



Catalyzing the future: recent advances in chemical synthesis using enzymes

Julia C. Reisenbauer¹, Kathleen M. Sicinski¹ and Frances H. Arnold

Biocatalysis has the potential to address the need for more sustainable organic synthesis routes. Protein engineering can tune enzymes to perform in cascade reactions and for efficient synthesis of enantiomerically enriched compounds, using both natural and new-to-nature reaction pathways. This review highlights recent achievements in biocatalysis, especially the development of novel enzymatic syntheses to access versatile small molecule intermediates and complex biomolecules. Biocatalytic strategies for the degradation of persistent pollutants and approaches for biomass valorization are also discussed. The transition of chemical synthesis to a greener future will be accelerated by implementing enzymes and engineering them for high performance and new activities.

Addresses

Division of Chemistry and Chemical Engineering, 210-41, California Institute of Technology, 1200 East California Blvd, Pasadena, CA 91125, United States

Corresponding author: Arnold, Frances H. (frances@cheme.caltech.edu)

¹ These authors contributed equally.

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Introduction

Biocatalytic transformations are becoming an essential part of the synthetic chemist's armamentarium, and these greener and more sustainable catalysts are transforming chemical process landscapes [1,2]. Enzymes can produce valuable compounds with high yields and exquisite selectivities, providing cost benefits compared to traditional organic synthesis and reducing waste production. The tunability and evolvability of biocatalysts, using protein engineering techniques such as directed evolution, mean that efficient catalysts can be generated for key synthetic steps [3,4]. The chemical

transformations catalyzed by enzymes are not limited to those found in nature, and thus exciting new trajectories such as strategies for the degradation of persistent pollutants and polymers as well as the atom-efficient synthesis of complex biomolecules are enabled. Here we highlight some of the latest advancements in biocatalysis, focusing on unique attributes that have been harnessed in industry and academic research (Fig. 1). We aim to showcase the ever-increasing opportunities that enzymes offer in devising solutions to long-standing challenges in organic synthesis.

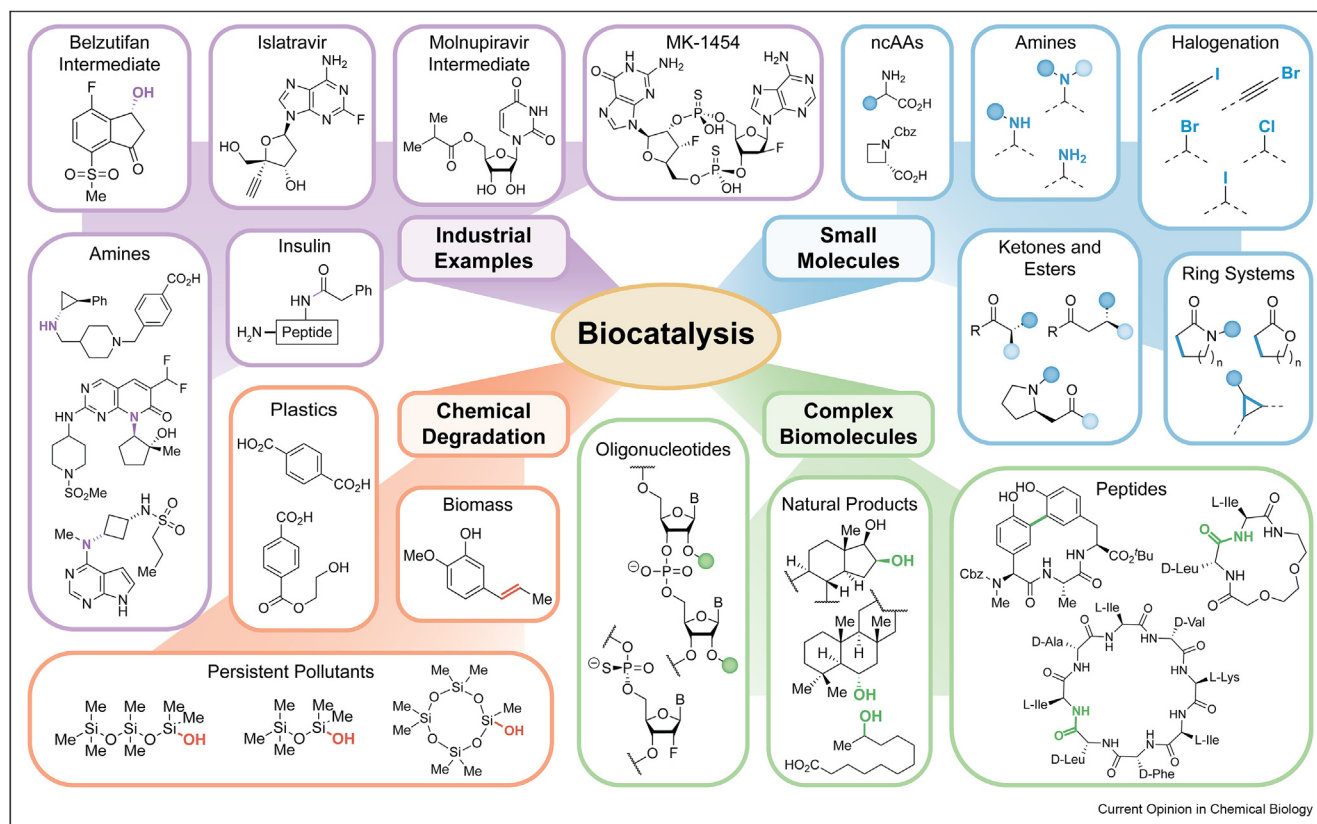
Application of biocatalysts in the pharmaceutical industry

