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Recent Advances in Developing Chemoenzymatic Processes for Active Pharmaceutical Ingredients

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Abstract:

Biocatalysis is becoming a transformational technology for chemical synthesis as a result of recent advances in large-scale DNA sequencing, structural biology, protein expression, high throughput screening, and enzyme evolution technologies. To truly impact chemical synthesis at the industrial scale, enzyme discovery, biocatalyst optimization, process design, and development must be integrated in order to deliver cost-effective and green chemistry solutions for drug manufacture. In this article, some recent applications of biocatalysis in developing chemoenzymatic processes were selected to illustrate the principle of this integrated approach. The emphasis will be directed to those examples where the chemoenzymatic route represents a second-generation process developed to replace the existing chemical one.

Introduction

Biocatalysis is an emerging and transformational technology uniquely suited to the manufacture of active ingredients in the pharmaceutical and related industries as a result of exponential growth in the publicly available gene bank for rapidly accessing diverse enzyme libraries, efficient molecular cloning and protein expression platforms to produce biocatalysts economically, and robust, directed enzyme evolution and screening technologies to improve an enzyme's specificity, selectivity, and stability. However, utilization of biotransformations at large scale is still limited, and most current applications for pharmaceutical synthesis have been limited to lab scales or to drug molecules at early stages before proof of concept (POC).[1−2] In order to develop cost-effective and environmentally friendly chemical syntheses at the industrial scale, it is essential to integrate biocatalysis and modern chemical research and development to deliver manufacturing routes with fewer synthetic steps, reduced waste streams, and improved overall synthetic efficiency in yields, regio- and stereoselectivities, process robustness, and safety.[3a,b] The importance of this strategy can be best illustrated from examples where a second-generation and chemoenzymatic process was developed to replace an existing and less efficient chemical route.

Results and Discussions

One of the primary driving forces in the current wave of biocatalysis is the rapid availability of diverse enzymes through bioinformatics and cloning from the exponentially growing gene bank. With greater numbers of enzymes becoming available, it is essential to develop an automated microtiter-based screening protocol that allows rapid identification of the desired biocatalyst hits from a family of enzyme libraries using minimal amounts of substrates and enzymes under good reaction control. As a result of comprehensive screening, the success rate can be significantly improved, and unique enzymes or conditions can be identified.[4]

For example, in the preparation of AG7404, a rhinovirus protease inhibitor for the treatment of common cold, the key intermediate is an acid precursor **4** (Scheme 1). In the first-generation synthesis, the key step involved alkylation of a P_2-triflate **2** by a pyridonyl amide **1** followed by ester (**3**) hydrolysis using LiI in pyridine. The major issue of this process is that the optically pure P_2-triflate is expensive, not readily available, and the existing chiral center in **3** is prone to racemization during chemical hydrolysis.[5]

To resolve these issues, an alternative chemoenzymatic process was developed where a racemic ester **5** was synthesized (Scheme 2). Through a 96-well plate-based screening of a comprehensive library of hydrolases, *Bacillus lentus* protease (BLP) was identified as the best hit.[6] Prior to this work, the synthetic applications of BLP for process development had never been reported in the literature. In the presence of 35% acetone and 100 g/L of the racemic ester, excellent enantiomeric purity (96% ee) was obtained at a conversion of 50% at pH 8.2 after 24 h (Scheme 2). Moreover, the wrong enantiomer **6** can be readily recycled back to the racemic substrate **5** under a catalytic amount of DBU. Overall, the second-generation chemoenzymatic process was higher yielding than the first-generation alkylation route.

Enzymatic catalysis is generally much more dynamic than small-molecular chemical catalysis, and reaction media including pH, solvent types and contents, and other additives

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(1) Leresche, J. E.; Meyer, H. P. *Org. Process Res. Dev.* **2006**, *10*, 572−580.
(2) Straathof, A. J.; Panke, S.; Schmid, A. *Curr. Opin. Biotechnol.* **2002**, *13*, 548−556.
(3) (a) Yazbeck, D.; Martinez, C.; Hu, S.; Tao, J. *Tetrahedron Asymmetry* **2004**, *15*, 2757−2763. (b) Thayer, A. M. Enzymes at Work. *Chem. Eng. News* **2006**, *84*(33), 15−25.
(4) Yazbeck, D.; Tao, J.; Martinez, C.; Kline, B.; Hu, S. *Adv. Synth. Catal.* **2003**, *4*, 524−532.
(5) Dragovich, P. S.; Prins, T. J.; Zhou, R.; Johnson, T. O.; Hua, Y.; Luu, H. T.; Sakata, S. K.; Brown, E. L.; Maldonado, F. C.; Tuntland, T.; Lee, C. A.; Fuhrman, S. A.; Zalman, L. S.; Patick, A. K.; Matthews, D. A.; Wu, E. Y.; Guo, M.; Borer, B. C.; Nayyar, N. K.; Moran, T.; Chen, L.; Rejto, P. A.; Rose, P. W.; Guzman, M. C.; Dovalsantos, E. Z.; Lee, S.; McGee, K.; Mohajeri, M.; Liese, A.; Tao, J.; Kosa, M. B.; Liu, B.; Batugo, M. R.; Gleeson, J.-P. R.; Wu, Z. P.; Liu, J.; Meador, J. W., III; Ferre, R. A. *J. Med. Chem.* **2003**, *46*, 4572−4585.
(6) Martinez, C.; Yazbeck, D.; Tao, J. *Tetrahedron* **2004**, *60*, 759−764.

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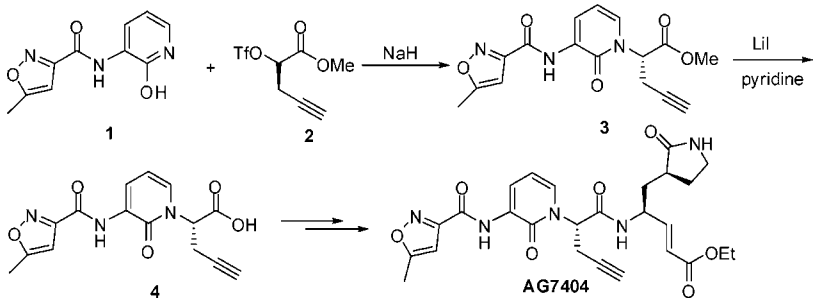
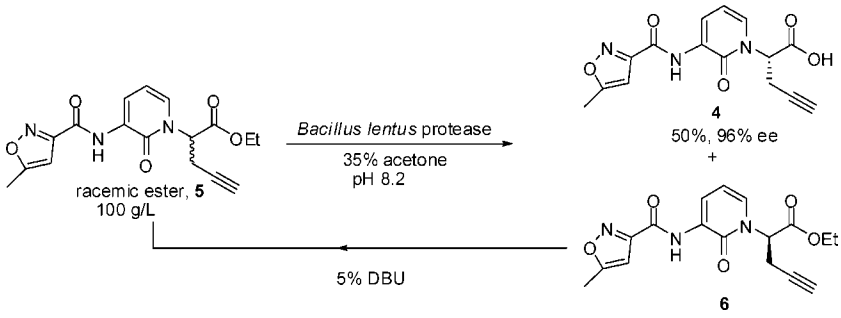
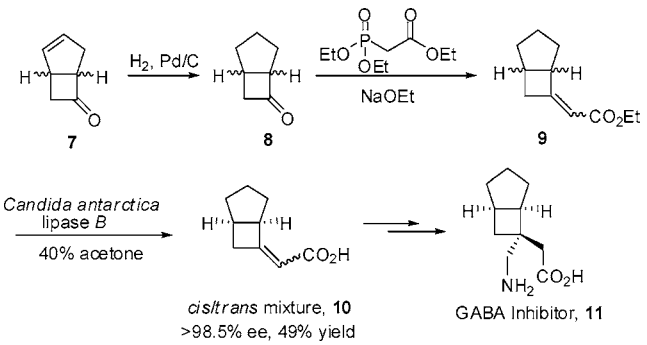
Scheme 1



Scheme 2



Scheme 3



often have a profound effect on protein conformation and its binding to substrates and products. It is therefore often beneficial to apply this principle in process optimization of enzymatic reactions once a suboptimal hit is identified from a comprehensive screening. For example, in the synthesis of a γ-amino butyric acid (GABA) inhibitor **11** for the treatment of neuropathic pain and epilepsy, the key intermediate is a racemic mixture of the *cis*- and *trans*-diastereomers **9** prepared from bicyclo[3.2.0]hept-2-en-6-one **7** upon hydrogenation (**8**) and Horner−Emmons reaction (Scheme 3). The ideal scenario is to identify an enzyme which can be enantioselective but not diastereoselective since both the *cis*- and *trans*-diastereomers of the desired enantiomer **10** can be converted into the final product in the downstream chemistry (Scheme 3).[7]

Initial screening of a large library of hydrolases produced only one hit, *Candida antarctica* lipase B. The enzyme has an *E* value of 10 making it essentially useless for a practical process. Through extensive solvent screening and pH optimization, an *E* value of >100 was obtained for the reaction in the presence of 40% acetone, and the chemoenzymatic route was quickly scaled up to prepare the GABA inhibitor for clinical trials (Scheme 3).[7] An alternative solution will be much more time-consuming by either cloning of a homologous enzyme or optimization of this enzyme through directed evolution. Preliminary 2D-TROSY NMR study using N^{15}-labeled *Candida antarctica* lipase B expressed from *Pichia* indicated the dramatic solvent effect was probably caused by a global conformational change of the protein.[7]

