

Power of Biocatalysis for Organic Synthesis

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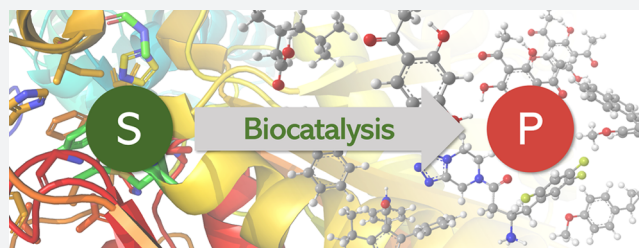
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ABSTRACT: Biocatalysis, using defined enzymes for organic transformations, has become a common tool in organic synthesis, which is also frequently applied in industry. The generally high activity and outstanding stereo-, regio-, and chemoselectivity observed in many biotransformations are the result of a precise control of the reaction in the active site of the biocatalyst. This control is achieved by exact positioning of the reagents relative to each other in a fine-tuned 3D environment, by specific activating interactions between reagents and the protein, and by subtle movements of the catalyst. Enzyme engineering enables one to adapt the catalyst to the desired reaction and process. A well-filled biocatalytic toolbox is ready to be used for various reactions. Providing nonnatural reagents and conditions and evolving biocatalysts enables one to play with the myriad of options for creating novel transformations and thereby opening new, short pathways to desired target molecules. Combining several biocatalysts in one pot to perform several reactions concurrently increases the efficiency of biocatalysis even further.



■ NEED FOR A BROAD TOOLBOX OF (CHIRAL) CATALYSTS FOR ORGANIC SYNTHESIS

Over almost two centuries, starting with Friedrich Wöhler,^{1,2} researchers have developed synthetic methods to prepare various natural products and derivatives thereof as well as completely new molecules.³ Consequently, organic synthesis has become a highly powerful art providing versatile protocols to prepare molecules of and for our life.^{4–14} Thus organic synthesis provides access to chemical compounds that cover our daily needs with respect to virtually every aspect of modern life, including clothing, hygiene, nutrition, transport, housing, and health.

Without doubt, organic synthesis has a major impact on our standard of living. Nevertheless, the economic, environmental, and social concerns of our society still urge chemistry to make synthetic routes even more efficient, more easily scalable, and more cost-effective. Ideally, chemical products should be accessible and affordable for all human beings, and their manufacture should allow the current state of the environment to be preserved or even improved. This goes in hand with and is part of the 12 principles of “Green Chemistry”.^{15–17}

Just recently, in 2018, K. C. Nicolaou requested improvements for organic synthesis, envisioning nature’s efficiency as an ideal goal:

“It is also important to re-emphasize that organic synthesis still has a long way to go before it can be considered competitive with biosynthesis, Nature’s way of making molecules. The standards of Nature (i. e., close to 100 % yield, little if any waste, ambient temperature, aqueous media) should be those we must seek in our drive to perfect our synthetic capabilities in order to enable the myriad of imaginable and unimaginable applications of organic synthesis of today and tomorrow.”¹³

Actually, in the “Organic Chemist’s Ode”, the power of nature’s synthesis sounds unreachable:

“Lord, I fall upon my knees / And pray that all my syntheses / May no longer be inferior / To those conducted by bacteria”¹⁸

We know that the natural catalysts, the enzymes present in all living beings, are responsible for these reactions. If these enzymes are so efficient, then is it not obvious that we should utilize them in organic synthesis? Why try to compete if there is the option to use them, learn how their mechanistic machinery works, and consequently tune and exploit them for different substrates? This is, of course, not a new idea, and about the last 25 years have initiated a slow change of paradigm in synthetic chemistry to incorporate some selected enzymes in the