The pharmaceutical industry has recognized the valuable role enzymes can play in manufacturing bioactive molecules, leveraging their advantages to circumvent obstacles encountered in traditional synthetic processes. This appreciation stems primarily from enzymes' remarkably high chemo-, regio-, enantio-, and substrate selectivities, which can enable multiple enzymes to work together in the same pot ('cascade' reactions) and make complex molecules with few side products, much as they do inside living cells. In this section, we will highlight recent examples that demonstrate the power of biocatalysts for the synthesis of enantioenriched building blocks, target molecules, or active pharmaceutical ingredients (APIs).

Biocatalysts confer high regio- and enantioselectivity

Enzyme properties can be tuned for industrial applications by engineering the protein sequence. Recent work by Merck researchers addressed challenges associated with α -ketoglutarate-dependent dioxygenases (α -KGD) on manufacturing scale, including low total turnover number (TTN), aerobic reaction conditions, low stability, enzyme inactivation by self-hydroxylation, and overoxidation of unnatural substrates. An engineered α -KGD replaced five synthetic steps with a direct enzymatic hydroxylation to produce chiral intermediate **2** from **1** used in the synthesis of belzutifan in high enantioselectivity and preparative yield (Fig. 2a) [*5]. Compared to heme-dependent oxygenases, α -KGDs require only iron in combination with α -ketoglutarate and do not necessitate complex cofactors or co-expression of reductase domains. Enzymes' high selectivity also enables them to target specific

Figure 1



Examples of chemoenzymatic approaches that pave the way to selectively construct synthetic targets in the pharmaceutical industry as well as in the context of small molecule and complex biomolecule synthesis. In addition to biosynthesis, biocatalysts provide new strategies for pollutant degradation and biomass valorization. ncAAs = noncanonical amino acids.

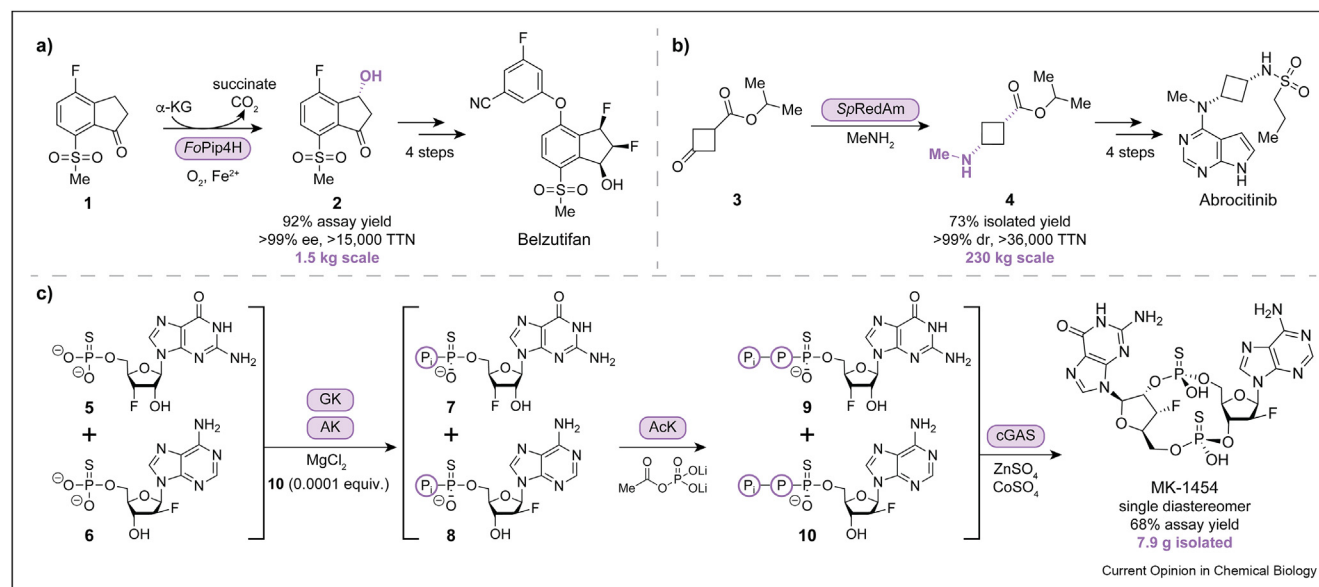
functional groups among others, and this selectivity can be engineered by directed evolution. Fryszkowska and co-workers engineered acylases for improved bioconjugation of insulin (consisting of both α - and β -peptides covalently bound by disulfide bonds). This facilitated the selective acylation of an internal or terminal amine, as well as selective hydrolysis of the phenylacetyl group [6].

Large scale production of APIs

Imine reductases (IREDs) and reductive aminases (RedAms) are well known for their scalability and have been successfully applied on ton scale for the synthesis of chiral amines from ketones [7]. Researchers at GlaxoSmithKline (GSK) engineered an IRED to perform a stereoselective reductive amination via kinetic resolution of a racemic mixture of *trans*-phenylcyclopropylamine (>38,000-fold greater TTN compared to the wild-type enzyme), reducing the generated waste by half — process mass intensity (PMI) improved from 355 to 178 [8]. Researchers at Pfizer improved on a prior chemoenzymatic synthesis of *cis*-cyclobutyl-*N*-methylamine intermediate **4**. Initially, a carbonyl-containing substrate was converted into the corresponding amine with a

transaminase, followed by chemical alkylation of the primary amine with iodomethane. Augmenting this preliminary process, they combined transamination and alkylation into a single enzyme-catalyzed reductive amination with methyl amine and a RedAm to selectively form the *cis* aminated cyclobutane **4** in 73% isolated yield from the corresponding carbonyl **3**, a >200-fold increase compared to the wild-type enzyme (Fig. 2b) [*9]. This step was optimized for large scale to afford 230 kg of the product. Cumulative batch processes generated >3.5 megatons of chiral intermediate **4** as the succinate salt for the synthesis of abrocitinib. Another RedAm was used to simplify production of an enantioenriched intermediate by directly installing a protected benzylamine on kilogram scale, with a substrate loading of 50 g/L, 98% *ee*, and 43% conversion [10,11]. These examples showcase the utility of IREDs and RedAms for chemical manufacturing on ton scale, due to their inherent stability, high activities, and broad applicability for installing amine functional groups. Future efforts need to focus on identifying and developing more enzyme families capable of performing useful transformations on industrial scale.

Figure 2



Examples of biocatalysis used to produce APIs. **(a)** Stereoselective hydroxylation of **1** with α -KGD FoPip4H (2 g/L) to generate enantioenriched intermediate **2** for the production of **belzutifan** [5]. **(b)** Biocatalytic reductive amination of ketone **3** catalyzed by SpRedAm (1.9 g/L) for the synthesis of enantioenriched amine building block **4** on 230 kg scale for the synthesis of **abrocitinib** [9]. **(c)** An enzymatic cascade to access **MK-1454** uses four enzymes and circumvents intermediate purification steps [14]. In one pot, guanylate kinase (GK, 0.1 g/L) and adenylate kinase (AK, 0.1 g/L) phosphorylate **5** and **6**, respectively to yield intermediates **7** and **8** which are then phosphorylated by the same acetate kinase (AcK, 75 mg/L). After subsequent addition of cyclic guanosine-adenosine synthase (cGAS, 2.2 g/L), the final cyclization between **9** and **10** yields **MK-1454**. $\text{P} = -\text{PO}_3^-$, $\text{P}_i = -\text{PO}_4^{2-}$.

One-pot enzymatic cascades

The high chemoselectivity of biocatalysts can suppress unproductive side reactions and allow multiple reactions to occur in a single pot. This typically results in a process with high atom economy, good step efficiency, limited waste generation, and no need for purification between chemical steps. Examples from Merck include extraordinary enzymatic systems for synthesis of APIs such as complex cyclic dinucleotides and nucleosides. Impressive demonstrations of enzyme cascade syntheses of nucleoside analogues **islatravir** and **molnupiravir** have generated the excitement and interest biocatalysis deserves [12,13]. Another example is the cascade synthesis of a stimulator of interferon genes (STING) protein activator, **MK-1454**, currently in clinical trials [14]. The original synthesis of this cyclic dinucleotide required nine synthetic steps — Merck researchers streamlined the synthesis into three concatenated biocatalytic reactions. The cascade consisted of two enzymatic phosphorylation events requiring three engineered kinases to produce activated thiotriphosphorylated nucleotides **9** and **10** (Fig. 2c). The final step involved an engineered cyclic guanosine-adenosine synthase (cGAS) and a bimetallic system (Zn^{2+} and Co^{2+}) necessary for the stereocontrolled cyclization of **9** and **10** to produce **MK-1454**. With these reported enzymatic cascades, API production was achieved in fewer steps with less waste generated during

synthesis and purification, improving the PMI. Protein engineering enabled inefficient, naturally occurring enzymes to become competent and robust catalysts with improved activity and diastereoselectivity.

Frontiers of biocatalysis for small molecule synthesis

Academic research groups are expanding the repertoire of biocatalysis by discovering novel enzyme-catalyzed natural product syntheses and developing non-natural transformations using engineered enzymes. Especially, new chiral synthetic intermediates have been accessed efficiently and stereoselectively using biocatalytic strategies. These novel and creative reaction designs are primed to be useful resources to consider in developing biocatalytic routes that produce fine chemicals and pharmaceuticals, after further improvement of process relevant properties such as enzyme stability, activity, and selectivity at scale.

Amination strategies to access important synthetic targets

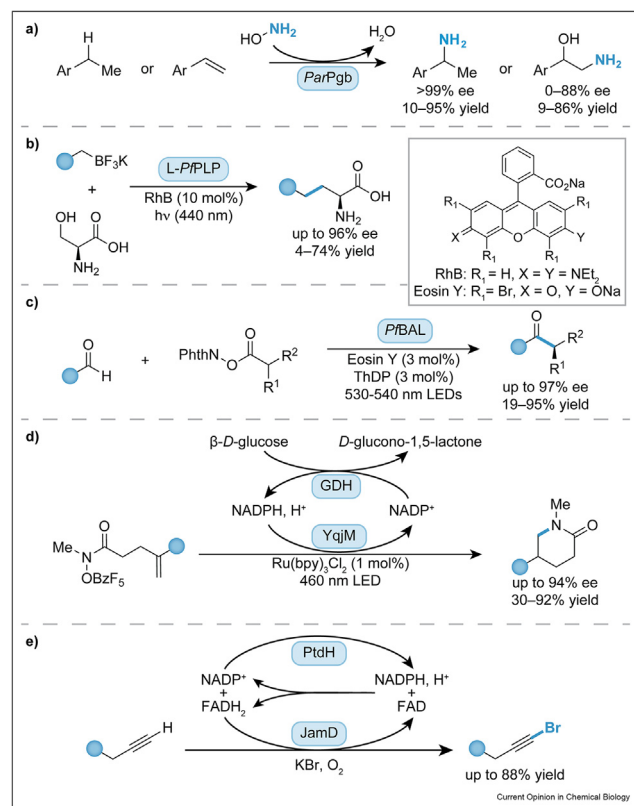
The efficient and stereoselective introduction of amine functional handles into target molecules is a fundamental synthetic task, given their importance in bioactive molecules. New-to-nature heme-containing enzymes have been shown to achieve direct amination by catalyzing C–H bond and alkene functionalization

reactions. Engineered *Pyrobaculum arsenaticum* protoglobins (ParPgb) variants can use inexpensive hydroxylamine hydrochloride ($\text{NH}_2\text{OH}\cdot\text{HCl}$) as a nitrene precursor, generating water as the sole byproduct (Fig. 3a) [15]. In the directed evolution campaign to develop these new enzymes, it was observed that the K_M values for hydroxylamine decreased (5.4 mM–0.30 mM), suggesting higher affinity of the aminating reagent with the heme enzyme resulting in improved activity (at low substrate concentrations). Also, an increase in the turnover number (k_{cat}) by 180-fold from the initial variant improved overall yield. The ability of enzymes to utilize hydroxylamine as an aminating reagent was then expanded to the biocatalytic conversion of boronic acids into the corresponding amines [16]. As another example, Zhao and coworkers

engineered flavin mononucleotide (FMN)-dependent ene-reductases to use N–O based reagents as amine precursors in the enantioselective hydroamination of alkenes under visible light [17]. The authors indicate that further directed evolution of the biocatalyst is required to improve on the low enzyme activity and expand the substrate scope.

Gathering mechanistic insights can help address the need for robust and novel biocatalytic processes. Hilvert and coworkers investigated a myoglobin model system to uncover the cause for the unproductive reduction of nitrenes generated from azide precursors [18]. Based on their findings, more competent heme-based nitrene transfer biocatalysts could be developed by fine-tuning the reduction potential of the enzyme, which in turn suppressed unproductive reduction pathways.

Figure 3



Biocatalytic transformations for the construction of small, versatile building blocks. (a) *Pyrobaculum arsenaticum* protoglobin (ParPgb) catalyzed amination of benzylic C–H bonds and alkenes using hydroxylamine as aminating reagent [15]. (b) A combined photoredox-PLP catalytic system for the construction of ncAAs relying on *Pyrococcus furiosus* tryptophan synthase β subunit (L-P/PLP) [22]. (c) Synthesis of α -chiral ketones relying on a dual organophotoredox and ThDP-dependent *Pseudomonas fluorescens* benzaldehyde lyase (PbAL) [30]. (d) Intramolecular hydroamination for the formation of lactams using *Bacillus subtilis* flavin-dependent ene-reductase (YqjM) [34]. (e) Bromination of terminal alkynes using *Moorena producens* flavin-dependent halogenase (JamD) [38]. GDH = glucose dehydrogenase, PtdH = phosphite dehydrogenase.

The selective introduction of azide groups as masked primary amine precursors presents another strategy to access nitrogen-decorated scaffolds. In this context, Huang and coworkers utilized a nonheme iron enzyme for benzylic azidation implementing sodium azide as the external azide source [19]. While all these examples showcase interesting directions for the design of novel synthetic routes, further protein engineering efforts to improve biocatalyst properties, including TTN and stability under process conditions, are necessary to facilitate their use at scale.

Emerging strategies for the efficient synthesis of non-canonical amino acids

Key pillars in modern small-molecule pharmaceuticals and peptidomimetics are finding and improving therapeutic targets by the incorporation of noncanonical amino acids (ncAAs) or isotopically labeled derivatives [20]. Selective access to C α and/or C β deuterated amino acids was achieved with a pyridoxal 5'-phosphate (PLP)-dependent two-enzyme system (DasD, DasE), providing labeled enantiopure amino acids on analytic and semi-preparative scales (>600 mg deuterated Ile, 0.5 mmol) [21]. Relying also on PLP-dependent enzymes, a synergistic photoredox-PLP biocatalytic approach was introduced by Yang and coworkers to facilitate the construction of ncAAs (Fig. 3b) [22]. In this reaction, photocatalytically generated radicals engage in a stereoselective C–C bond forming reaction with a covalently bound substrate-enzyme intermediate. Extension of their work to glycine and α -branched amino acids substrates enabled the enantioselective synthesis of α -tri and tetrasubstituted ncAAs exhibiting up to two contiguous stereocenters [23]. Related work by Hyster and coworkers also demonstrated the utility of engineered threonine aldolases as a platform to synthetically access α -tertiary amino acids using pyridinium salts as alkylating agents [24]. In a different study, Arnold and coworkers achieved enantioselective

synthesis of azetidine products, bioisosteres of proline, using an engineered P450 variant with an axial serine substitution, classified as P411 enzymes, to mediate a one-carbon ring expansion of aziridines [25]. To further expand the set of chemically available proline bioisosteres, Renata and coworkers reported the construction of five- to eight-membered cyclic ncAAs based on a two-step chemoenzymatic cascade relying on a transaminase-mediated cyclization followed by a stereocontrolled PtO_2 hydrogenation [26]. Again, using these catalysts to make other ncAAs for industrial purposes will require further enzyme engineering.

Synthesis and functionalization of carbonyl intermediates

Several enzyme classes have been explored to expand the toolkit of organic transformations that can access and functionalize carbonyl-containing substrates and products. Flavin-dependent ene-reductases could be harnessed for the challenging chemo- and enantioselective $\text{C}(\text{sp}^3)\text{--C}(\text{sp}^3)$ cross coupling of alkyl halides and nitroalkenes to access derivatized ketone products [27]. This reaction is proposed to proceed via a quaternary charge transfer complex in the presence of all reaction partners in the FMN active site. The same enzyme class was also utilized in the desymmetrization of enones to access enantioenriched cyclohexanones from either cyclohexanone or cyclohexadienone substrates [28]. Recently, engineered cytochrome P450s were further improved to enable $\alpha\text{-C--H}$ functionalization of cyclic amines via a carbene transfer reaction using diazo precursors on gram scales to attach carbonyl functional handles [29]. Also, a dual system comprised of a thiamine diphosphate (ThDP)-dependent radical acyl transferase and an organophotoredox catalyst facilitated the synthesis of α -chiral ketones (Fig. 3c) [*30]. The reaction is proposed to proceed via the formation of a Breslow intermediate by the condensation of the aldehyde with the ThDP cofactor. This intermediate is prone to oxidation and subsequent radical rebound with the alkyl radical to generate the carbonyl product. While these examples represent exciting new directions of implementing new-to-nature enzyme-catalyzed transformations, scale-up challenges associated with photochemical processes must be overcome to enable efficient application at scale.

Construction of ring systems through C–C bond formation

The synthesis of cyclic compounds via carbon-carbon bond formation represents a fundamental strategy to introduce complexity to molecular scaffolds. Lactones and lactams were accessed by P411 enzymes via an intramolecular carbene insertion strategy and by B12- and heme-dependent enzymes via a radical cyclization mechanism of tertiary alkyl halides with alkenes or arenes [31–33]. Hyster and coworkers reported a

hydroamination reaction using ene-reductases to access a variety of enantiopure lactams and amides via a synergistic photo- and biocatalytic strategy (Fig. 3d) [*34]. High enantioselectivities are obtained by generation and subsequent coupling of the reactive amidyl radical with the alkene in the protein active site. Besides lactones and lactams, the efficient and stereoselective construction of small carbo-cyclic scaffolds is also synthetically relevant. In most cases, relatively reactive carbene precursors (e.g. diazoacetate) are necessary to enable efficient construction of cyclopropane rings from alkenes [35]. On larger scales, the implementation of more stable carbene precursors for transfer reactions may be attractive due to their improved safety profiles. Arnold and coworkers reported the use of diazirine reagents in biocatalytic cyclopropanations as well as N–H, B–H, and Si–H insertion reactions [36]. Together with further improvement of enzyme stability and activities at high substrate and product concentrations, these developments increase the potential for ‘carbene transferases’ to be established in safe and sustainable industrial processes.

Halogenations for the installation of functional handles

Derivatization of molecular scaffolds by the installation of halogen atoms is an important strategy to increase complexity and introduce functional groups. Lewis and coworkers demonstrated that the bromination and iodination of several arenes and an alkene can be catalyzed by a flavin-dependent halogenase AetF [37]. In a study by Lukowski and coworkers, another flavin-dependent halogenase, JamD, was identified to catalyze chemoselective bromination and iodination of terminal alkynes over electron-rich arenes (Fig. 3e) [38]. Impressively, the naturally occurring enzyme was shown to exhibit a broad substrate scope, affording simple and late-stage haloalkynes. The intramolecular construction of benzylic $\text{C}(\text{sp}^3)\text{--F}$ bonds using *N*-fluoroamide substrates was enabled by repurposing a non-heme iron epoxidase from *Streptomyces viridochromogenes* (SvHppE) [39]. In a different study, a native α -KGD hydroxylase was engineered to perform selective halogenation over hydroxylation of $\text{C}(\text{sp}^3)\text{--H}$ bonds in lysine [40]. Even though a variety of naturally occurring and engineered halogenases have been identified to perform useful transformations on aromatic scaffolds, enzymatic halogenation of unactivated C–H bonds is still in its infancy, with further biocatalyst engineering required in many cases.

Biocatalysts for streamlined synthesis of complex biomolecules

Synthesizing complex biomolecules often requires preactivated substrates and orthogonal protecting groups. These approaches generate waste and necessitate chromatographic purification of target compounds. Enzymes can circumvent these disadvantages by

specific substrate orientation and thus selectively reacting with a single functional group among others. This molecular precision results in fewer synthetic steps, increased atom efficiency, and more sustainable processes. Below we highlight strategies that use enzymes for the synthesis of complex biomolecules such as natural products, peptides, and oligonucleotides.

Late-stage oxidation of natural products

Natural product synthesis is a complex, multistep process usually requiring the manipulation of protecting groups. Biocatalysts provide a useful alternative to enable regio- and stereoselective control in the presence of bare functional groups (Fig. 1, Natural Products). Examples of late-stage modifications of natural products are inspired by native pathways, utilizing P450s or other oxygenases for oxidation of steroids and fatty acids. Alcalde and coworkers evolved a fungal peroxxygenase to selectively hydroxylate fatty acids at the ω -1 position [41]. A single mutation caused a narrowing of the active site, resulting in tighter binding of the fatty acid substrate and allowing for targeted hydroxylation. Similarly, a steroid hydroxylase, CYP109B4, was discovered through genome mining to oxidize steroids at the C16 position. This regioselectivity was then altered using directed evolution to instead precisely hydroxylate at C15 [42]. In another example, Renata and coworkers streamlined the total synthesis of ansellone B by discovering and engineering a P450 monooxygenase capable of hydroxylating the C6 position of sclareol with six-fold improved catalytic activity compared to their initial variant [43].

Cyclization of peptides

Cyclization of peptides is synthetically challenging because intramolecular reactions must compete with intermolecular couplings, and unprotected amino acid side chains can lead to undesired side reactions (Fig. 1, Peptides). In native systems, nonribosomal peptide (NRP) synthetases utilize thioester-linked peptides for cyclization. Wakimoto and coworkers designed an alternative leaving group, ethylene glycol, which could be easily installed on the resin support during solid-phase peptide synthesis [44]. Using this strategy, they discovered a wild type NRP cyclase (SurE) capable of selective head-to-tail cyclization of peptides containing *N*-terminal L-isoleucine and *C*-terminal D-leucine. After protein engineering, a SurE variant cyclized peptides without the terminal amino acid requirements, illustrating the evolvability of SurE. Another cyclization strategy, biaryl cross coupling with tyrosine side chains, was investigated by a group at Genentech [45] inspired by a complementary example for naphthol cross coupling by the Narayan lab [46]. These few examples begin to set the stage for new and selective late-stage peptide modifications without the need for noncanonical amino acid incorporation.

Chemoenzymatic oligonucleotide synthesis

Modified nucleic acid therapeutics are a class of molecules traditionally synthesized by solid-phase phosphoramidite-based synthesis. This process requires densely protected monomers, generates significant waste during synthesis and purification, and yield deteriorates as oligo length increases. The Lovelock lab discovered a cascade of native enzymes that allows for polymerization of oligonucleotides and incorporation of unnatural monomers [*47]. Even without protein engineering, the scope of the native polymerases supports many noncanonical oligonucleotide building blocks (thiophosphate, locked, and 2'-modified nucleotides) and could serve as starting points for future directed evolution campaigns (Fig. 1, Oligonucleotides), similar to what was achieved for incorporating 2'-*O*-(2-methoxyethyl) nucleic acids in oligo synthesis [48]. To date, most oligonucleotide syntheses require a template strand for polymerization, and ways to incorporate unnatural nucleotide monomers remain to be explored.

Pollutant degradation and biomass valorization using biocatalysts

Advances in biocatalysis also include using enzymes to break down environmentally persistent compounds and polymers. Man-made volatile methyl siloxanes (VMS) (Fig. 1, Persistent Pollutants) used in consumer products such as detergents and cosmetics are produced on megaton scale and are not naturally degraded by microorganisms. Arnold and coworkers engineered a cytochrome P450 to oxidize terminal carbons in selected VMS, leading to Si–C bond cleavage via the carbinol intermediates. Enzymatic cleavage of this critical bond hints that biodegradation of siloxanes may one day be possible [*49]. Depolymerization of polyethylene terephthalate (PET) has been achieved using an engineered thermostable hydrolase capable of catalysis at the glass transition temperature of PET [50] (Fig. 1, Plastics). A major component of biomass, lignin can be depolymerized by chemical strategies, but feasible methods to convert those degradation products into valuable chemicals are needed. An engineered eugenol oxidase (EUGO) converts the most abundant lignin monomer, 4-*n*-propylguaiaicol, into isoeugenol, a commodity chemical used for industrial production of fragrances, flavors, and polymers [51] (Fig. 1, Biomass).

Outlook

Biocatalytic strategies are increasingly recognized as attractive, sustainable alternatives to traditional organic chemistry approaches. Enzymes offer new solutions to synthetic challenges, enabling access to small, enantioenriched intermediates and complex biomolecules. Protein engineering efforts have facilitated the development of simplified synthetic routes that implement resilient, highly selective enzymes in cascade reactions. Beyond transforming the organic chemistry landscape,

degradation of persistent pollutants and polymers have also been addressed with enzymes. Most of the biocatalysts described in recent reports, however, are of limited applicability. General shortcomings such as enzyme robustness, substrate scope, ability to work with high concentrations of substrate and product, and reaction yield need to be improved to successfully implement biocatalysis in industrial manufacturing campaigns [52]. The feasibility of this approach is amply illustrated by the examples reviewed here. Performing directed evolution more efficiently in combination with process engineering to streamline scale up will accelerate the implementation of biocatalysis. In general, the field will advance by exploiting new technologies, such as assay automation and machine learning-assisted directed evolution [53], all pointing toward the goal of making these processes more accessible, cost-effective, and easier to implement.

Declaration of competing interest

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Data availability

No data was used for the research described in the article.

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