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Perspective

The Evolving Nature of Biocatalysis in Pharmaceutical Research and Development

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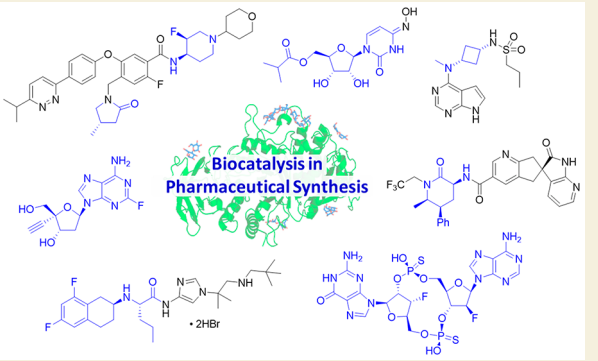
ABSTRACT: Biocatalysis is a highly valued enabling technology for pharmaceutical research and development as it can unlock synthetic routes to complex chiral motifs with unparalleled selectivity and efficiency. This perspective aims to review recent advances in the pharmaceutical implementation of biocatalysis across early and late-stage development with a focus on the implementation of processes for preparative-scale syntheses.



KEYWORDS: *Biocatalysis, Pharmaceuticals, Enzymes, Chirality, Synthesis, Industry, Manufacturing*

INTRODUCTION

Over the past decade, the uptake and incorporation of biocatalysis in the pharmaceutical sector has seen tremendous growth with broader implementation and increased sophistication of its usage. Enzymatic catalysis has several major benefits as an enabling synthetic technology, including the ability to mediate chemical transformations with exquisite chemo-, regio-, and enantioselectivity.[1]

The significant expansion in biocatalytic implementation in pharma is heavily tied to the ability to evolve enzymes. This is critical for improving the scope of wild-type enzymes toward non-natural substrates and increasing their activity and tolerance to the demanding process parameters needed to make syntheses commercially and environmentally viable. Additionally, the breadth of the biocatalysis toolbox continues to grow as a result of academic developments in the field, key industrial-academic collaborations, and in-house enzyme discovery efforts.

The sustainable nature of biocatalysis also helps support the ever-growing drive toward wider use of green chemistry.[2−7] These initiatives aim to reduce the impact on the environment, both in terms of raw material usage and waste disposal, as well as improve the safety profile of reactions, especially on a large scale. Biocatalytic reactions inherently begin to address these challenges as they typically operate in aqueous solvent under mild temperature and atmospheric pressure. However, for biocatalysis to live up to the label of being a sustainable technology, there is also a strong requirement to maintain process intensification metrics that match typical synthetic organic chemistry. High utilization factors for biocatalysts (large substrate:enzyme ratios) and reduction of process mass intensity (PMI)[5] can ensure biocatalytic reactions are truly green processes.[6] With this aim, many pharmaceutical companies are members of the American Chemical Society, Green Chemistry Institute, Pharmaceutical Roundtable to share best practices in a noncompetitive way as well as author several helpful guides including one with a biocatalysis focus.[8]

One of the more recent capabilities of biocatalysis that has now been implemented on scale, is the combination of multiple enzyme-catalyzed steps in one-pot, which avoids the need for time-consuming, material-intensive, and costly isolation procedures.[9−11] As such, these cascade processes can also significantly reduce the PMI of synthetic routes.[5]

There is a rich history of biocatalysis having impact in late-stage development when there is time and focus to invest in enzyme engineering strategies and dedicated process chemistry optimization for scale up.[12] However, as the breadth of implementation of biocatalysis continues to grow, there is an increasing reach of its impact into earlier discovery space, characterized by vast numbers of investigational compounds, rapid development timelines, and high attrition.[13] As well as

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biocatalysis for large-scale implementation, there is also the valuable application of enzymatic transformations in small-scale metabolite synthesis[14] for pharmacokinetics and dynamics studies and lead diversification in discovery chemistry (Figure 1).[15−17]

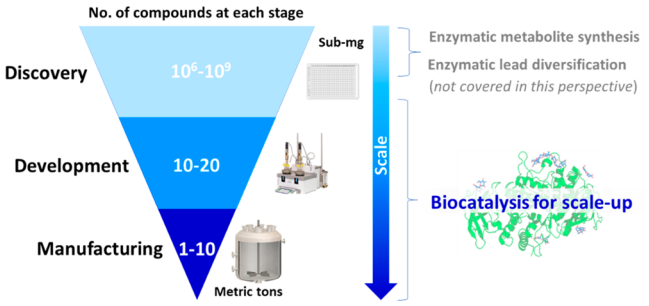


Figure 1. General overview of pharmaceutical development from discovery chemistry through to commercial manufacturing.

This perspective specifically aims to focus on recent key examples of implementation of biocatalysis for scalable syntheses of pharmaceutical intermediates. Several excellent reviews have been published on industrial biocatalysis covering various aspects of enzymatic transformations for drug development.[11,12,18−27] As such, this perspective does not aim to be comprehensive but rather highlight the current state-of-the-art of preparative enzymatic synthesis in an industrial setting. Therefore, the use of biocatalysis for small-scale pharmaceutical metabolite synthesis,[28] lead diversification,[15,16,23,24] bioconjugation,[29] and selective protein modification[30−32] will not be focused upon in this article.

As a means of highlighting common themes in enzyme sourcing, reactions throughout are denoted as either catalyzed using Enzyme^{WT} (wild-type enzyme), Enzyme^{Eng} (engineered enzyme specifically for the process), or Enzyme^{Comm} (commercially available enzyme that may or may not have been previously engineered).

RECENT ADVANCES IN BIOCATALYSIS ACROSS EARLY AND LATE-STAGE DEVELOPMENT

Traditional application of biocatalysis in late-stage development has continued to be a staple of this technology in recent years. Chemistry at this stage of development does not suffer the same rate of project attrition compared to the early development space. This is typically due to having positive readouts of key clinical data in hand that give confidence to invest in dedicated process optimization. As such, bespoke biocatalysts can be developed via enzyme engineering to suit the intended process.

Due to the time, cost, and resources required to pursue dedicated enzyme engineering, there is a strong desire to identify transformations that could be executed with biocatalysis as soon as possible in route design and synthesis. However, in earlier development phases, time frames and resources are typically tighter as there are many projects in progress simultaneously and the risk of attrition of investigational candidate drugs is significantly higher. Therefore, for biocatalysis to have an impact in this space, synthetic solutions must be rapidly scaled up to deliver material forearly biological testing and ultimately for phase 1 studies in which the investigational drug is first dosed to humans. This leads to reactions being enabled with "off-the-shelf" enzymes (i.e., wild-type, commercially available or previously engineered and have become commercially available) with processes that are fit-for-purpose to deliver material for testing but are perhaps still not ideal for final manufacturing routes.

At this stage of development, it can be difficult to get a true snapshot of the processes developed because IP restrictions in medicinal chemistry are more prohibitive due to the absence of fully approved composition of matter patents. Projects also rarely stand still and, if biological data is positive, then these drugs successfully progress into late stage dedicated process optimization.

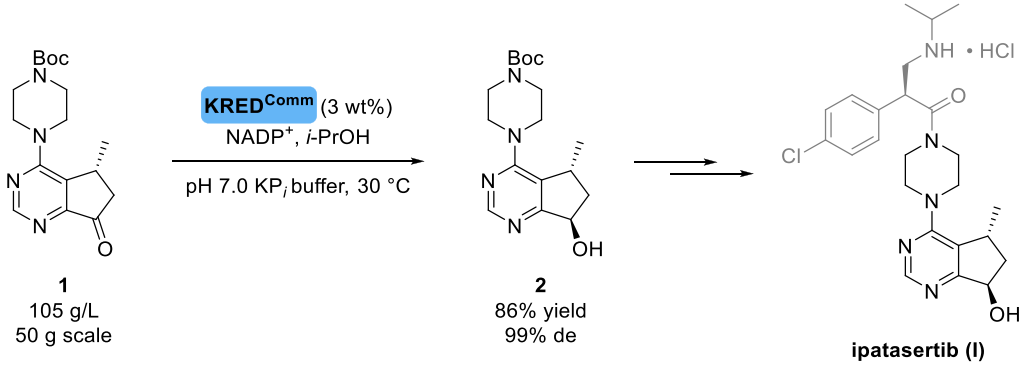
Several recent biocatalytic processes implemented by various companies spanning the early and late-development space are described below with key process features and considerations highlighted.

SINGLE-STEP ENZYMATIC TRANSFORMATIONS

Alcohol Dehydrogenases and Ketoreductases [EC 1.1.1.X]

Alcohol dehydrogenases (ADHs) and ketoreductases (KREDs) have become go-to biocatalysts for chiral alcohol synthesis in pharma with many enzymes available commercially or in the literature. These enzymes utilize NAD(P)H as reductant which requires an added enzymatic recycling system to avoid the use of stoichiometric cofactor. Frequently, either *i*-PrOH is used as a cosubstrate to regenerate NAD(P)H, or a glucose dehydrogenase (GDH)/glucose recycling system is implemented.

Scheme 1. Asymmetric KRED Process to Access a Chiral Alcohol Intermediate 2 for the Synthesis of the Akt Kinase Inhibitor Ipatasertib (I)[33]



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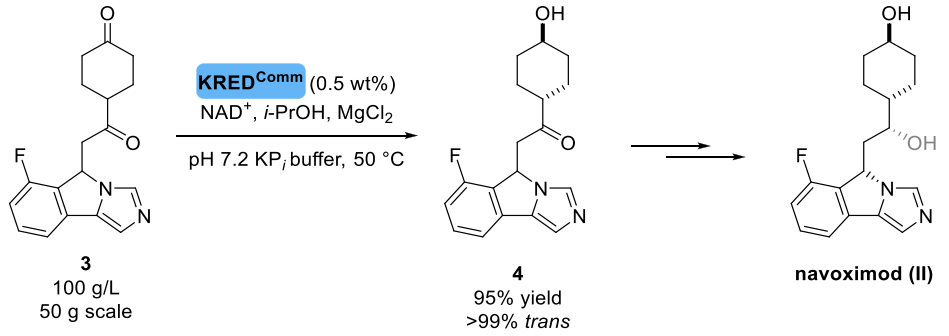
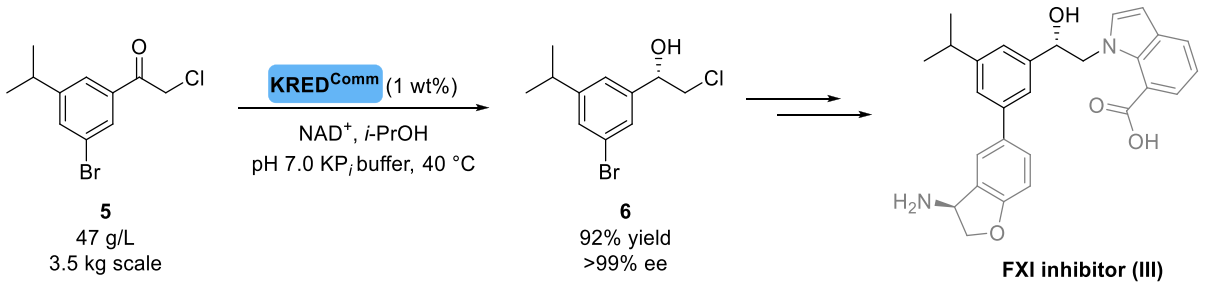
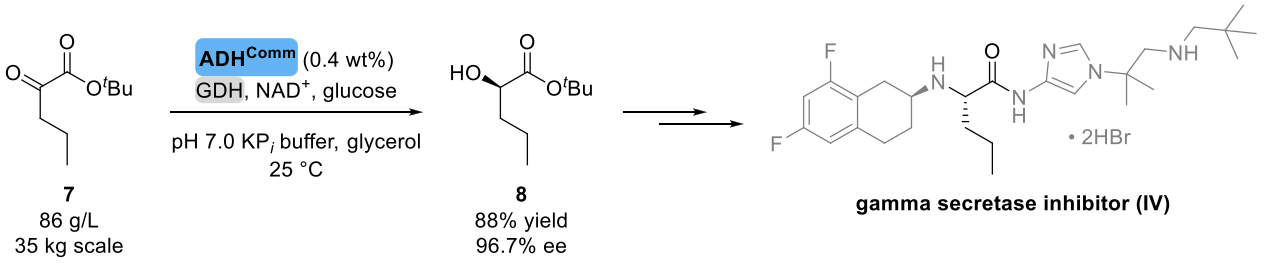
Scheme 2. Asymmetric KRED Process to Access a *trans*-Configured Cyclohexanol Intermediate 4 for the Synthesis of the IDO Inhibitor Navoximod (II)[34]



Scheme 3. Asymmetric KRED Process to Access a Chiral Alcohol Intermediate 6 for the Synthesis of an FXI Inhibitor (III)[35]



Scheme 4. Asymmetric KRED Process to Access a Hydroxy Ester Intermediate 8 for the Production of a Gamma Secretase Inhibitor (IV)[36]



The teams at Genentech and Roche developed a KRED process that was part of the synthesis of the Akt inhibitor ipatasertib (I) (Scheme 1).[33] A commercially available enzyme from Codexis was identified for this transformation that was able to perform the highly diastereoselective reduction to access intermediate **2** as well as regenerate the cofactor NADPH from *i*-PrOH as a terminal reductant. Early iterations of this process had employed a GDH/glucose cofactor recycling system; however, the one enzyme system improved operational simplicity. To overcome some observed substrate degradation, the process was operated at high substrate loading as a slurry-to-slurry reaction and was shown to achieve high yield. This approach was favored over an alternative Ru-catalyzed asymmetric transfer hydrogenation route because of the high diastereoselectivity of the enzymatic process and the challenges associated with purging of residual Ru.

Another selective enzymatic ketone reduction was developed by Genentech and Roche in which ketone **3** was stereoselectively converted to alcohol **4**, an intermediate in the synthesis of the indoleamine 2,3-dioxygenase (IDO) inhibitor, navoximod (II).[34] After screening more than 500 enzymes, a commercially available KRED was identified that was able to act on the cyclohexanone motif. However, no enzymes were found that could react with the more sterically hindered central ketone. Process chemistry optimization led to a reaction that was demonstrated on 50 g scale with elevated temperature and low enzyme loading, achieving the desired *trans*-configured product in high yield and selectivity (Scheme 2). For the sterically challenging central ketone, a diastereoselective chemical reduction was executed to access the desired diol of navoximod (II). This highlights a current gap in the biocatalytic toolbox for the reduction of ketones with flanking bulky groups.

Researchers at Novartis investigated the use of a KRED asymmetric alcohol synthesis to access an intermediate en route to a Factor XI (FXI) inhibitor (III).[35] Over 300 commercially available KREDs were screened and a Codexis enzyme taken forward for full process optimization and implementation. It was noted that although a GDH/glucose cofactor recycling system could be used with this transformation, the need to continually adjust the pH of the reaction was undesirable on scale because of the increased complexity of the operations. Therefore, the use of *i*-PrOH as cosubstrate, sacrificial reductant, and cosolvent was pursued.

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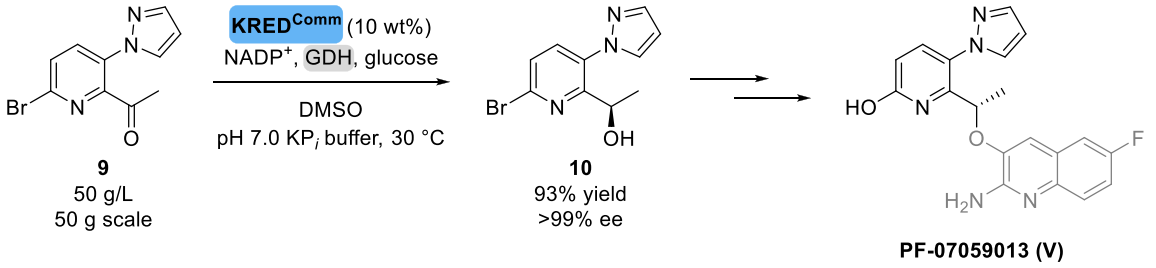
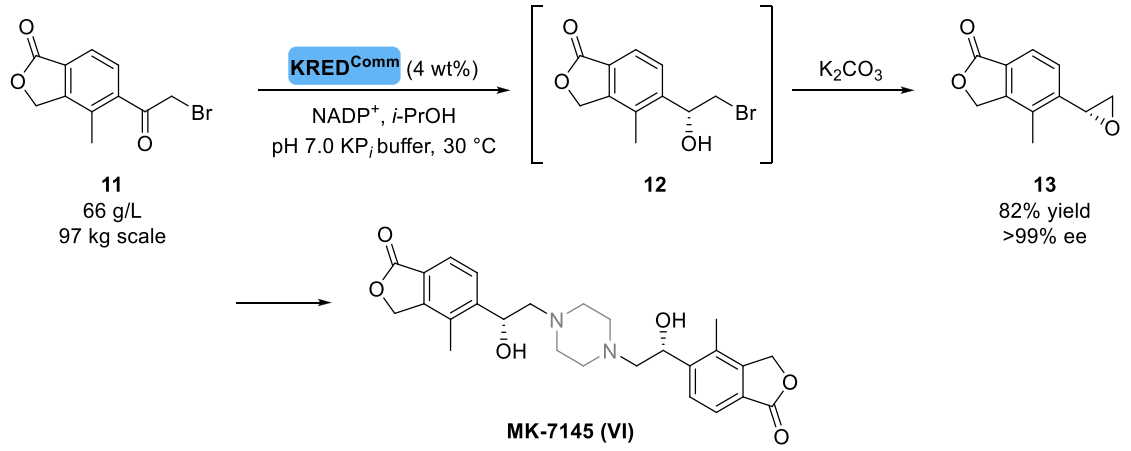
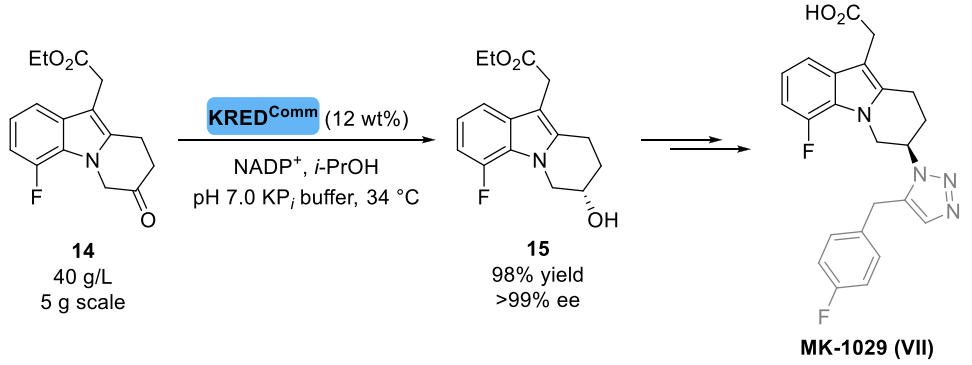
Scheme 5. KRED-Catalyzed Asymmetric Synthesis of an Intermediate 10 for the Production of the Novel Sickle Cell Disease Treatment PF-07059013 (V)[37]



Scheme 6. KRED-Catalyzed Asymmetric Synthesis of a Chiral Intermediate 13 for the Production of ROMK Inhibitors Including MK-7145 (VI)[38]



Scheme 7. KRED-Catalyzed Asymmetric Synthesis of a Chiral Intermediate 15 for the Production of the CRTH2 Antagonist MK-1029 (VII)[39]



The final process was implemented on a multikilogram scale to achieve the desired chiral alcohol in excellent yield and enantioselectivity (Scheme 3).

A chiral hydroxy ester **8** required to produce a gamma secretase inhibitor (IV) was accessed using an ADH process.[36] Researchers at Pfizer screened 190 commercially available enzymes for this transformation, and a c-LEcta ADH enzyme was chosen for large scale enablement. The use of *i*-PrOH was evaluated as a terminal reductant for this process for its operational simplicity; however, due to enzyme instability at high *i*-PrOH concentrations and the appearance of an unknown impurity, the process was implemented with a GDH/glucose cofactor recycling system and pH maintained with base titration. The reaction was operated on a multikilogram scale with high substrate and low enzyme loading to generate the desired product **8** in good yield and high *ee* (Scheme 4).

For a separate project, scientists at Pfizer applied a commercially available Codexis KRED for the asymmetric reduction of ketone **9** (Scheme 5).[37] Fast process enablement gave rise to implementation of this reaction on a 50 g scale to achieve enantiopure alcohol **10** in high yield which was used for early clinical deliveries of the noncovalent hemoglobin modulator PF-07059013 (V).

As part of the structure−activity relationship (SAR) investigations of a potential renal outer medullary potassium channel (ROMK) inhibitor, the team at Merck & Co. was interested in accessing the chiral epoxide **13** (Scheme 6).[38] Synthetic investment was made in this building block as it could be readily diversified upon ring opening by various

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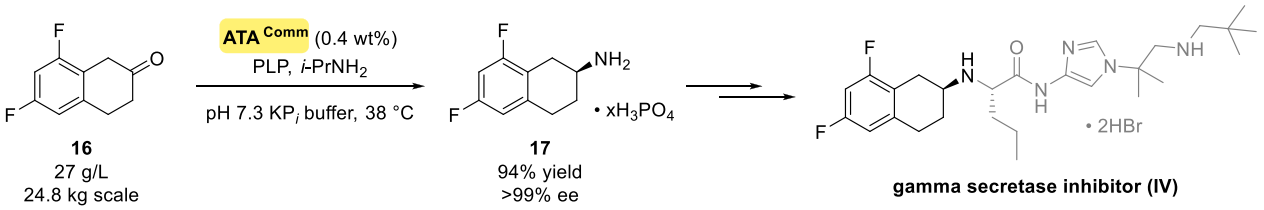
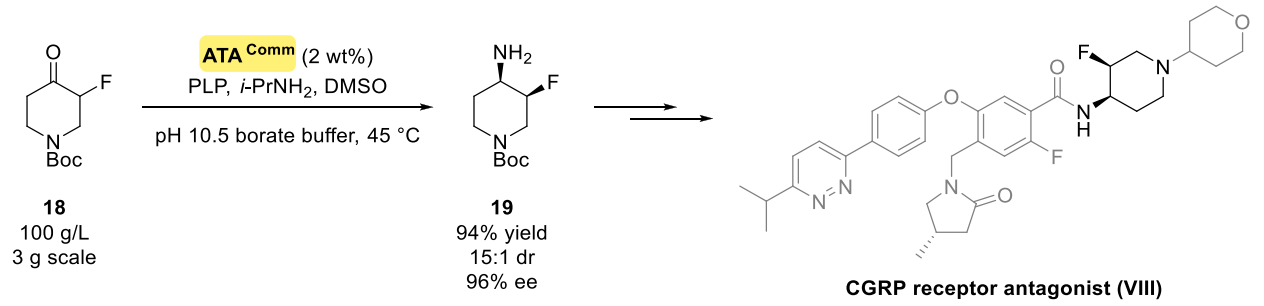
Scheme 8. Asymmetric Transaminase Process to Synthesize a Chiral Amine Intermediate 17 for the Preparation of a Gamma Secretase inhibitor (IV)[36]



Scheme 9. Asymmetric Transaminase Process to Synthesize a Chiral Amine Intermediate 19 for the Preparation of a CGRP Receptor Antagonist (VIII)[47]



nucleophiles and, therefore, represented a versatile intermediate for the drug project. In this process, a commercially available Codexis KRED performed the asymmetric reduction of α-bromo ketone **11** to afford the corresponding chiral alcohol **12**. At the end of the reaction, basification with K_2CO_3 triggered the ring closure to form desired epoxide **13** in high yield and excellent *ee*. As well as multigram deliveries to enable SAR, this process was then scaled to multikilograms for use in the synthesis of the lead clinical candidate MK-7145 (VI).

A KRED process was also developed by Merck & Co. for the synthesis of chiral alcohol intermediate **15**, used to access the chemo-attractant receptor expressed on Th2 cells (CRTH2) antagonist MK-1029 (VII).[39] One of the challenges with this transformation was instability of the ketone substrate **14** in the buffer system which was hypothesized to degrade via enol/enamine formation and oxidation pathways. This was overcome by degassing the reaction mixture and running the reaction under nitrogen as well as increasing the loading of the commercially available Codexis enzyme. This process was described on a 5 g scale and used for early clinical development of MK-1029 (VII) (Scheme 7).

Amine Transaminases [EC 2.6.1.X]

Amine transaminases (ATAs) catalyze the asymmetric transfer of an amine group from one substrate to another using pyridoxal phosphate (PLP) as a cofactor.[40] Often, transaminase reactions suffer from unfavorable equilibria necessitating large excesses of an amine donor, removal of the ketone byproduct, use of smart amine donors,[41−43] or additional enzymatic systems[44,45] to force the equilibrium toward desired amine formation. For industrial use, isopropylamine is typically favored as the amine donor because it is cheap and readily available. It can also be supplied in excess to a reaction to help drive the transaminase equilibrium to the desired product, with concomitant removal of the acetone byproduct with a nitrogen sweep or under vacuum.[40,46] Isopropylamine tolerance is often an engineered trait of transaminases, with wild-type enzymes utilizing other amine sources such as alanine. These enzymes have become a workhorse family for a variety of chiral primary amine syntheses in pharma.

A transaminase process was recently developed by researchers at Pfizer for the asymmetric amination of β-tetralone **16** to access a key chiral amine intermediate **17** for the synthesis of the gamma secretase inhibitor (IV).[36] A commercially available enzyme from c-LEcta was chosen for process enablement based on initial screening and evaluation of various enzyme panels. This enzyme possessed the desired selectivity and had high activity on the β-tetralone **16**. The reaction was ultimately demonstrated on a multikilogram scale with low enzyme loading (0.4 wt %) and excellent yield and selectivity (Scheme 8). This process was operated without any organic cosolvents as a slurry-to-slurry reaction as it was found that the desired amine product precipitated out of the reaction as the phosphate salt upon formation. β-Tetralones are prone to oxidation in air, therefore, the reaction was operated under a sweep of nitrogen which protected the starting material from spontaneous oxidation as well as serving to remove the acetone byproduct of the reaction and hence drive the equilibrium to high conversion.

A chiral amine intermediate **19** required for the preparation of a calcitonin gene-related peptide (CGRP) antagonist (VIII) was accessed via a transaminase approach. The team at Merck & Co. developed an elegant dynamic kinetic resolution (DKR) process starting from racemic ketone **18**, bearing a fluoro substituent at the α position.[47] For the DKR process to be feasible, a high pH was needed to facilitate the *in situ* epimerization of the α stereocenter. Therefore, an enzyme with the desired selectivity was needed that also was sufficiently stable at elevated pH. A suitable transaminase was identified by screening the commercially available Codexis panel. This enzyme was demonstrated to operate at high substrate and low enzyme loading at pH 10.5 to access the desired chiral molecule in high stereoselectivity and high isolated yield (Scheme 9).

Another CGRP project at Merck & Co. involved the evolution of a transaminase to enable a dynamic kinetic

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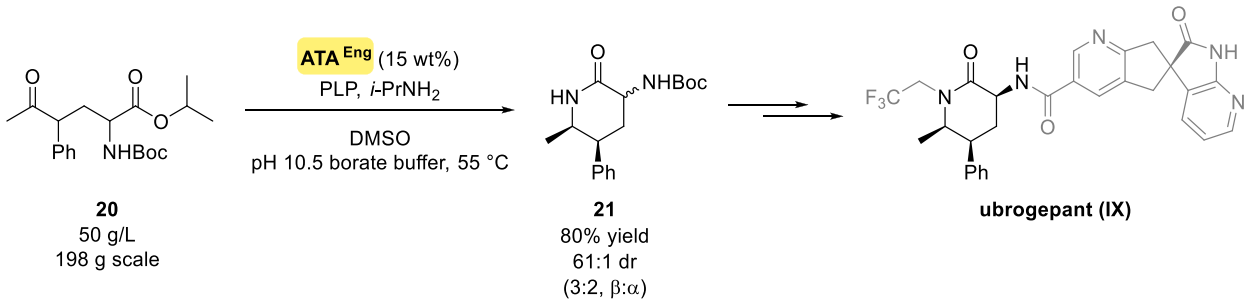
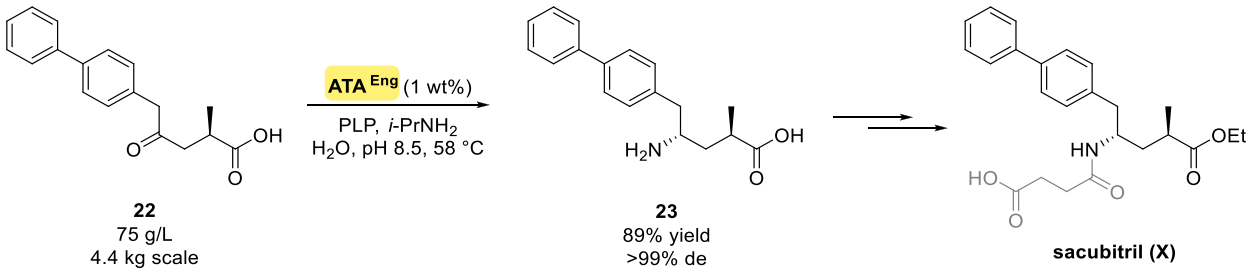
Scheme 10. Dynamic Kinetic Transaminase Resolution Process to Synthesize a Chiral Lactam Intermediate 21 for the Preparation of Ubrogepant (IX)[48]



Scheme 11. Transaminase-Catalyzed Asymmetric Synthesis of a Chiral Amino Acid Intermediate 23 for the Production of Sacubitril (X), an Active Ingredient in the Heart Failure Treatment LCZ696 (Entresto®)[49,50]

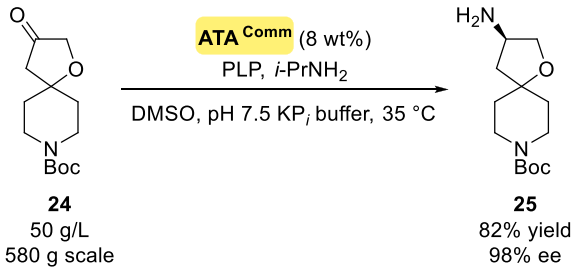


resolution process of ketone **20** to access the chiral lactam building block **21** for the synthesis of ubrogepant (IX).[48] Partnering with Codexis, screening of the Codexis enzyme panel was performed at pH 10.5 to enable epimerization of the ketone*α*stereocenter. A transaminase was selected that gave proof-of-concept for the DKR process which was then subjected to several rounds of evolution to improve its characteristics for this transformation: namely improving selectivity at the C-5 and C-6 chiral centers as well as its tolerance to high DMSO cosolvent concentrations. Through this evolution program, the enzyme selectivity was improved from 7:1 to 61:1 *syn*:*anti*, the substrate **20** loading could be increased from 5 g/L to 50 g/L, enzyme loading could be decreased from 300 wt % to 15 wt %, and the enzyme could operate in a 1:1 DMSO:buffer reaction solvent at 55 °C. During the reaction, as the ketone was transaminated, the compound spontaneously cyclized to the lactam **21**, promoted by the basic reaction pH and elevated temperature, which helped to drive the transaminase equilibrium to the desired product. The optimized process was executed on a 198 g scale to isolate **21** in high yield and selectivity (Scheme 10).

Novartis, in collaboration with Codexis, developed a transaminase process for the asymmetric synthesis of a key chiral, non-natural, amino acid intermediate for the manufacture of sacubitril (X), one of the active ingredients in the heart failure treatment LCZ696 (Entresto®).[49,50] Starting from a transaminase variant that gave trace activity for the wrong diastereomer, one round of enzyme engineering identified an enzyme with the opposite, desired selectivity, and a subsequent 10 rounds of evolution were performed to improve activity (500,000-fold), substrate tolerance, and thermal stability. The final developed process utilized this highly evolved enzyme that incorporated 26 amino acid mutations at high substrate and low enzyme weight percent (1 wt %) loading at elevated temperature in a slurry-to-slurry reaction. Cosolvents did not improve substrate solubility and had a negative effect on enzyme activity. Ultimately, the process was run in water in the absence of a buffer system as there was minimal pH change over the course of the reaction (Scheme 11).

The team at Pfizer applied a commercial Codexis transaminase for the synthesis of a chiral amine fragment **25** for application in medicinal chemistry synthesis (Scheme 12).[51]

Scheme 12. Transaminase-Catalyzed Asymmetric Synthesis of the Chiral Pharmaceutical Intermediate 25[51]



Rapid process enablement resulted in reaction condtions that were demonstrated on a 580 g scale with high yield and *ee*. During this work, it was observed that starting material aggregation became significant upon upscaling the reaction which was addressed by increasing the DMSO content to 10% v/v, improving the fluidity of mixing of the reaction.

A recent example from Merck & Co. described the application of a transaminase for the synthesis of amine **27** which is a precursor to the chiral tetrahydropyran motif present in the reversible Bruton's tyrosine kinase inhibitor nemtabrutinib (XI).[52] Starting from the sustainable feedstock material Cyrene **26** which already possessed one of the desired stereocenters, a commercially available Codexis enzyme was employed to install the amine and set the desired

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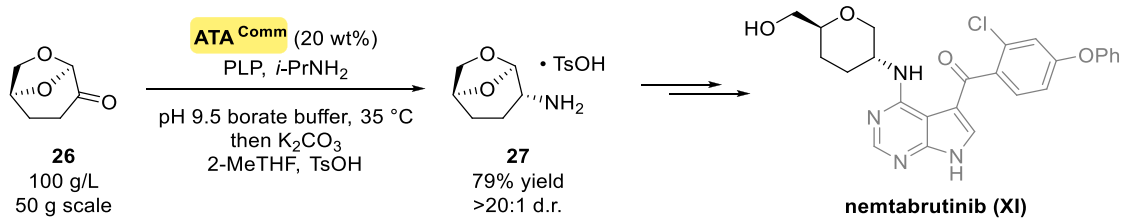
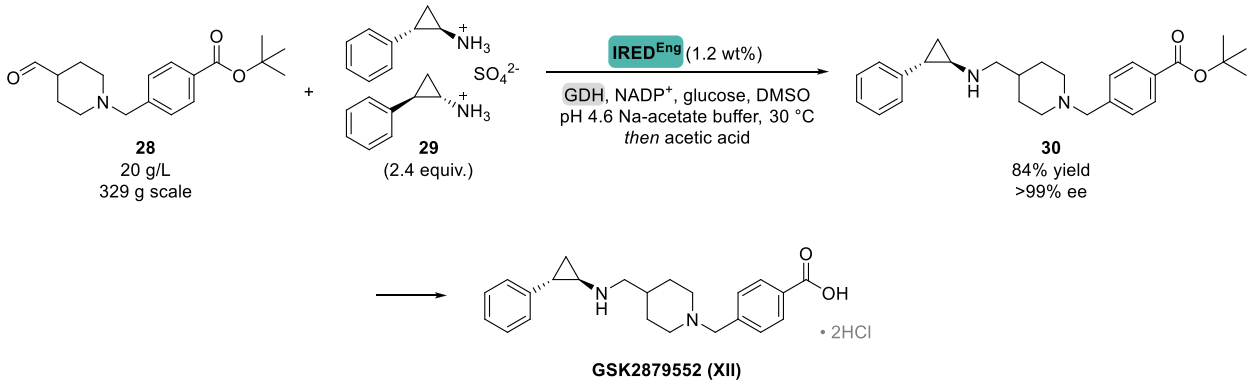
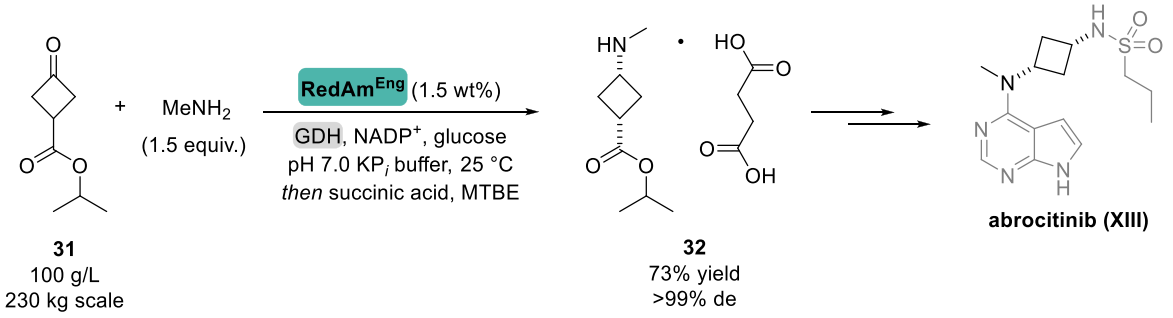
Scheme 13. Transaminase-Catalyzed Asymmetric Synthesis of a Chiral Amine Intermediate 27 Starting from Cyrene 26 for the Production of Nemtabrutinib (XI)[52]



Scheme 14. IRED-Catalyzed Reductive Amination Process for the Synthesis of an Intermediate 30 for the LSD1 Inhibitor GSK2879552 (XII)[56]



Scheme 15. IRED-Catalyzed Reductive Amination Process for the Synthesis of an Intermediate 32 for the JAK1 Inhibitor Abrocitinib (XIII)[57,58]



diastereomeric center. Notably, this enzyme was capable of overriding the inherent substrate-controlled selectivity of reductive amination compared to traditional chemical methods with NH_4OAc and $NaBH_3CN$ in MeOH. High substrate loading was utilized (100 g/L) in the final process which was demonstrated on a 50 g scale to isolate the desired amine **27** in high yield and selectivity (Scheme 13).

Imine Reductases and Reductive Aminases [EC 1.5.1.X]

Over the past decade, imine reductases (IREDs) have undergone a successful transition from the realm of academia into true application on industrial scale with several pharmaceutical companies describing the incorporation of IRED-catalyzed transformations into syntheses of key pipeline assets. Within the broader family of IREDs that catalyze imine reduction, there are enzymes that possess reductive amination activity to couple ketone or aldehyde substrates with an amine cosubstrate. Enzymes that are capable of catalyzing the imine formation step of the overall reductive amination reaction can be termed reductive aminases (RedAms).[53−55]

One of the first industrial examples described was from the team at GSK in which an IRED was engineered for the reductive amination of an aldehyde **28** to access an intermediate **30** of the investigational lysine-specific demethylase-1 (LSD1) inhibitor GSK2879552 (XII).[56] Although the IRED in this process did not directly set a chiral center, its value was in the enantioselective recognition of the amine reacting partner which was supplied as a racemate. Therefore, as well as achieving the desired bond forming reaction, the racemic amine **29** was also simultaneously resolved. IREDs typically operate at a neutral to basic pH; however, the IRED in this process was evolved to tolerate low pH at which the substrates and desired product had a better stability profile. In total, three rounds of evolution were performed, achieving >38,000-fold improvement over the parent enzyme. The final process was demonstrated on over 300 g scale to achieve high isolated yield of product **30** with excellent enantioselectivity (Scheme 14).

The team at Pfizer developed an enzymatic reductive amination of ketone **31** with methylamine which was utilized

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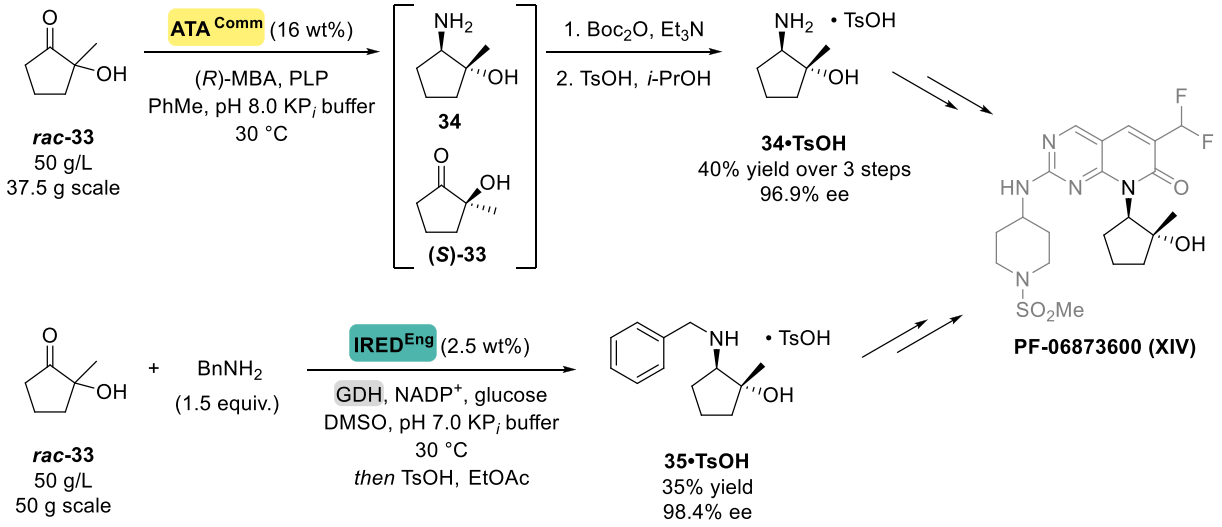
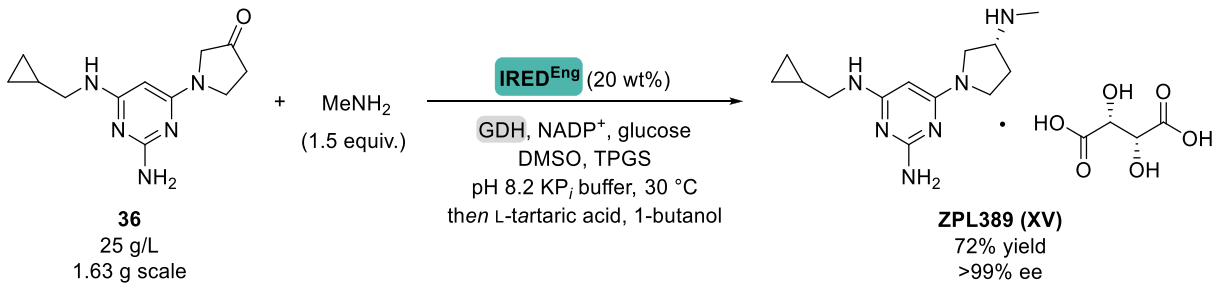
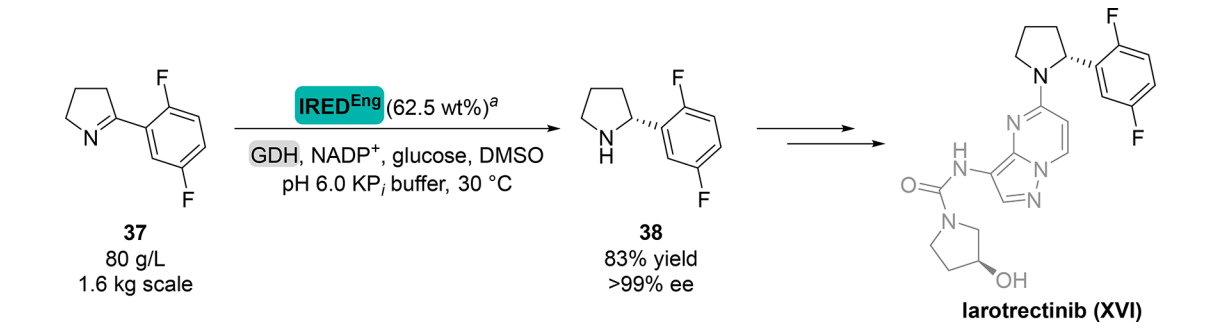
Scheme 16. Transaminase-Catalyzed and IRED-Catalyzed Resolution Processes for the Synthesis of Intermediates for an Investigational CDK2/4/6 Inhibitor, PF-06873600 (XIV)[59,60]



Scheme 17. IRED-Catalyzed Reductive Amination Process for the Synthesis of the H4 Receptor Antagonist ZPL389 (XV)[61]



Scheme 18. IRED-Catalyzed Cyclic Imine Reduction to Access a Chiral Pyrrolidine Intermediate 38 En Route to the Kinase Inhibitor Larotrectinib (XVI)[62a]



[a]Based on wet whole cell weight. Enzyme utilized as cell free lysate.

in the synthetic route to abrocitinib (XIII) (CIBINQO®).[57,58] A wild-type reductive aminase (RedAm) was identified from initial screening and subjected to several rounds of engineering. For the abrocitinib process, the evolved RedAm possessed >200-fold improvement over the wild-type enzyme and could perform with high substrate loading (100 g/L) and low enzyme loading (1.5 wt %) with excellent diastereoselectivity for the desired *cis*-configured cyclobutane system. Notably, the process was scaled to hundreds of kilograms and delivered metric tons of desired amine succinate salt **32** for the commercial manufacturing of this drug (Scheme 15).

Additionally, the Pfizer team utilized an IRED to access a chiral amine fragment used for the synthesis of an investigational CDK2/4/6 inhibitor (XIV).[59,60] Initially, the desired product was the primary amine **34** which could be made via transaminase-catalyzed resolution of the racemic ketone *rac*-**33** utilizing a commercially available enzyme. However, the high aqueous solubility of the primary amine product necessitated a Boc-protection/deprotection isolation strategy. This led the team to switch gears to develop an IRED-catalyzed reductive amination process with benzylamine as the amine reacting partner, to form a benzyl-protected amine **35** which was simpler to isolate than the free amine. An IRED was specifically

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evolved for this reaction, and the final process was demonstrated on a 50 g scale, achieving good yield and higher *ee* than the transaminase process (Scheme 16).

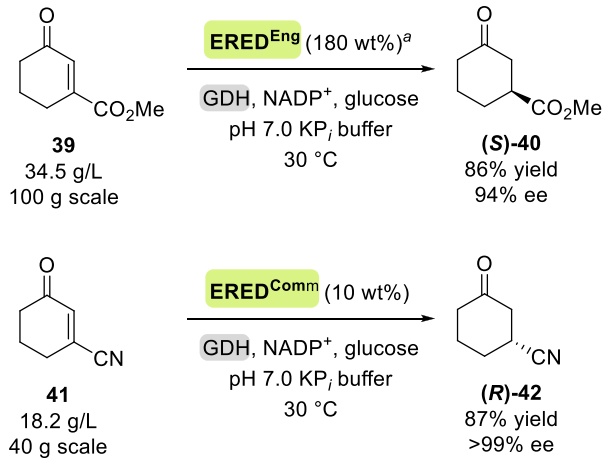
Scientists at Novartis developed an IRED-catalyzed reductive amination of a bulky pyrrolidinone substrate **36** with methylamine as a route to access the H4 receptor antagonist ZPL389 (XV).[61] In this work, a machine learning approach to the enzyme evolution was evaluated alongside strategies including deep mutational scanning and error prone PCR. From these efforts an improved, engineered IRED was successfully demonstrated on gram scale at 25 g/L. The *ee* of the IRED step was 98% which was upgraded to >99% in the isolation of the product amine as the L-tartrate salt (Scheme 17).

Recently the academic groups of Guo and Zheng described the evolution of an IRED enzyme for the asymmetric reduction of cyclic imine **37** to produce chiral pyrrolidine **38**,[62] an intermediate en route to the kinase inhibitor larotrectinib (XVI) (Vitrakvi®).[63] Cyclic imine substrates are generally stable in water, and therefore, enzymatic catalysis is focused on the reduction step rather than imine formation. In this work, a wild-type enzyme was subjected to three rounds of evolution to improve the specific activity for substrate **37** >100-fold. The engineered enzyme was demonstrated on a kilogram scale to access **38** in high yield and excellent enantioselectivity (Scheme 18). Furthermore, the evolved enzyme was also tested with a variety of cyclic imine substrates, showing improved scope compared to the wild-type enzyme.

Ene Reductases [EC 1.3.1.X]

Enablement of various biocatalytic approaches to chiral 3-substituted cyclohexanones was performed at GSK to have several enzymatic options to access these valuable building blocks for medicinal chemistry usage. Enantiocomplementary options were developed to access either the (*R*)- or (*S*)-, methyl-3-carboxylate, or 3-nitrile compounds [(*R*)-**40**, (*S*)-**40**, (*R*)-**42**, (*S*)-**42**]. The enzymes employed for these single-step transformations included ene-reductases (EREDs)[64,65] to perform the asymmetric reductions of the cyclohexenone derivatives **39** and **41** (Scheme 19) or lipase or nitrilases for resolutions of the corresponding racemic ester *rac*-**40** or nitrile *rac*-**42** precursors (*vide infra*, Scheme 22).[66] For the ERED-catalyzed synthesis of (*S*)-**40**, a reaction pH of 7.0 was important to minimize formation of a disproportionation byproduct that was observed at pH 7.5 and above. Also, the use of a GDH/glucose cofactor recycling system necessitated further pH control to neutralize the equivalent of gluconic acid formed over the course of the reaction. This was achieved on a small scale and at lower substrate loadings by increasing the buffer strength to 200 mM, but on a larger scale, it was achieved with active base titration with NaOH. Additionally, some background ERED activity was observed in the crude cell preparation of the enzyme which was speculated to be the cause of the lower *ee* of 94%. A similar process was developed starting with the nitrile analogue **41**. These processes were each successfully scaled to multigram quantities to deliver material for medicinal chemistry studies (Scheme 19).

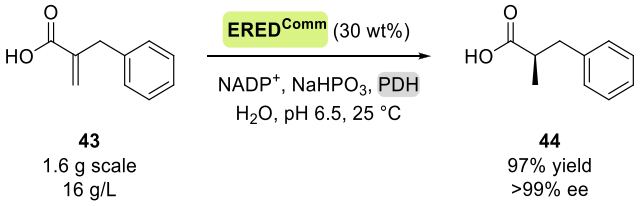
Scheme 19. ERED-Catalyzed Approach to Synthesize Chiral 3-Substituted Cyclohexanone Building Blocks[66,a]



[a]Frozen whole cells used.

Researchers at Merck & Co. utilized EREDs to synthesize α-chiral carboxylic acids for pharmaceutical development (Scheme 20).[67] In this work, a commercially available

Scheme 20. ERED-Catalyzed Asymmetric Reduction of Acrylic Acid 43 to Generated Chiral Acid Product 44[67]



Prozomix ERED was identified that was able to reduce a panel of aromatic and aliphatic acrylic acids with excellent enantioselectivity. A phosphite dehydrogenase was employed for NADPH recycling which was advantageous as it did not require pH monitoring and control compared to more traditional GDH/glucose recycling which generates gluconic acid as a byproduct. ERED activity on α,β-unsaturated acids is rare and, therefore, the characterization of this ERED to achieve this transformation for a diverse set of acrylic acid substrates is a valuable addition to the toolbox of biocatalysts. The process developed was demonstrated on gram scale with substrate **43** to achieve excellent yield of enantiopure **44**.

Hydrolases [EC 3.X.X.X]

As well as a KRED process (*vide supra*, Scheme 1), Genentech and Roche also utilized biocatalysis to access an enantiopure nitrile building block for ipatasertib (I) (Scheme 21).[33] As part of their efforts to develop a long-term manufacturing synthesis of this API, a nitrile resolution process was developed in which the (*R*)-enantiomer of racemic nitrile *rac*-**45** was selectively hydrolyzed to the corresponding acid **46**, leaving the desired (*S*)-enantiomer (*S*)-**45** unreacted. A commercial enzyme from c-LEcta was employed in this transformation as a liquid preparation. It was found that high concentrations of sulfate and phosphate ions enhanced the enzyme's activity, and polar organic cosolvents had a detrimental effect on enzyme performance. This led to a process being implemented on a 200 g scale at very high substrate loading (235 g/L) without organic cosolvents (Scheme 21).

Alongside ERED-catalyzed approaches to the medicinal chemistry building blocks (*S*)-**40** and (*R*)-**42** (*vide supra*, Scheme 19), researchers at GSK also developed hydrolase resolutions to the enantiocomplementary compounds (*R*)-**40**

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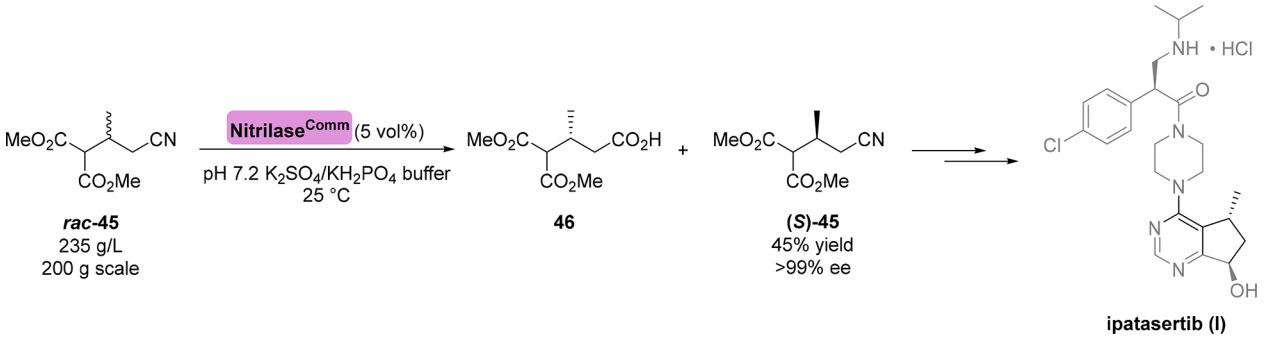
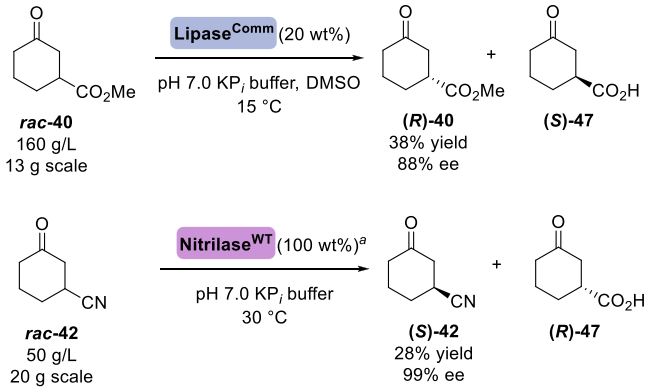
Scheme 21. Nitrilase Resolution Process to Access an Enantiopure Nitrile Intermediate (*S*)-45 for the Synthesis of the Akt Kinase Inhibitor Ipatasertib (I)[33]



and (*S*)-**42** (Scheme 22).[66] A commercial lipase and a wild-type nitrilase were selected from screening for rapid process enablement to deliver multigram quantities of each material. The varying biocatalytic approaches to these compounds was the result of not finding the desired high selectivity in any one class of enzyme that could afford both enantiomers of each building block in high purity.

Scheme 22. Lipase and Nitrilase Biocatalytic Approaches to Synthesize Chiral 3-Substituted Cyclohexanone Building Blocks[66,a]



[a]Cell lysate used.

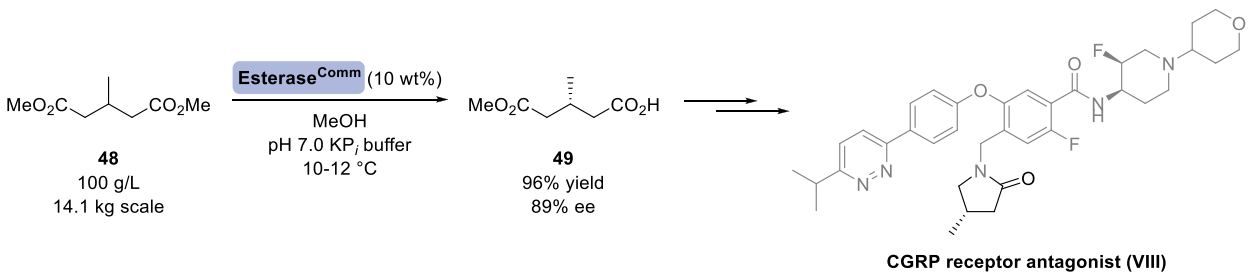
A biocatalytic desymmetrization was employed in the synthesis of the chiral lactam motif of the CGRP receptor antagonist (VIII). Researchers at Merck & Co. developed an esterase-catalyzed hydrolysis of the symmetrical diester **48** to achieve the chiral monoacid **49** in high yield (96%) (Scheme 23).[47] Commercially available pig liver esterase was used for this transformation which was successfully scaled to a multikilogram scale at high substrate loading (100 g/L). Only a modest *ee* of 89% was achieved from this enzymatic process; however, this could be efficiently upgraded to 99% *ee* by a chiral salt resolution in a subsequent synthetic step. This synthesis, as well as the transaminase-catalyzed DKR synthesis of the chiral piperidine fragment of (VIII) (*vide supra*, Scheme 9), enabled all early clinical supplies of this investigational compound.

An epoxide hydrolase (EH) process was developed by GSK to resolve racemic epoxide *rac*-**50**, a key intermediate used to access the ileal bile acid transport (iBAT) inhibitor GSK2330672 (XVII) for type II diabetes and cholestatic pruritus (Scheme 24).[68] The enzyme utilized for this reaction was a wild-type identified from a panel comprised of literature and metagenomically sourced biocatalysts. The wild-type enzyme had sufficient activity and substrate tolerance to enable a 22 g scale up with very high substrate loading (338 g/L). It was noted that enzyme engineering may be able to improve the selectivity of the enzyme to increase the yield of (*R*)-**50** while maintaining high enantiomeric excess.

Oxygenases [EC 1.14.X.X]

Scientists at AstraZeneca developed an asymmetric sulfoxidation process to produce a chiral sulfoxide derivative of the kinase inhibitor AZD6738.[69] Early synthetic deliveries of compound **53** were achieved via unselective *m*-CPBA oxidation of sulfide **52** and chromatography; however, a more efficient asymmetric option was desired for subsequent deliveries. Screening of a selection of commercially available Codexis Bayer-Villiger monooxygenases (BVMOs)[70] identified an enzyme with activity for the desired sulfoxidation, utilizing a KRED and *i*-PrOH for NADPH recycling. The best BVMO hit from screening was taken forward into dedicated process chemistry optimization where it was found that oxygen mass

Scheme 23. Esterase-Catalyzed Desymmetrization Process to Access a Chiral Precursor 49 of the Lactam Fragment of the CGRP Receptor Antagonist (VIII)[47]



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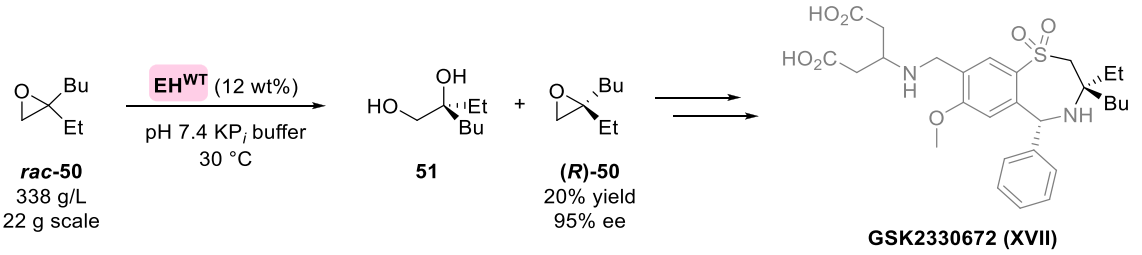
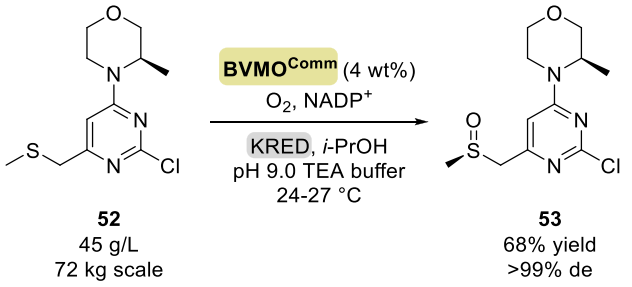
Scheme 24. Epoxide Hydrolase-Catalyzed Resolution Process to Access an Enantiopure Epoxide Intermediate (*R*)-50 for the Synthesis of the iBAT Inhibitor GSK2330672 (XVII)[68]



transfer was key for the success of this reaction on scale. A combination of optimizing air sparging and fast mixing speed were able to resolve mass-transfer limitations, and following 29 batches on a ∼1 kg scale, the process was ultimately run on a 72 kg pilot plant scale with good yield and excellent diastereoselectivity (Scheme 25).

Scheme 25. BVMO-Catalyzed Asymmetric Sulfoxidation to Access a Derivative 53 of the Investigational Kinase Inhibitor AZD6738[69]



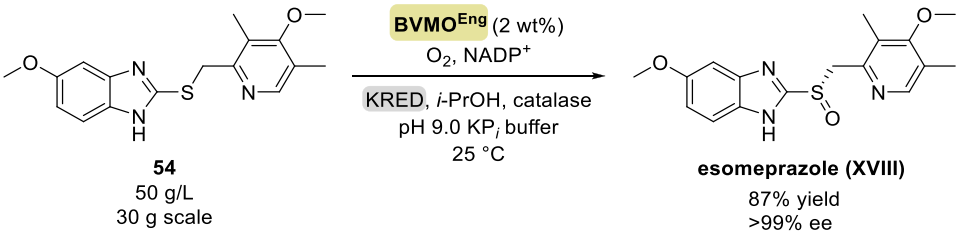
The development of engineered BVMOs for industrial chiral sulfoxidation is also evidenced by the work described by Codexis to access esomeprazole (XVIII), the blockbuster proton pump inhibitor to treat gastric reflux.[71] A wild-type enzyme was subjected to 19 rounds of enzyme engineering in which the enantioselectivity was switched to form the desired enantiomer, and an overall 140,000-fold improvement over wild-type was achieved over the course of the evolution. Interestingly, it was noted that the BVMOs native ability to catalyze Bayer-Villiger oxidations was diminished, suggesting a fundamental change in the inherent catalytic mechanism. From a process development perspective, the reaction was run slurry-to-slurry, and efficient mixing was again crucial to avoid limitations from oxygen mass transfer. Additionally, catalase was supplemented in this reaction to rapidly breakdown any hydrogen peroxide generated from uncoupled turnover of the enzyme which could otherwise oxidatively deactivate the enzyme or form a racemic product by reacting with starting material nonselectively. Overall, this process was able to access esomeprazole (XVIII) in high yield and excellent enantioselectivity (Scheme 26).

As part of efforts to develop synthetic routes to important pharmaceutical building blocks for medicinal chemistry exploration, researchers at Novartis embarked on enzyme discovery to identify new proline hydroxylases (PHs) to access hydroxylated proline compounds.[72] These wild-type enzymes were found via genome mining and applied on a multimilligram scale for the preparative synthesis of different regioisomers and enantiomers of hydroxyproline, isolated as the Fmoc derivatives and purified via chromatography (Scheme 27).

Lyases [EC 4.X.X.X]

To access a non-natural phenylalanine intermediate **62** for the synthesis of the angiotensin II type 2 (AT_2) antagonist olodanrigan (XIX) (EMA401), Novartis partnered with Codexis to develop a scalable phenylalanine ammonia lyase (PAL) route (Scheme 28).[73] PALs catalyze the reversible, asymmetric, and regioselective hydroamination of cinnamic acid derivatives with the equilibrium lying in favor of the cinnamic acid.[74] Therefore, an excess of ammonia is required in these reactions to drive the reaction to amino acid according to Le Chatelier's principle. Wild-type PALs that were screened were not able to convert cinnamic acid **61**. However, a previously evolved enzyme was subjected to multiple rounds of further engineering to arrive at an enzyme suitable for the process with more than 20 mutations incorporated from the starting point. As well as protein engineering, extensive process chemistry studies and optimization were carried out. Calculations of Gibbs free energy of the reaction led to the implementation of a temperature ramp from 45 to 20 °C over the course of the reaction to help favor product formation by decreasing $\Delta\Delta S$ for the transformation. The process utilized ammonium carbonate as the optimal ammonia source for the reaction and was operated as an emulsion of the starting cinnamic acid. At the end of the reaction, distillation to remove

Scheme 26. BVMO-Catalyzed Asymmetric Sulfoxidation to Access the Proton Pump Inhibitor, Esomeprazole (XVIII)[71]



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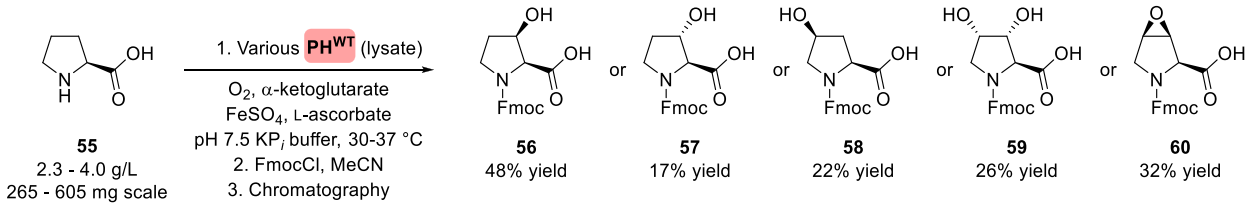
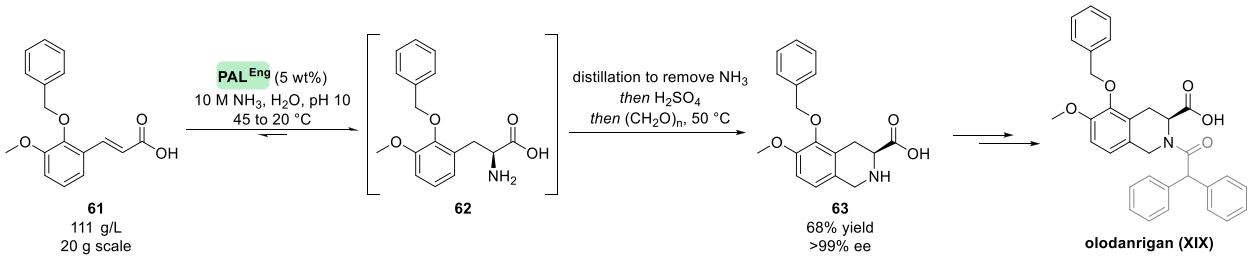
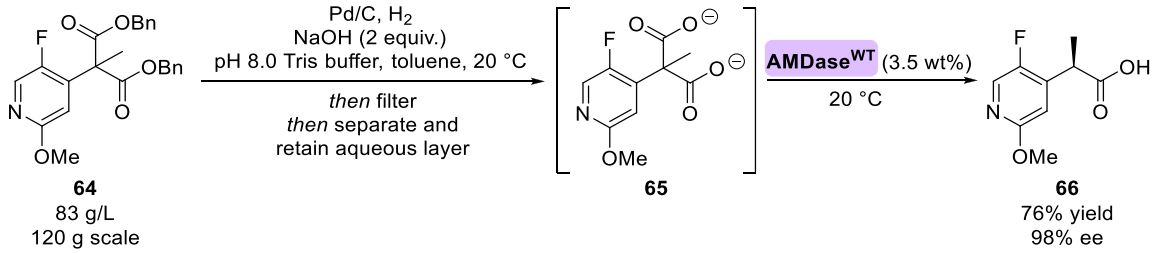
Scheme 27. Proline Hydroxylase-Catalyzed Asymmetric Oxidations to Access a Variety of Hydroxylated Proline Motifs for Medicinal Chemistry Applications[72]



Scheme 28. PAL-Pictet-Spengler Telescoped Process for the Synthesis of an Intermediate 63 of the AT_2 Antagonist Olodanrigan (XIX)[73]



Scheme 29. Telescoped Hydrogenolysis-Enzymatic Decarboxylation Process to Access the Pharmaceutical Intermediate 66[75]



excess ammonia followed by acidification and Pictet-Spengler cyclization with paraformaldehyde was performed to yield desired product **63** in excellent enantioselectivity. The distillation to remove ammonia and CO_2 from the reaction mixture was shown to be suitable for recycling this stream back into a subsequent reaction batch.

A recent example from Pfizer highlights the use of biocatalysis for medicinally relevant small molecule α chiral propionic acid building blocks.[75] These compounds belong to the profen family of pharmacologically active structures and are therefore of interest in drug discovery. In this work, an aryl malonate decarboxylase (AMDase) was employed for the asymmetric synthesis of several (*R*)-propionic acids from the corresponding dibenzyl malonates with one compound **64** scaled to 120 g (Scheme 29). A telescoped approach from the dibenzyl malonates was developed to overcome instabilities of the *N*-heterocyclic malonic acids which were prone to spontaneous decarboxylation. By performing a Pd/C catalyzed hydrogenolysis reaction in a basic, biphasic medium, the intermediate malonates could be accessed with minimal spontaneous decarboxylation. The use of two equivalents of NaOH effectively neutralized the two equivalents of acid generated in the hydrogenolysis to maintain a pH > 8.0, which was found to be critical to avoid compound decomposition. Following filtration of the Pd/C and removal of the toluene organic layer (required to solubilize the starting material **64**), the aqueous layer containing the malonate **65** at pH 8.0 was subjected to the AMDase, furnishing the desired propionic acid **66** in high *ee*. A wild-type enzyme was used which was already highly active for this substrate and allowed for low enzyme loadings to be used.

MULTI-STEP ENZYMATIC CASCADES

Over the past years, there has been an explosion in the academic literature describing novel cascade syntheses in which various enzymes have been combined with other enzymes as well as in conjunction with chemo-catalytic reactions.[9−11,21] These processes are extremely attractive especially in the context of reducing PMI, streamlining syntheses, and avoiding purifications and isolations. However, cascade processes require several additional considerations for successful implementation on scale. The addition of more enzymes into the same pot increases the total protein concentration of the reaction which can cause challenges from the perspective of final isolations. High protein concentrations can lead to problematic emulsions and slow filtrations which become difficult on scale without strategies for protein removal. Therefore, there in an increased drive to use biocatalysts at low weight percent loadings. Also, mutual incompatibilities of different enzymes or reaction conditions can still be a challenge, necessitating the operation of cascades in a sequential or telescoped manner.

Despite these challenges, the advantages for cascade syntheses to make complex products is significant, with recent examples in this area highlighted below.

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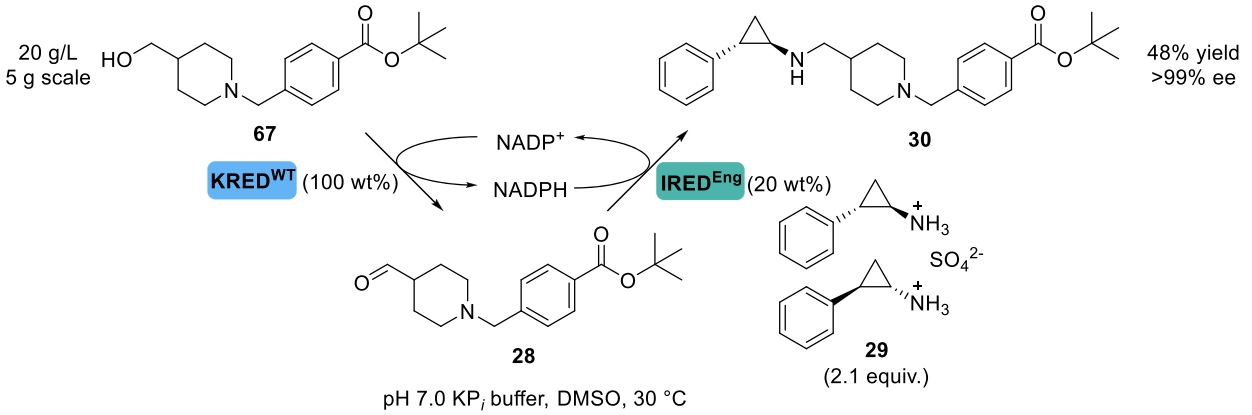
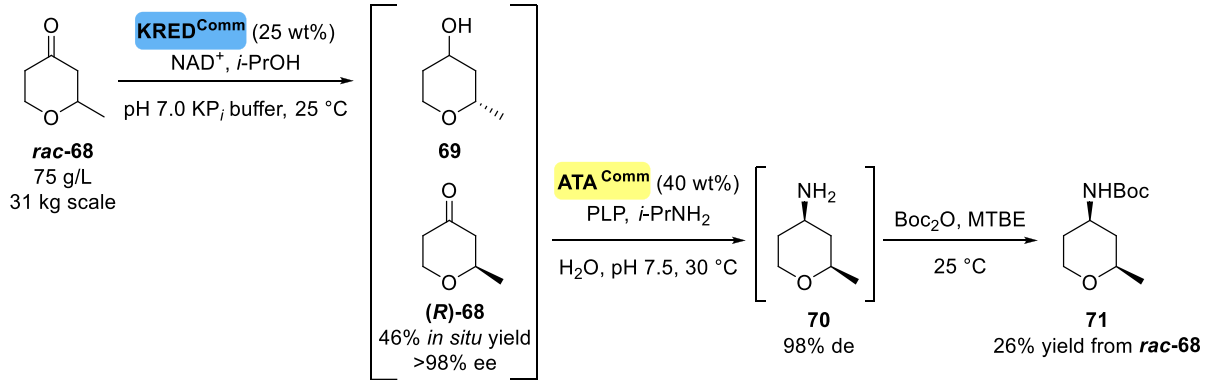
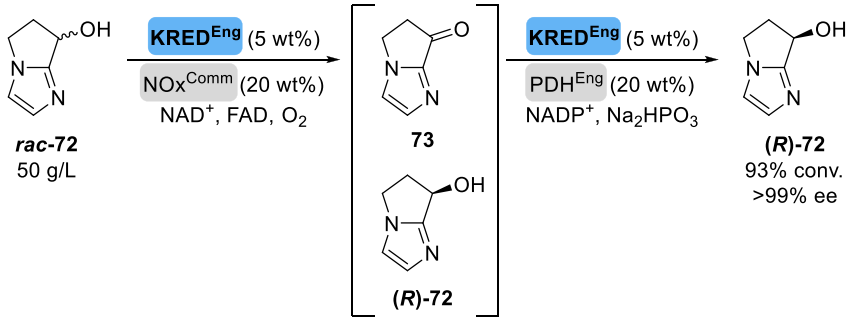
Scheme 30. Enzymatic KRED-IRED Hydrogen Borrowing Cascade Synthesis of an Intermediate for the LSD1 Inhibitor GSK2879552 (XII)[56]



Scheme 31. KRED-ATA Cascade Synthesis of the Chiral Pharmaceutical Intermediate 71[76]



Scheme 32. Cascade Deracemization of Racemic Alcohol *rac*-72 by Two Engineered KREDs Operating with Orthogonal Cofactor Recycling Systems[77]



As well as a single step transformation of aldehyde **28** to amine **30** catalyzed by an IRED (*vide supra*, Scheme 14), the team at GSK also evaluated a cascade to start with the corresponding alcohol **67** and introduce an extra enzymatic oxidation step (Scheme 30).[56] This approach was desirable as it would remove the need for a separate copper oxidation of the alcohol **67** to generate the aldehyde **28** and avoid an additional isolation and purification step. Screening of >400 KREDs from the GSK collection identified a wild-type enzyme that enabled proof of concept of this cascade. During the reaction, the NADPH generated from the KRED-catalyzed oxidation of the alcohol **67** subsequently reduced the imine in the IRED-catalyzed reductive amination in a "hydrogen-borrowing" fashion. A modest yield of 48% was reported that was believed to be the result of operating the cascade with a wild-type KRED at pH 7.0 which was a compromise between KRED activity and product stability. Improvements were envisaged with a more active KRED and further process optimization. It was also noted that, although increasing the equivalents of the racemic amine cosubstrate was likely to help drive the reaction to higher conversion, this was not an economically viable solution.

A one-pot sequential cascade was developed by the team at Pfizer for the synthesis of a chiral disubstituted tetrahydropyran **70** (Scheme 31).[76] This approach superseded a five-step synthesis used for initial medicinal chemistry deliveries that included individual lipase resolution and transaminase steps. A transaminase that was able to perform the simultaneous direct

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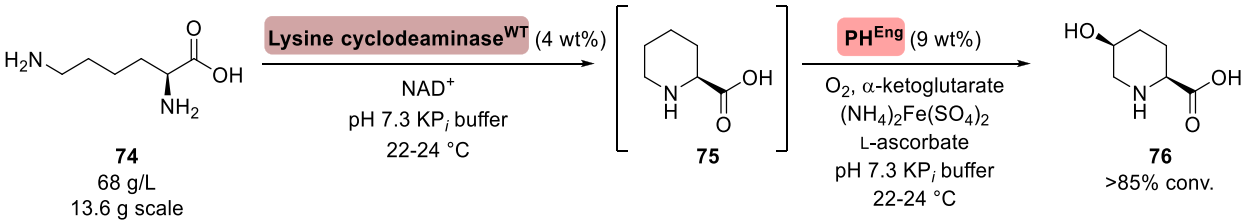
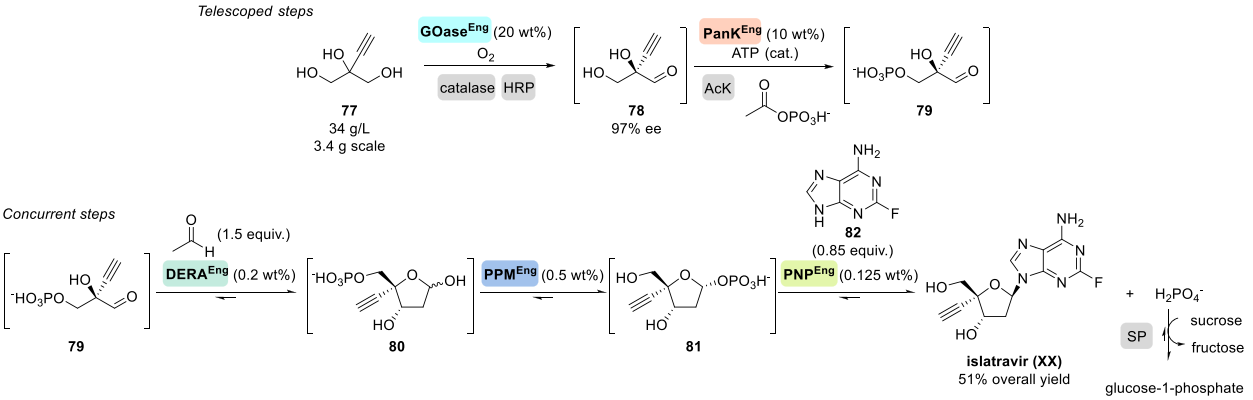
Scheme 33. Cascade Synthesis of (2*S*,5*S*)-Hydroxypipecolic Acid 76 from L-Lysine 74 Using a Wild-Type Lysine Cyclodeaminase and Engineered Proline Hydroxylase[78]



Scheme 34. Biocatalytic Cascade Synthesis of the Investigational HIV Drug, Islatravir (XX)[79]



amination and resolution of the ketone *rac*-**68** could not be identified, and therefore, a two-enzyme solution was developed. The first step of the cascade involved the resolution of the racemic ketone starting material *rac*-**68** with a KRED. The absolute selectivity of the KRED for setting the chiral alcohol stereocenter was not important so long as the desired stereodifferentiation of the remote chiral methyl center was achieved. Following an acidification-pH readjustment and Celite filtration to remove the protein catalyst from the first step, the reaction mixture was then directly telescoped into the subsequent transamination step to generate amine **70**. Once complete, the enzyme was again removed by acidification-basification and Celite filtration and excess *i*-PrNH$_2$ removed by distillation. Finally, an *in situ* Boc protection enabled isolation of the final product **71**. At the gram-scale and during initial development, a screen of in-house and vendor KREDs and transaminases led to the selection of commercially available enzymes (c-LEcta and Codexis) for this process. Once this process was transferred to an external CRO for multikilogram scale implementation, these enzymes were replaced with the CROs own proprietary enzymes to reduce costs and improve freedom to operate. However, this necessitated the use of higher enzyme loadings than was demonstrated on lab scale and required intermediate enzyme removal steps to avoid problematic emulsions.

Researchers at Merck & Co. developed a deracemization process to generate an enantiopure alcohol (*R*)-**72** from a racemic mixture (Scheme 32).[77] This cascade employed two engineered KREDs with different cofactor preferences, acting orthogonally to enable this transformation with high conversion and *ee*. The first KRED selectively oxidized the (*S*)-enantiomer of the alcohol to the corresponding ketone **73** which was driven by the turnover of NAD by NADH oxidase (NOx). A second KRED then selectively reduced the ketone to the desired (*R*)-enantiomer with NADPH recycling achieved using a phosphite dehydrogenase (PDH)/phosphite system. The use of two enzymes with orthogonal cofactor preferences allowed for these enzymatic steps to operate successfully in a concurrent fashion.

A two-enzyme cascade was described in a patent from Codexis in collaboration with Merck & Co. starting from L-lysine **74** to synthesize (2*S*,5*S*)-hydroxypipecolic acid **76**.[78] The first step of the cascade utilized a lysine cyclodeaminase catalyst to generate the corresponding pipecolic acid **75**. A wild-type enzyme was employed for this transformation as L-lysine was its native substrate, and therefore, the biocatalyst was already highly active. The subsequent step was achieved with proline hydroxylase (PH) which was engineered for improved activity and selectivity for pipecolic acid. The PH was Fe^{2+}-dependent, and this metal cofactor was supplied to the reaction as $(NH_4)_2Fe(SO_4)_2$ (Mohr's salt). It was found that the presence of ascorbic acid in the reaction improved the hydroxylase activity which was believed to help maintain or regenerate the active enzyme-Fe^{2+} catalytic form. The reaction was also sparged with air to supply the O_2 needed for the oxidation. This process was demonstrated on a 13.6 g scale to achieve high overall conversion (>85%) (Scheme 33).

In pioneering work, the team at Merck & Co. developed an elegant enzymatic cascade to access an investigational nucleoside reverse transcriptase translocation inhibitor, islatravir (XX), for the treatment of HIV.[79] The process started with a galactose oxidase (GOase)-mediated desymmetrizing oxidation to generate aldehyde intermediate **78**. This was then telescoped into a phosphorylation of the primary alcohol facilitated by the kinase PanK. The ATP required for PanK was regenerated *in situ* using acetate kinase (AcK) and acetyl phosphate as the ultimate phosphate source. The unisolated intermediate **79** was then subjected to a one-pot, concurrent, three-enzyme cascade in which a deoxyribose phosphate aldolase (DERA) initially constructed the ribose ring frame-

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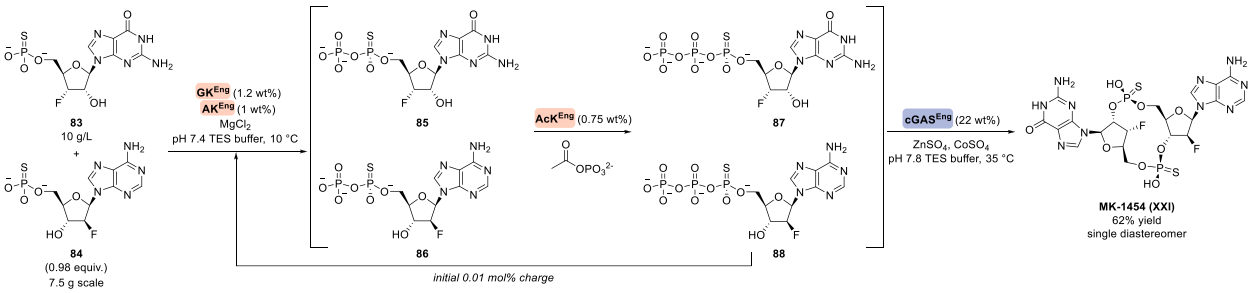
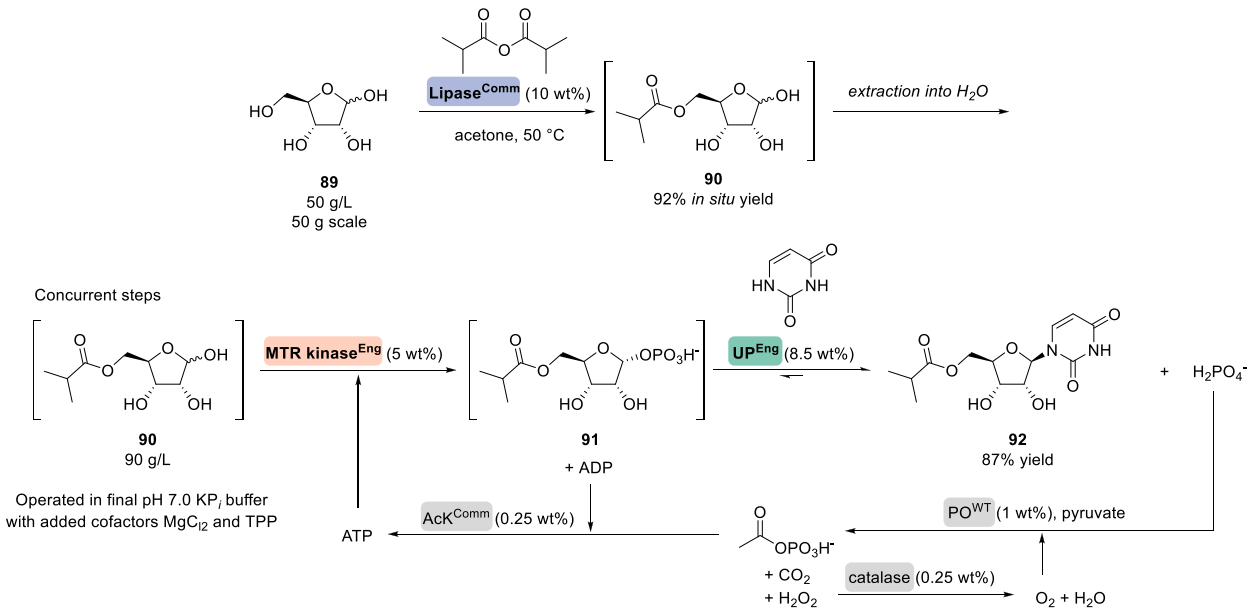
Scheme 35. Biocatalytic Cascade Synthesis of the Novel STING Antagonist MK-1454 (XXI)[80,81]



Scheme 36. Cascade Synthesis of the Key Chiral Intermediate 92 for the Production of the Antiviral, Molnupiravir[82]



work. A subsequent phosphopentamutase (PPM)-catalyzed transfer of the phosphate from the 5- to the 1-position generated compound **81**, which was then a substrate for the final purine nucleoside phosphorylase (PNP)-catalyzed attachment of the purine base **82**. The equilibrium of this concurrent cascade was pulled through to the final product by the introduction of an orthogonal enzymatic step in which the phosphate byproduct was removed with sucrose and sucrose phosphorylase (SP) (Scheme 34). The five key enzymes involved in this cascade, GOase, PanK, DERA, PPM, and PNP were all highly engineered to improve their performance in terms of activity and selectivity.

Scientists at Merck & Co. also established a novel cascade synthesis of their investigational stimulator of interferon genes (STING) activator, MK-1454 (XXI) (Scheme 35).[80,81] This complex, cyclic dinucleotide was constructed via a multienzymatic synthesis starting from non-natural nucleotide thiomonophosphates **83** and **84**. An engineered guanylate kinase (GK) and adenylate kinase (AK) were used to install an additional phosphate unit to **83** and **84**, respectively, to generate thiodiphosphates **85** and **86**. This was followed by an acetate kinase (AcK) step which formed the thiotriphosphates **87** and **88** using the high energy phosphate source, acetyl phosphate. This formation of the thiotriphosphates from the thiomonophosphates was operated as a three-enzyme cascade that was initiated with 0.01 mol % **88** before becoming self-catalytic. Once this portion of the cascade was complete, the final cyclic guanosine-adenosine synthase (cGAS) step was started by charging additional metal cofactors, adjusting pH, and finally adding the cGAS enzyme. All the enzymes in this cascade were highly engineered for improved activity and substrate tolerance, and this process was described on a multigram scale to achieve good yield and excellent diastereoselectivity. The cGAS step transformation in this process originated from efforts in discovery chemistry to access novel cyclic dinucleotides. This highlights the significant impact biocatalysis can have in the drug discovery space by creating access to structurally complex molecules that would otherwise be challenging to make by traditional synthetic organic chemistry.

Following on from the landmark cascade synthesis of islatravir (XX), the team at Merck & Co. developed a novel biocatalytic cascade for the non-natural nucleoside **92** which is one synthetic step away from the COVID-19 drug molnupiravir (Scheme 36).[82] Key drivers for the development of this process were speed and sustainability of production of this therapy to address the global health crisis of the COVID-19 pandemic. Starting from ribose **89** as a commodity building block, the formation of the isopropyl ester at the 5-hydroxyl position was catalyzed by the commercially available lipase Novozymes 435. This reaction stream was then telescoped into a concurrent cascade to install the uracil base. The first step involved the selective phosphorylation of the 1-position of **90** by an engineered 5-*S*-methylthioribose (MTR) kinase with

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Table 1. Summary of Limitations and Challenges for Biocatalytic Implementation and the Solutions Developed from the Highlighted Examples Herein

limitation	challenge	solution	see example (scheme number)
enzyme	poor enzyme activity	increase enzyme loading	7, 13, 17, 19, 20, 22, 30, 31
		evolve enzyme for improved activity	10, 11, 14, 15, 16, 17, 18, 19, 26, 28, 32, 33, 34, 35, 36
	poor enzyme selectivity	evolve enzyme for improved selectivity	10, 26, 34, 35, 36
substrate	low aqueous solubility of substrate	use an organic cosolvent	1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16, 17, 18, 22
		operate reaction as slurry	8, 11, 28
		operate reaction at high organic cosolvent concentration (may require enzyme evolution)	10
		operate reaction at elevated temperature (may require enzyme evolution)	2, 3, 10, 11
	substrate instability	operate reaction as slurry	1
		operate reaction degassed under nitrogen	7, 8
		adjust pH range to minimize degradation (may require enzyme evolution)	14, 19, 29
		increase enzyme loading	7
	poor oxygen mass transfer (for O_2 dependent reactions)	efficient sparging of reaction with air	25, 26, 33
		efficient mixing	25, 26
product	product instability	cascade or telescope reaction into next synthetic step	29
	high aqueous solubility of product	in situ protection to aid isolation	16, 31
reaction conditions	unfavorable reaction equilibrium	have one starting material in excess	8, 9, 10, 12, 13, 28, 31
		remove product (or byproduct) by subsequent irreversible reaction (or cascade)	8, 10, 12, 34, 35
		use orthogonal cofactor recycling systems to drive reaction	32, 36
	acidification of reaction mixture	titrate in base over the course of reaction to adjust pH	4, 15, 17, 18, 19, 29
		use higher buffer strength	19
		use cofactor recycling systems that do not generate acidic byproducts	1, 2, 3, 20
timelines	short timelines for development	use commercially available or wild-type enzymes in fit-for-purpose processes	5, 6, 12, 20, 23, 24, 25, 27, 29, 31

ATP as the high energy phosphate source. An engineered uridine phosphorylase (UP) then catalyzed the displacement of the 1-phosphate with added uridine which was formally a reversible reaction. To drive this cascade to desired product formation, an elegant solution was developed in which the final phosphate byproduct was used to regenerate ATP via an acetyl phosphate intermediate catalyzed by an engineered pyruvate oxidase (PO) and acetate kinase (AcK). The use of this system avoided the need to use stochiometric phosphoryl donor or the use of phosphate sequestration strategies to drive the equilibrium, as was used for islatravir (XX). Additionally, catalase was employed to breakdown the hydrogen peroxide byproduct of the PO step to generate oxygen which could be used for a subsequent turnover of PO. The cascade was demonstrated on a 50 g scale starting from ribose **89** to achieve nucleoside **92** in 87% isolated yield which was 70% shorter than the previous chemical route as well as being almost 7-fold higher yielding. Recently, an alternative electrochemical method for ATP was described under air-free conditions that could be successfully implemented in this cascade to avoid the challenges of oxygen mass transfer.[83]

CURRENT STATE-OF-THE-ART

Biocatalysis continues to be highly impactful for key synthetic challenges in the pharmaceutical industry. In all the industrial examples highlighted, there have been significant challenges to the implementation of biocatalytic transformations. These challenges and the solutions developed are summarized in Table 1 and can be divided into the areas of enzyme, substrate, product, reaction conditions, and timeline-related problems.

More well-established enzyme classes such as KREDs and transaminases have become the go-to methods for chiral alcohol or amine synthesis, respectively, as well as hydrolases for desymmetrizations or resolution processes. For commercial syntheses, highly engineered enzymes are typically implemented in final processes, whether that is from a bespoke evolution campaign or a previously evolved, commercial enzyme. As starting points for evolution programs and development of new industrial enzyme platforms, companies are increasingly embarking on in-house and external enzyme discovery efforts to find suitable biocatalysts.

From a process chemistry implementation perspective, several examples have demonstrated that poorly aqueous soluble substrates and processes at high substrate loading can be operated successfully as slurried reactions. Additionally, the efficient removal of protein from end-of-reaction streams overall remains a challenge and is especially heightened in cascade processes when there are many enzymes present in the system. To some extent, process chemistry solutions can be developed to overcome issues associated with enzymatic work-

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ups such as denaturing proteins at low pH and/or adsorption of residual protein on to diatomaceous earth. These types of process solutions are particularly important for early development when using an off-the-shelf biocatalyst or an enzyme that has not been subjected to evolution to improve its compatibility for the reaction. Ultimately, enzymes with high specific activity are desired so these can be charged at low wt % loadings to improve downstream processing as well as reducing overall cost of enzyme production or procurement.

From a large-scale manufacturing viewpoint, it is common to apply enzymes as freeze-dried powders in processes instead of cells or lysates due to the ease of transportation and storage when enzyme production is not collocated with the chemistry facility. Also, enhanced processing of enzyme preparations such as coagulation, flocculation, and ultrafiltration (use of 0.2 μm filters) to remove genetically modified organisms (GMOs) can enable less restrictive requirements for international shipping of enzymes and benefit reaction work up by minimizing additional cellular debris. Furthermore, having multiple options and vendors for production of an enzyme helps secure the supply chain for commercial manufacturing. The ideal scenario is to be able to apply an evolved in-house enzyme for the desired processes in which the IP is entirely owned by the company and, therefore, there is the most freedom-to-operate for choosing enzyme production and synthetic implementation.

It is clear that in most cases a combination of approaches is required for successful realization of efficient biocatalytic reactions on scale, ranging from expertise in protein engineering through process chemistry and chemical engineering. It also emphasizes the need for a truly multidisciplinary group of researchers, interacting on the interfaces of molecular biology, chemistry, and engineering in order to develop and deliver these processes in the ever-accelerating time frame of pharmaceutical development.

FUTURE OUTLOOK

The sophistication of enzyme evolution continues to grow with exciting opportunities for machine-learning directed evolution as well as diversifying enzyme libraries through approaches such as ancestral sequence reconstruction.[84] The speed of enzyme engineering remains a bottleneck for development, and therefore, efforts to improve high-throughput platforms such as microfluidic workflows[85] and accelerated screening protocols[86] have the potential to make game-changing leaps in how biocatalysis development is performed. Furthermore, as well as wild-type enzyme sequences found in nature, exciting advances continue to be made in *de novo* designed proteins[87,88] and enzymes that incorporate noncanonical amino acids[89,90] which could further expand the suite of biocatalysts available as starting points for evolution.

As the field evolves and the scope of biocatalysis in the pharmaceutical sector becomes more ambitious, the trend toward more complex multienzymatic syntheses is also apparent. Over the past few years there has been significant growth in computational resources for biocatalytic retrosynthesis[91−93] and cascade design such as RetroBioCat[94] from the Turner and Flitsch groups[95] and a similarity-based enzymatic retrosynthesis tool from the Jensen group.[96] Computer-aided synthesis tools such as these continue to help broaden the uptake of biocatalysis across the synthetic chemistry community and can assist in the construction of single and multistep biocatalytic transformations for complex molecules.

There is also great promise for the successful combination of flow chemistry and biocatalysis,[97] but it has yet to be widely implemented in the pharmaceutical industry because of the challenges associated with heterogeneous reactions that areoften associated with biocatalysis and dealing with enzymes that have cofactor recycling requirements. As a result, there still remains huge potential for growth in the flow-biocatalysis arena.[98]

As a result of significant efforts in late-stage development, the number of off-the-shelf, commercially available enzymes for early process development continues to expand, providing more options for rapid synthetic enablement.[99] Furthermore, improvements being made in accelerating enzyme evolution have the potential to expand the reach and impact of biocatalysis in the early development space where timelines are increasingly tight.

Looking ahead to the continued growth of biocatalysis implementation in the pharmaceutical industry, certain gaps in the toolbox remain. For example, the synthesis of amide bonds which are present in many pharmaceutical drugs is often achieved with stoichiometric and potentially toxic coupling reagents. Advances are being made in the use of ligases for these transformations,[100,101] as well as engineered carboxylic acid reductases (CARs);[102,103] however, to avoid the use of stoichiometric ATP in these reactions, more robust methods for ATP recycling are still required. Excellent headway has been made in this area[104−106] and will surely continue to develop as ATP-dependent reactions become more mainstream. Another area of growth is that of enzymatic halogenation, which has great potential for pharmaceutical synthesis.[107−111] Implementable enzymatic fluorination remains elusive yet would be a highly valuable addition to the toolbox,[112] especially as fluorine incorporation has become more widespread in medicinal chemistry. Finally, there are still limited options for general C−C bond forming biocatalytic transformations without major substrate limitations or handles. Opening up this capability could have significant impact across the breadth of enzyme implementation in industry. The biocatalysis community is currently addressing these challenges with advances in convergent biocatalysis[113] and strategies such as photobiocatalysis,[114−116] providing promise for the realization of these technologies on the manufacturing scale.

SUMMARY

Continued breakthroughs and advances in enzyme discovery and protein engineering have the potential to unlock new chemistries and enable even greater numbers of biocatalytic transformations available to the synthetic chemist. The role of biocatalysis in the pharmaceutical industry looks poised for exciting growth in scope and impact for many years to come.

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Notes

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■ ABBREVIATIONS

AcK, acetate kinase; AK, adenylated kinase; AMP, adenosine monophosphate; ATA, amine transaminase; ATP, adenosine triphosphate; BVMO, Bayer-Villiger monooxygenase; cGAS, cyclic GMP-AMP synthase; DERA, deoxyribose-phosphate aldolase; DMSO, dimethylsulfoxide; EH, epoxide hydrolase; Enzyme^{Comm}, commercial enzyme; Enzyme^{Eng}, engineered enzyme; Enzyme^{WT}, wild-type enzyme; Fmoc, fluorenylmethyloxycarbonyl; GDH, glucose dehydrogenase; GK, guanylate kinase; GMP, guanosine monophosphate; GOase, galactose oxidase; IRED, imine reductase; KP_i, potassium phosphate; KRED, ketoreductase; MTBE, methyl tert-butyl ether; MTR, 5-*S*-methylthioribose; NAD(P)+, nicotinamide adenine dinucleotide (phosphate); NOx, NADH-oxidase; PAL, phenylalanine ammonia lyase; PanK, pantothenate kinase; PDH, phosphite dehydrogenase; PH, proline hydroxylase; PLP, pyridoxal phosphate; PMI, process mass intensity; PNP, purine nucleoside phosphorylase; PO, pyruvate oxidase; PPM, phosphopentamutase; SP, sucrose phosphorylase; TPGS, tocopheryl polyethylene glycol succinate; TPP, thiamine pyrophosphate; UP, uridine phosphorylase

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