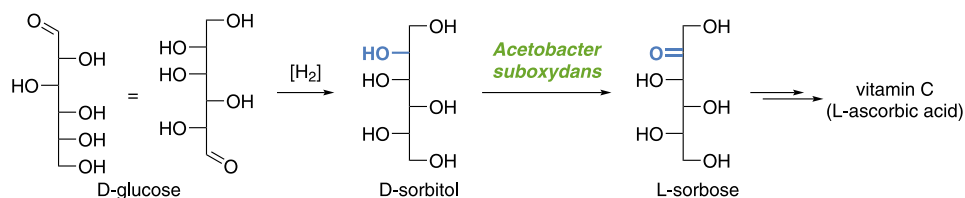
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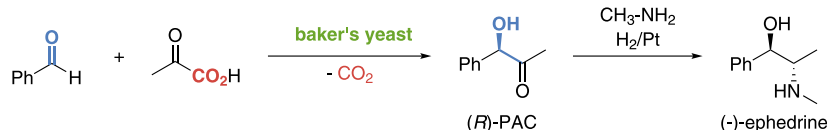


Scheme 1. Early Examples of Enzyme-Catalyzed Biotransformations (Using the Wild-Type Whole-Cell Organisms) as Part of a Longer Synthetic Route for Industrial Chemical Production^a

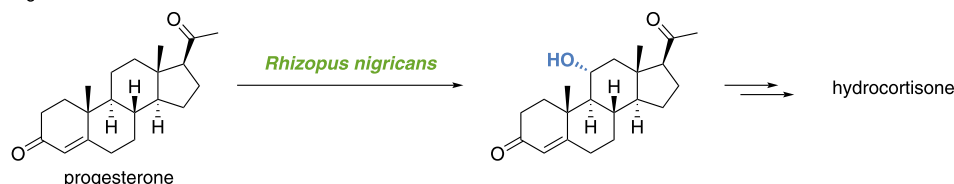
(a) Regioselective oxidation



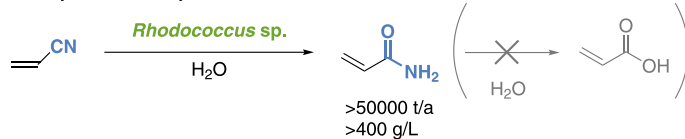
(b) Stereoselective benzoin reaction



(c) Regio- and stereoselective C-H oxidation



(d) Monohydration of acrylonitrile



^a(a) Vitamin C, (b) (–)-ephedrine, (c) hydrocortisone, and (d) acrylamide.

repertoire of catalysis. Enzymes, made up of a chain of L- α -amino acids, may be considered as huge organocatalysts if there is no metal involved or as organometallic catalysts with a huge ligand in case a metal is involved. The enzymes used today in synthesis are typically composed of a peptide chain of 200–600 amino acids, consequently possessing a molecular weight between 20 and 60 kDa. If metals are present, then these usually have a high natural abundance and low toxicity, such as iron, copper, manganese, zinc, magnesium, cobalt, or molybdenum.¹⁹

The outstanding properties of nature's catalysts were already exploited in chemical synthetic routes about 100 years ago, at a time when nothing was known about the structure of these catalysts or even their amino acid sequence. At this time, the whole (living) organism was employed as a black box catalyst for a single chemical step without knowing what was going on inside. For instance, a bacterium named *Acetobacter suboxydans* was used for the regio- and chemoselective oxidation of a single *sec*-alcohol moiety in D-sorbitol in the neighborhood of three more *sec*-OHs and two primary OHs to give the corresponding ketone, L-sorbose (Scheme 1a).^{20–22} This microbial oxidation step is part of a reaction sequence involving several chemical steps starting from D-glucose and leading to vitamin C. This overall process is basically still used today on an industrial scale, albeit with some improvements.

A stereoselective benzoin reaction has been the asymmetric key step in the industrial synthesis of (–)-ephedrine since 1921 until today, originally performed using living cells of baker's yeast (Scheme 1b).^{23,24} Thereby, a new C–C bond is formed

between benzaldehyde and decarboxylated pyruvate, giving (R)-phenylacetyl carbinol ((R)-PAC) as the key intermediate.

A regio- and stereoselective C–H oxidation accomplished by a fungus (e.g., *Rhizopus*; Scheme 1c)²⁵ is part of the large-scale synthesis of hydrocortisone, an important anti-inflammatory agent for the treatment of debilitating diseases including rheumatoid arthritis. These three examples nicely illustrate the exquisite regio-, chemo-, and stereoselectivity of enzymes. Just to also show an example for a bulk chemical, the production of acrylamide by the monohydration of acrylonitrile on a > 50 000 tons per year scale is one of the most impressive industrial applications of enzymes, reaching the productivity levels of heterogeneous catalysts (Scheme 1d; space–time yields 53–93 g L^{–1} h^{–1}).^{26,27} The advantage of the last reaction over the chemical alternative is its high chemoselectivity, exclusively giving the amide, as well as the easier separation of the catalyst from the product compared with metal catalysts. Various other examples throughout the history of biocatalysis have been reviewed.^{28,29}

■ ENZYMES AS AN EXTENSION OF THE TOOLBOX OF CATALYSTS

Transformations using living bacterial or fungal strains may have had the flavor of a nonscientific method for an organic chemist, who would have wanted to precisely describe the mechanism of the reaction and wanted to know the molecular structure of the catalyst. Fortunately, the specific enzymes responsible for catalyzing the reactions above have been identified in the meantime. Thus light was brought into the darkness of the black

Table 1. Individual Steps in an Enzyme Catalytic Cycle

step	step	chemistry	purpose
i	binding of substrate(s)	Binding may be covalent but always relies on several weak interactions (≥ 3): hydrogen bonding, π - π -stacking, ionic interactions, hydrophobic interactions Enzyme backbone may adapt to a substrate structure	Precise positioning of substrates and, if required, cofactors in a productive pose to each other in 3D. The more interactions, the tighter the binding. Too tight binding has to be avoided because it may lead to substrate inhibition
ii	activation of substrate(s)	Activation may be achieved by acid–base catalysis, metals, cofactors, etc. Enzyme backbone may move during catalysis	Initiating the reaction
iii	stabilization of transition state	Various residues in the active site may provide an appropriate environment	Lowering energy for transition state
iv	product release	Lower binding affinity than for the substrate	To expel the product quickly after the reaction is important to minimize product inhibition. This is easier the more the substrate and the product differ from each other

box, and we know what the catalysts are. Over the last several decades, many enzymes in biosynthetic pathways have been identified, and their 3D structures have been solved and deposited in databases like the Protein Data Bank (PDB),³⁰ which currently contains more than 160 000 structures and grows by more than 10 000 entries every year. Thus the enzymes are available and awaiting their exploitation. Thanks to cocrystallization or soaking of the proteins with substrates/products, crystal structures were obtained, showing the binding modes of the substrate/product in the catalyst, which is rarely possible with organo- or organometallic catalysts. Such protein structures containing the substrate/product help us to understand the mechanism as well as the reactivity and stereo- and regioselectivity of enzymes. Alternatively, computational tools^{31–34} for modeling of enzyme structures and the mode of substrate binding support the elucidation of mechanisms and the understanding of these biocatalysts.

With the growing understanding of the structure and function followed by searching for applications, the need to produce the biocatalyst outside of its natural source organism arose. In this context, three practical aspects are worth mentioning: First, the preparation of the catalyst requires, in general, the machinery of the protein synthesis of microorganisms via fermentation, which ultimately consumes nutrients such as sugars and amino acids. Thus it relies on renewable resources. Second, the production of a new batch happens, in general, within a few hours, maybe up to a few days, in one process step; thus it is relatively quick in comparison with, for example, multistep syntheses of nonnatural ligands. Third, because the biocatalyst is made of amino acids, it is also naturally degradable; one might even eat it. This clearly underlines that such catalysts can be easily integrated in a circular economy.^{35,36}

The term biocatalyst is very often used, and unfortunately, this term is rather imprecise, as it is employed, for example, for a wild-type organism, a single enzyme, a crude enzyme preparation, or an enzyme (preparation) immobilized on a carrier. Most often, the term biocatalyst refers to a crude cell extract from the production strain (e.g., *E. coli*), which may contain many other enzymes that, in general, do not interfere with the intended reaction. Enzyme purification by chromatography is prohibitively expensive on a large scale, explaining the prevalence of crude preparations provided by commercial sources. However, certain production strains (e.g., *Komagataella phaffii*, previously known as *Pichia pastoris*) allow the secretion of enzymes into the aqueous medium in which the production strain grows, making separation straightforward and leading right away to a rather pure enzyme preparation. Actually, for example, when produced in *E. coli*, even the whole cells

containing the heterologously expressed enzyme may be used as catalyst preparation. When we speak in the following about a “biocatalyst”, this refers to a single enzyme, which may be used in purified form, as a crude preparation, or immobilized on a carrier.

Consequently, the word biocatalysis is also used for many approaches. For this outlook in connection to organic synthesis, we consider biocatalysis as the use of either a single enzyme (also when provided as crude preparation) or combinations of different enzymes in one pot for the transformation of any well-defined organic compound; thus we do not deal with living organisms here. Living organisms present additional challenges for biotechnologists because they contain thousands of enzymes and countless metabolites at changing concentrations within a cell. For organic chemistry, we prefer a simpler, more defined system. Biocatalysis in this sense of the word enables the sustainable production of chemicals by catalyzing specific steps while avoiding the restrictions associated with microbial strains.³⁷

■ HIGH-TECH FEATURES OF A BIOCATALYST

In the early days of biocatalysis, it was assumed that the acceleration of the reaction rate achieved by enzymes is mainly due to the stabilization of transition states. However, this view was falsified when the performance of antibodies that possess an ideal complement structure to the transition state was clearly inferior to that of enzymes.³⁸ It turned out that efficient catalysis requires more than just transition-state stabilization. Following an enzymatic catalytic cycle in detail, one notices that in an ideal case, each step is controlled by several interactions between the substrate and the enzyme. The main steps of a general catalytic cycle are (i) binding of substrate(s), (ii) activation of substrate(s), (iii) stabilization of transition state(s), and (iv) product release (Table 1). The substrate binding relies on not just one or two interactions between the enzyme and the substrate (Table 1) but several, which are required for the precise positioning of the reaction partners to each other in 3D space in a productive pose. The interaction between the substrate and the biocatalyst at (at least) three points is also important for the differentiation between enantiomers.³⁹ One might argue that a significant contribution to the efficiency of enzymes is the controlled precise orientation of the reactants in space, in addition to the strategically ideal location of various catalytically active residues. Thus several residues usually contribute to enabling the reaction; this well-established concept in enzymes has recently been described as synergistic catalysis.⁴⁰ The residues catalyzing the reaction or binding the substrate shape the active site of the catalyst, providing the

confinement.⁴¹ Nevertheless, for an enzyme, this shape is adapting in size to the substrate; the enzyme backbone is flexible/dynamic during the catalytic cycle, adapting for ideal positions.^{42–47} Thus the conformational dynamics also influenced by allosteric regulation (i.e., tuning enzyme function by distal positions) play a crucial role in the substrate binding as well as for product release. In comparison, a heterogeneous catalyst like a zeolite will absorb a substrate so that it is completely surrounded by the rigid structure of the zeolite. An organocatalyst is more flexible but can control only a limited number of degrees of freedom of the substrate due to its smaller size. The enzyme combines the best from both, the pocket formation and flexibility, which are considered important for substrate binding and catalytic turnover.^{48,49} Furthermore, whereas a low-molecular-weight organocatalyst is, in general, not controlling the approach of the substrate to the catalytically active residue, and the zeolite is differentiating mainly by size, the flexible loops and domains of a biocatalyst gate the access of the substrate to the active site.^{45,50,51}

The biocatalyst combines the best from both (organocatalysts and, e.g., zeolites), the pocket formation and flexibility, which are considered important for substrate binding and catalytic turnover.

In Scheme 2a, the important features of an enzyme contributing to the different steps are exemplified for an enzyme enabling a C–C bond formation, namely, the acylation of resorcinol.^{52–58} The natural reaction of this acyltransferase is the disproportionation of monoacetyl-phloroglucinol (Scheme 2b);^{59,60} however, it turned out that the natural catalyst shows promiscuous activity to also accept esters (e.g., phenyl or vinyl) as acyl donors and to link the acyl group to (substituted) resorcinol via the formation of a new C–C bond. This is remarkable because O-acylation occurs under chemical catalysis. To ensure this chemoselectivity, precise binding is required.

Looking at the structure of the catalyst, one can differentiate between certain regions of the enzyme (Figure 1): Obviously, the most important one is the place where the reaction occurs,

the active site. Often, a tunnel, maybe via a gate,⁵⁰ leading to the active site preselects and preorientates the substrates.^{51,61,62} There might be several tunnels to the active site, for example, one for bigger molecules and another for smaller molecules. The active-site pocket accommodates the catalytically active residues, whereof one residue may bind the substrate covalently. (In the example, a cysteine, Cys88, forms a reactive thioester intermediate; this covalent interaction may also occur with a cofactor.) Other residues contribute to substrate activation and its correct positioning in the active site (e.g., His56 and Tyr124 in Figure 1 in addition to others not highlighted).

Alternatively, cofactors extending the repertoire of catalysis may be accommodated in the active site, enabling reductions/oxidations via hydride transfer (NAD(P)⁺/NAD(P)H, flavins) or, for example, oxygenation (iron bound either in porphyrin or directly to the enzyme), methyl transfer, or methyl shuttling (S-adenosyl methionine or cobalamin) and various others.^{63–65}

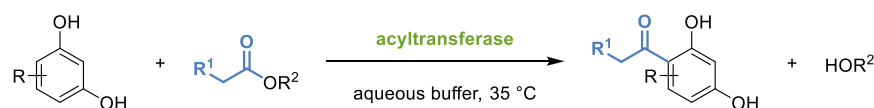
The amino acid side chains are oriented by the folding of the protein and fine-positioned by the residues surrounding them. Consequently, not only the residues in the active site but also those in the next outer shell contribute to the 3D structure of the active site. Countless interactions within the densely packed protein core, together with conformational restrictions of the peptide backbone, lead to a defined but flexible arrangement of the active-site residues.

The outermost layer is responsible for the contact with the medium and, in some cases, for enabling the formation of dimers or even oligomers of the catalyst. In summary, the large molecular size of enzymes in combination with numerous intramolecular interactions leads to a 3D structure that is well-defined but also dynamic, thereby meeting the requirements of substrate binding and catalysis. Unfortunately, protein dynamics may also lead to conformational states that trigger unfolding and therefore loss of activity. Indeed, enzyme stability, for example, toward elevated temperatures, cosolvent concentrations, or substrate concentrations, may be an issue for certain biocatalysts. Nevertheless, it has been shown that enzymes can be stabilized by various approaches, such as ancestral sequence reconstruction,⁶⁶ enzyme engineering, including the introduction of salt bridges^{67,68} and disulfide bonds,^{32,51,69–72} or glycosylation.⁷³

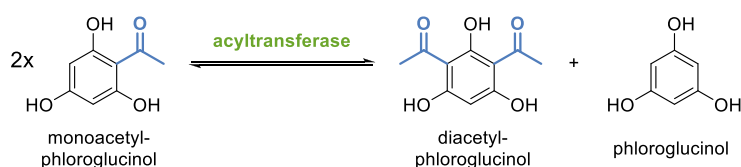
The above example gives a glimpse of the complexity of enzymes as catalysts and shows that there are many screws to turn. The high number of triggers promoting a reaction and the

Scheme 2. Biocatalytic C-Acylation of Resorcinol Derivatives Using an Acyltransferase^a

a) Biocatalytic C-acylation of resorcinol



b) