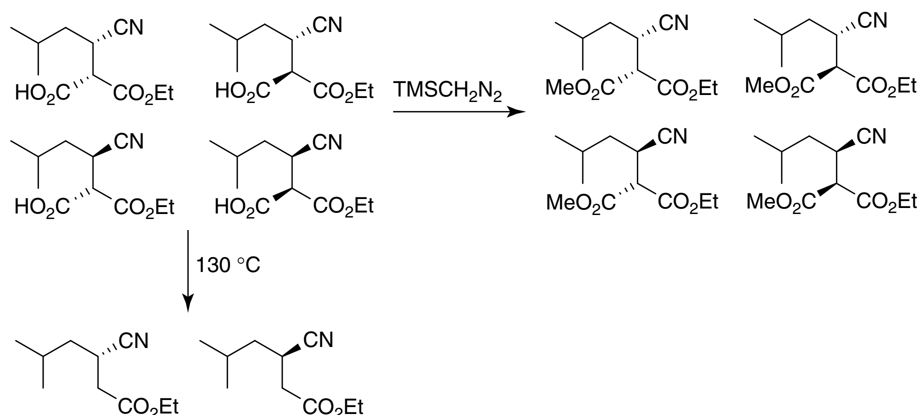


Scheme 1.7 Biocatalytic process for the production of pregabalin.

reported involving diastereomeric resolution, asymmetric hydrogenation, or chiral desymmetrization followed Hoffman degradation (Scheme 1.6) [15–17]. The main issue with late-stage resolution of the racemic amino acid is that the undesired enantiomer could not be recycled efficiently and as a result the route suffers from poor atom economy.

To address the green chemistry and cost issues, an enzymatic resolution route was recently developed with excellent process efficiency (Scheme 1.7) [18]. The key step in this process is stereoselective hydrolysis of a cyano diester (CNDE), which was prepared from isovaleraldehyde by Knoevenagel condensation and cyanation.

One of the most difficult issues in this route development is to identify reliable analytical methods. Here both direct and indirect chiral gas chromatographic (GC) methods were developed using reference standards (Scheme 1.8). Under



Scheme 1.8 Synthesis of reference standards for chiral GC analysis.

GC conditions at 130 °C, the resulting carboxylic acids from enzymatic hydrolysis are decarboxylated to give cyano esters, allowing the measurement of *ee*s. Alternatively, all four thermostable diastereomers could be prepared by esterification (Scheme 1.8).

Initial screening of commercially available hydrolases showed that seven enzymes showed reasonable to good enantioselectivity with *E*-values >35, including lipases from *Thermomyces lanuginosus, Rhizopus delemar, R. niveus, Pseudomonas* sp., *Mucor miehei, R. rryzae,* and an esterase from *M. miehei*. Both lipases from *T. lanuginosus* and *R. delemar* have *E*-values >200 (Table 1.2). For the final optimization, the lipase from *T. lanuginosus*, known commercially as *Lipolase,* was selected due to high enantioselectivity and superior activity, and its commercial availability at large scale. Although there is no substrate inhibition, significant product inhibition was observed at a high substrate (CNDE) loading of 1–3 M. Extensive optimization showed that calcium acetate was effective in overcoming product inhibition. At a substrate loading of 3 M, only 170 mM of calcium was needed. High temperatures tend to deactivate the enzyme, and the best pH appears to be 8.0.

Table 1.2 Active enzymes screened from the hydrolase library.

Enzyme	***E*-value**	**Selectivity**
Lipolase	>200	*S*
Rhizopus delemar lipase	>200	*S*
Rhizopus niveus lipase	66	*S*
Pseudomonas sp. lipase	51	*S*
Mucor miehei lipase	41	*S*
Rhizopus oryzae lipase	35	*S*
Candida lipase A or B	3–5	*S*
Streptomyces griseus protease	20	*R*

The optimized process parameters are 1.5% (w/w) enzyme loading, pH 8.0, 25 °C, and 170 mM $Ca(OAc)_2$. Under these conditions, the enzymatic step has an excellent volumetric activity with a substrate loading of 765 g l^{-1}. The resulting (*S*)-monoacid was obtained in high enantioselectivity (>98% *ee*) at a conversion of 40–45%, which could be readily converted to the final API upon thermal decarboxylation followed by saponification and hydrogenation. Since the undesired *R*-enantiomer could be readily racemized to CNDE by NaOEt in toluene, the overall yield was improved to over 40–45% after one recycling from <30% by the chemical resolution. Moreover, the final three steps were conducted in water and intermediates were telescoped, resulting in significant reductions in wastes and process costs. This process has currently been adopted for the manufacture of pregabalin [18].

1.5 Conclusion

Nature makes all sorts of small molecules and polymeric materials and their conjugates by enzymes, which catalyze a wide range of transformations that are challenging by chemical methods. Recent advances in large-scale DNA sequencing and enzyme-directed evolution rendered biocatalysis a more practical technology than ever for industrial applications [19]. The demand for sustainable products and efficient process technologies provides a great opportunity to advance enzyme technologies further for the chemical synthesis of fuels, chemicals, and materials. As illustrated in the example of levetiracetam, atorvastatin, and pregabalin, biotransformations provide a new dimension in route design to satisfy both the process and green chemistry metrics [20, 21].

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