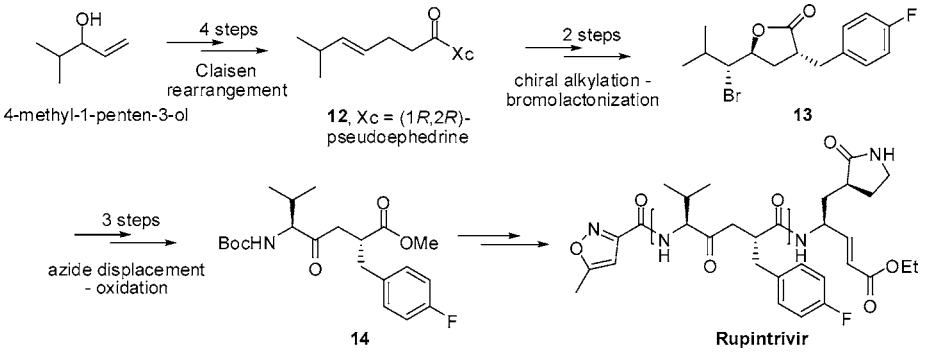
One of the most important aspects in developing chemoenzymatic processes is to integrate biocatalysis and chemical synthesis at the retrosynthetic level to design or redesign a highly efficient reaction sequence. In general it is often difficult to fix a poor reaction sequence by simply replacing one problematic chemical step with an enzymatic transformation. For example, the first-generation synthesis of rupintrivir, a rhinovirus inhibitor, was lengthy and suffered from low overall yields and inefficient chromatographic separation (Scheme 4).[8] In this route, the key steps involved installation of the chiral auxiliary (1*R*,2*R*)-pseudoephedrine in **12** from 4-methyl-1-penten-3-ol, followed by chiral alkylation/bromolactonization to install the 4-fluorobenzyl side chain in **13**, then azide displacement of the bromide to obtain the protected ketomethylene dipeptide isostere (KDI) **14** upon oxidation (Scheme 4). The overall synthesis of the KDI requires nine steps with only 7% in yield.

As a result, a second-generation synthesis was developed involving an uncommon bond disconnection for the ketomethylene peptidomimetic moiety (Scheme 5), and no inter-

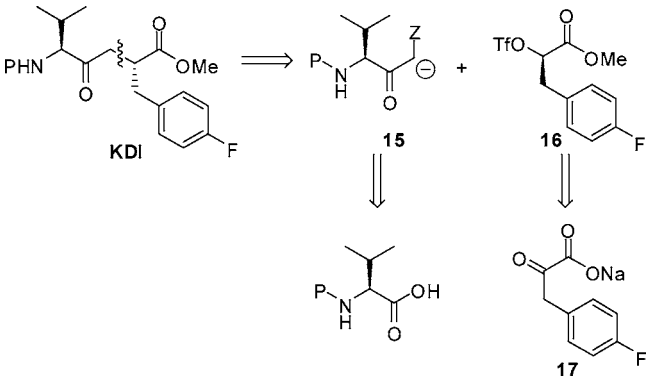
(7) Yazbeck, D.; Derrick, A.; Panesar, M.; Deese, A.; Gujral, A.; Tao, J. *Org. Process Res. Dev.* **2006**, *10*, 655−660.

(8) Dragovich, P. S.; Webber, S. E.; Babine, R. E.; Fuhrman, S. A.; Patick, A. K.; Matthews, D. A.; Reich, S. H.; Marakovits, J. T.; Prins, T. J.; Zhou, R.; Tikhe, J.; Littlefield, E. S.; Bleckman, T. M.; Wallace, M. B.; Little, T. L.; Ford, C. E.; Meador, J. W., III; Ferre, R. A.; Brown, E. L.; Binford, S. L.; DeLisle, D. M.; Worland, S. T. *J. Med. Chem.* **1998**, *41*, 2819−2834.

Scheme 4



Scheme 5



mediates from the first-generation route were shared in the new route. The key to the success of the second-generation process is the availability of an optically pure P_2-triflate **16** at large scale, which can be used for chiral alklyation by a stabilized enolate **15** to furnish the KDI. Chemical syntheses of this deceptively simple molecule have not been successful by asymmetric hydrogenation, chiral reduction, or chiral pool approaches from chiral epoxides or amino acids.[9] Eventually the 2-hydroxy acid precursor of the P_2-triflate was obtained in high yields (80−88%) and ee's (>99.9%) from the corresponding keto acid salt **17** through a continuous process, using enzymatic reduction and cofactor regeneration in a stirred tank reactor (Scheme 5).[10] As a result of the successful biocatalytic conversion, the synthesis of the KDI was four steps shorter than the previous route with the overall yield being improved to about 25%, resulting in significant cost savings.

Another strategy to integrate process chemistry and biocatalysis is to change or modify poor substrates, which are not ideal for biotransformations, without sacrificing overall process efficiency. For example, a different protecting group may significantly change the reactivity, solubility, lipophilicity, and polarity of a molecule, leading to high selectivity and/or productivity in enzymatic catalysis. To optimize biocatalytic reactions, substrate modulation can be a much faster and more economic alternative to the enzyme-directed evolution approach.

For example, a standard bond disconnection of an HIV protease inhibitor **23** led to two key precursors: 3-amino-2-hydroxy-4-phenylbutyric acid, **22** (AHPBA), and *N*-Boc-3,3-difluoro-4,4-dimethylproline, **21** (DFDMP), (Scheme 6).[11] Since it took nine steps to produce the racemic ester **20** from *N*-Boc-glycine through a vinyl glycine intermediate **18** followed by an expensive fluorination on the ketone precursor **19**, it is essential to find an efficient and high-yielding method to convert the racemic substrate **20** into (*S*)-DFDMP, which can then be coupled with AHPBA to give the end product.

It should be noted that an enantioselective chemical synthesis of this unusual amino acid DFDMP has been problematic. For the *N*-Boc protected ester **20** (Scheme 6), only *subtilisin Carlsberg* was identified to be active from a library of over 150 hydrolases. The reaction was slow, requiring 4−5 days even under a low substrate loading of 10 g/L rendering the process impractical at large scale. However, by introducing a benzyl protecting group instead of the Boc into the racemic ester **26** (Scheme 7), pig liver esterase was selected from the same enzyme library, which catalyzes the hydrolytic kinetic resolution of **26** with excellent efficiency to give **27** in 44% isolated yield and >99% ee in 24 h at a substrate loading of 100 g/L. Moreover, it took only seven steps to prepare the racemic substrate for enzymatic resolution from *N*-Boc-glycine, and the wrong enantiomer **28** could be readily recycled. The benzyl group was removed by hydrogenolysis afterwards (not shown) without introducing additional synthetic steps in the downstream chemistry.[12] Directed evolution of *subtilisin Carlsberg* can be challenging and time-consuming due to the intrinsic toxicity of this protease to *Escherichia coli*.

Once a chemoenzymatic process is conceptualized and a biocatalyst is discovered from a library screening, substrate modulation and medium optimization should be used synergistically. For example, in the synthesis of the glycinamide ribonucleotide formyltransferase (GARFT) inhibitor pelitrexol, the first-generation process required 20 linear chemical transformations with an overall yield of only 2% starting from 2-methyl thiophene (Scheme 8).[13] The key steps involve the installation of the Evans' chiral auxiliary 4-benzyl-2-oxazolidinone in **29** after 11 steps of linear chain extension, chiral alyklation to introduce an amino methylene side chain

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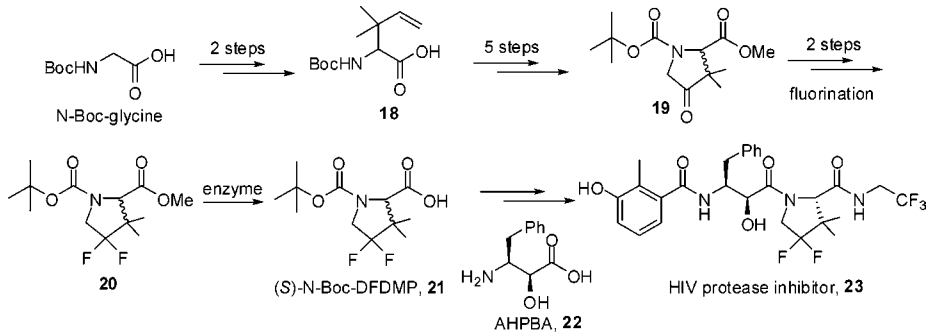
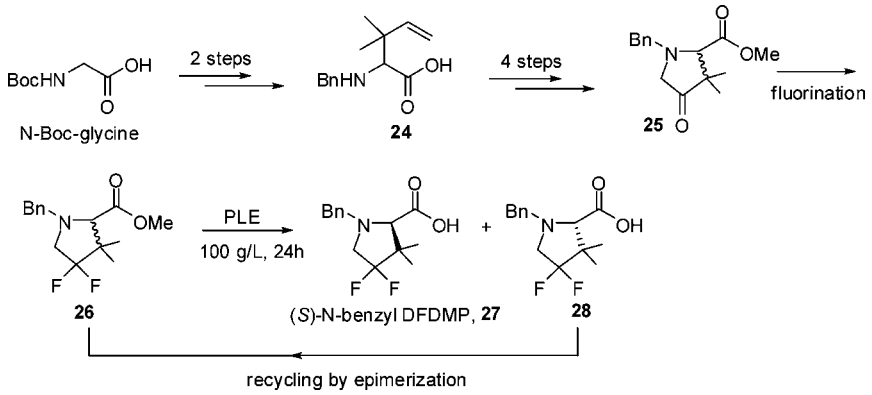
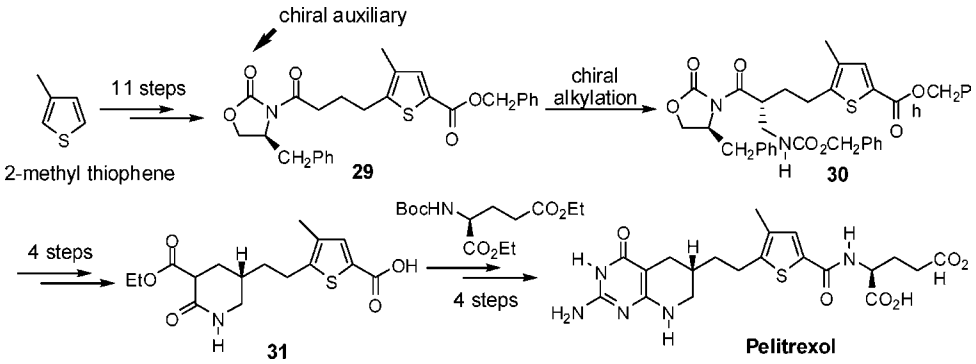
Scheme 6



Scheme 7



Scheme 8



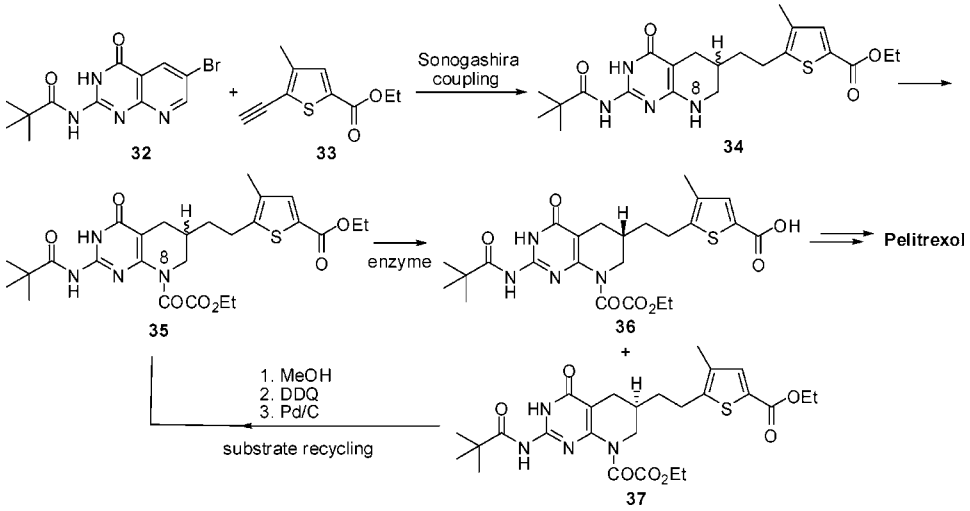
(**30**), intramolecular cyclization (**31**), and construction of the dihydro pyridopyrimidine moiety by fusion to guanidine to provide the end molecule upon coupling with the glutamic ester followed by global deprotection.

Through route redesign incorporating biotransformations, retrosynthetic analyses led to a new process combining Sonogashira coupling with an enzymatic resolution of the resulting adduct **34** (Scheme 9).[14a,b] Asymmetric chemical synthesis of **34** had proved to be exceedingly difficult despite extensive efforts. In this molecule, the chiral center is six bonds away from the ester group, and not surprisingly, direct enzymatic hydrolysis of the substrate gave low selectivity. However, when a labile, oxalamic ester protecting group was introduced (**35**), the desired enantiomer **36** was obtained with excellent resolution efficiency (100 g/L substrate, 95% ee, 45% conversion within 4−5 h) (Scheme 9).[14] The use of 30% DMF was crucial to this process, which increases both the solubility of the substrate in water and the enantioselectivity of the enzyme. It is worth noting that enzymatic hydrolysis takes place at the thiophene ester rather than the oxalamic ester. The oxalamic ester group was cleaved in the workup, and the resulting intermediate converted to pelitrexol after coupling with the glutamic ester followed by deprotection. Interestingly, the wrong enantiomer **37** could be efficiently recycled under oxidation and hydrogenation conditions. In this fashion, the new process required only nine steps, and the overall yield was increased to 10−15% from the original 2%.

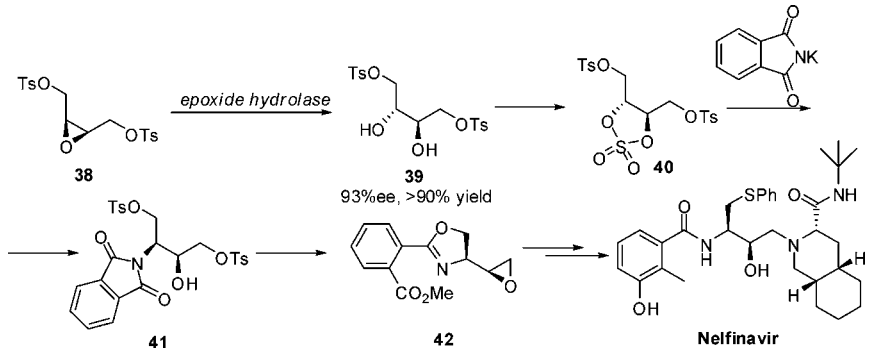
As DNA sequences in the gene bank grow rapidly and metagenomics advances, discovery of novel enzymes has become an important part of the solution of an integrated approach to developing chemoenzymatic processes when existing enzymes do not possess the desired activity or specificity. For example, epoxide hydrolases are known to catalyze the resolution of terminal and di- and trisubstituted

(14) (a) Hu, S; Kelly, S.; Lee, S.; Tao, J.; Flahive, E. *Org. Lett.* **2006**, *8*, 1653−1655. (b) Hu, S.; Tat, D.; Martinez, C.; Yazbeck, D.; Tao, J. *Org. Lett.* ***2005***, *7*, 4329−4331.

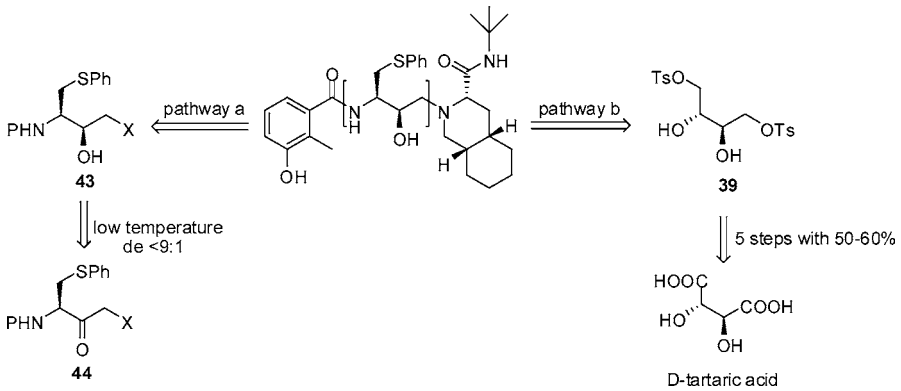
Scheme 9



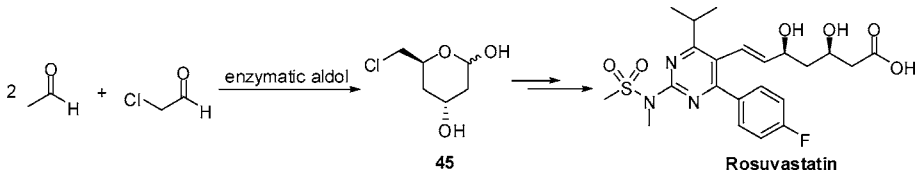
Scheme 10



Scheme 11



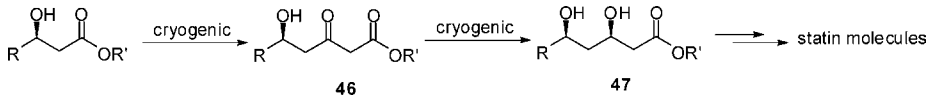
Scheme 12



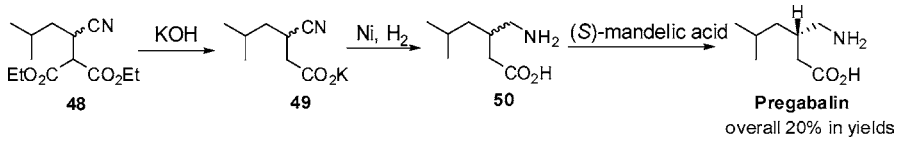
epoxides. However, known epoxide hydrolases reported in the literature were unable to hydrolyze *meso*-epoxides selectively. A library of novel epoxide hydrolases discovered from environmental libraries was found to be able to desymmetrize various *meso*-epoxides in high ee and yields.[15]

A particularly interesting epoxide hydrolase in this library was found to be able to desymmetrize the *meso*-epoxide **38** (Scheme 10) to afford the (*R*,*R*)-diol **39** in 90% yields and 93% ee,[16a] a key intermediate toward the synthesis of nelfinavir, the API of the anti-HIV drug Viracept. The

Scheme 13



Scheme 14



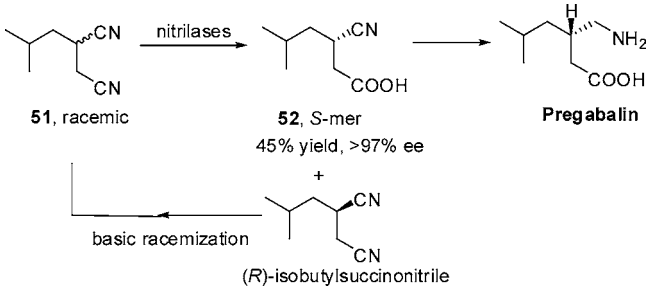
resulting diol bistosylate **39** can then be converted to the final API through the formation of a cyclic sulfate **40**, introduction of the nitrogen by S_N2 displacement of a phathalimide salt to afford **41**, formation of an oxazolinyl epoxide **42**, and then its coupling with the 3-isoquinolinecarboxamide (Scheme 10).[16b]

This biocatalytic route is superior to the existing chemical processes by either reduction of a chiral ketone **44** (pathway a, Scheme 11) or using D-tartaric acid (pathway b, Scheme 11).[16b] The reduction approach required low temperature, and the amino alcohol **43** was obtained in only modest diastereoselectivity (de <9:1), while in the D-tartaric acid route, the preparation of the *meso*-diol required five steps, and the overall yield was only 50−60% compared to about 80% by the enzymatic method.

Another example illustrating the value of enzyme discovery was the synthesis of a lactol precursor **45** for statin-type HMG-CoA reductase inhibitors including rosuvastatin, the API of Crestor and atorvastatin, the API of Lipitor to reduce low-density lipoprotein (LDL) cholesterol. The most efficient way to install this type of dihydroxy acid is to use deoxyribose-5-phosphate aldolase (DERA), which catalyzes the sequential aldol condensation between one equivalent of 2-chloroacetaldehyde and two equivalents of acetaldehyde (Scheme 12).[17] In the initial process, a recombinant *E. coli* DERA was used, and the reaction was run with high enzyme loading (20 % wt/wt) with low volumetric productivity (2 g/L per day).[17b] By a combination of activity- and sequence-based screening, a novel DERA was discovered from environmental DNA libraries by the metagenomic approach. Using the newly identified aldolase, the volumetric productivity of the aldol reaction was improved to 720 g/L per day, and the enzyme loading was reduced to 2 % wt/wt.[17b]

The aldolase route is much more efficient than existing chemical routes to the synthesis of statin side chain, where a typical Claisen condensation (**46**) and chelation-controlled chiral reduction (**47**) sequence were applied under cryogenic

Scheme 15



conditions (Scheme 13). Many other methods have also been developed but mostly to target earlier intermediates used in the current chemical route to statin drugs.[18−19]

Directed evolution shall be part of the integrated solution when the best wild-type enzyme is still suboptimal after comprehensive screening and optimization. Over the last 5 years, directed evolution has proved to be a robust technology for improving enzyme specificity and reactivity. As more and more practical and high throughput enzymatic assays are established, the time required to implement this technology has been shortened significantly, and a 10−30 fold functional improvement can be often achieved within 6−12 months. While this timeline may not fit well for drug molecules at preclinical or early clinical stages where time is often stringent, the evolution methodology can be integrated powerfully into process development for compounds in late stages or on the market. For example, in the first-generation manufacturing route to pregabalin, the API of the neuropathic pain drug Lyrica, the synthesis started with a cyano diester (CNDE) **48** (Scheme 14). After basic hydrolysis (**49**) and Ni-catalyzed hydrogenation (**50**), a classic resolution was applied at the end of the synthesis using (*S*)-mandelic acid. The resolution was followed by a crystallization to enrich the optical purity, and the yield is only around 30%. Unfortunately, the wrong enantiomer (not shown) of the final API could not be recycled back to the racemic compound (Scheme 14).[20] As a result, the route suffers from an overall low yield of about 20%.

To overcome these issues, a new route was developed using a nitrilase-catalyzed resolution of racemic isobutylsuccinonitrile (Scheme 15).[21] In this approach, a targeted nitrilase

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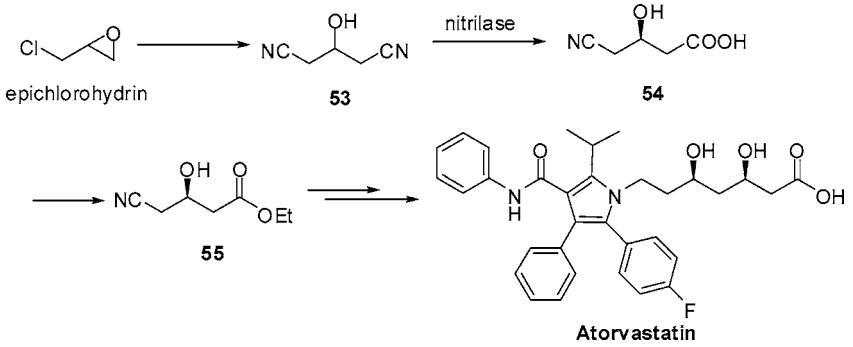
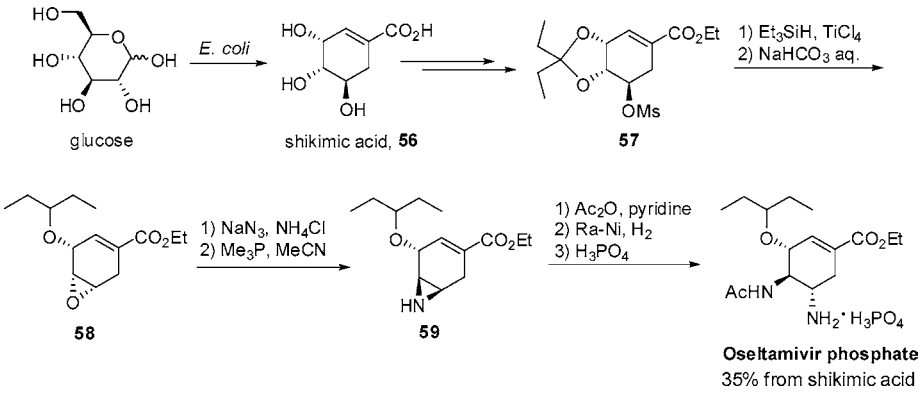
Scheme 16



Scheme 17



library was initially discovered from the gene bank allowing the identification a nitrilase from *Arabidopsis thaliana*, which regio- and stereoselectively hydrolyzes one of (*S*)-isobutylsuccinonitrile (>97% ee, 45% yield), and the (*R*)-enantiomer can be recycled under basic conditions. To improve the activity of the enzyme, directed evolution was applied by using error-prone PCR. In the first round of evolution, a single C236S mutation led to a mutant with 3-fold increase in activity.[22−23]

In addition to reactivity, directed evolution can also be used to improve enantioselectivity as illustrated in the development of a nitrilase-catalyzed synthesis of (*R*)-4-cyano-3-hydroxybutyric acid **54**, a key intermediate in the current manufacturing process of atorvastatin (Scheme 16). In this route, the *meso*-3-hydroxyglutaronitrile **53** was prepared from inexpensive racemic epichlorohydrin. With the wild-type nitrilase identified from the initial screening, the ee of the desired product was 88% under a loading of 3 M of the substrate.[24] Gene site-saturation mutagenesis was then applied to improve the stereoselectivity of the lead enzyme, and a resulting Ala190His mutant from high throughput mass spectrometric screening gave an excellent enantioselectivity of 99% under the same substrate loading.[25−26] The synthesis is short with high throughput as a result of the stereospecific enzymatic desymmetrization, and the compound **54** can be tagged into the current manufacturing route of atorvastatin through the intermediate **55**.

Since a large library of enzyme mutants needs to be screened, directed evolution is often much more time-consuming and costly than reaction engineering, substrate modulation, medium optimization, and enzyme kinetics studies. It is therefore essential to build a high-quality, focused library and develop a robust, high throughput screening assay to reduce the time and cost to identify the best mutant.[27−28] Alternatively, if two enzymes have high sequence homology, a homologous 3-D model for an enzyme, whose structure is unknown, can be constructed from an existing structure solved by either crystallography or NMR. Molecule docking can then be used to guide a subset of amino acids for mutations to reduce the size of the mutant library to be screened.[29−31]

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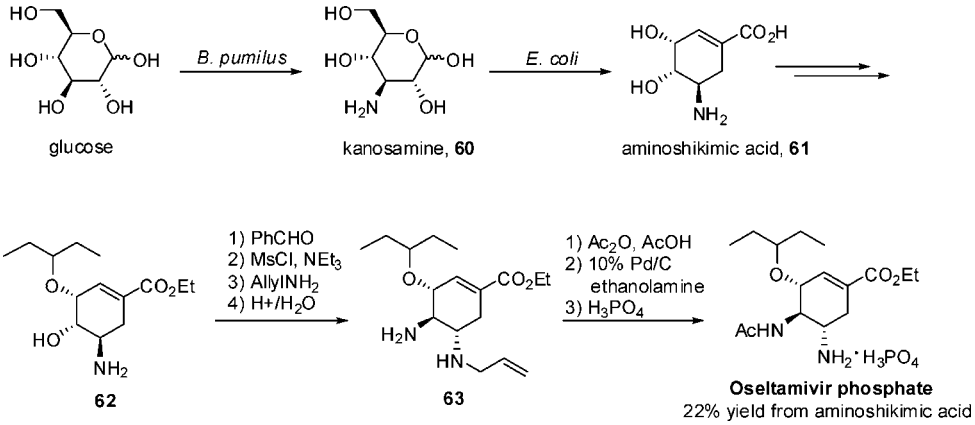
Scheme 18



One of the holy grails in biocatalysis is to design cascade reactions using multiple enzymes in a cell factory as nature does. Recent advances in metabolic engineering, combinatorial biosynthesis and systems biology have led to the development of new and robust biocatalysts and engineered cells suitable for industrial-scale applications. A prominent example is the production of shikimic acid as a key intermediate for the production of the neuraminidase inhibitor oseltamivir phosphate (Tamiflu) (Scheme 17), used for prevention of influenza virus infections and as part of the prevention plan against an influenza pandemic. In the current 10-step commercial synthesis (Scheme 17),[32−33] all three stereogenic centers and the cyclohexene ring backbone in the final oseltamivir phosphate were derived from shikimic acid **56**. Chemical synthesis of shikimic acid is not efficient enough for commercial production. On the other hand, this molecule is an intermediate in the common pathway of aromatic amino acid biosynthesis. Metabolic engineering efforts have led to the development of a genetically engineered *E. coli* strain, which is deficient in both shikimate kinase isozymes, capable of producing shikimic acid with a titer of 84 g/L and a yield of 33% from glucose.[34] Subsequently, this acid was converted into a diethyl ketal **57**, followed by the installation of the 3-pentyl ether side chain by reductive opening of the ketal and base-catalyzed epoxide ring closure to afford **58**, which was then transformed to the oseltamivir phosphate involving the potentially explosive sodium azide and formation of the aziridine intermediate **59**. The overall yield from shikimic acid is 35%. As demand for Tamiflu rapidly expands, biocatalytic synthesis provides most of shikimic acid for Tamiflu production.[34−36]

One drawback of the above process is the use of azides. To avoid the use of potentially explosive azides and azido intermediates, several azide-free syntheses have been recently reported.[37−40] One of them comprised fewer synthetic steps than the commercial process (Scheme 18).[40] This new route starts from aminoshikimic acid **61** which can be produced from glucose using a two-step microbial process involving the synthesis of kanosamine **60** by *Bacillus pumilus* ATCC 21143.[41] A derivative of the shikimic acid-producing *E. coli* strain expressing an aminoDAHP synthase isolated from *Amycolatopsis mediterranei* was employed to produce aminoshikimic acid from kanosamine in an overall yield of 5% from glucose.[41] The new synthesis gives oseltamivir phosphate in 22% yield from aminoshikimic acid after installation of the 3-pentyl ether side chain in **62** and stereoinversion to introduce the second amino group in **63**.[40] This route holds great potential if the biocatalytic synthesis of aminoshikimic acid can be further improved.

Conclusions

Biotransformation has emerged as a powerful technology for chemical synthesis and is uniquely suited for the development of cost-effective and environmentally friendly solutions for drug manufacture.[42] The successful implementation of biotransformations at industrial scale requires the strategic use of medium screening, substrate modulation, reaction engineering, enzymology, biocatalyst discovery, and evolution. Most importantly, biocatalysis and modern chemical research and development need to be integrated at the retrosynthetic level to deliver efficient and practical routes with fewer synthetic steps and significantly reduced waste streams.[43a,b] To be "right the first time" to save resources, reduce costs, and accelerate a drug molecule to the market, it is imperative to integrate both the chemical and biocata-

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lytic retrosynthetic analyses at the beginning of route scouting.

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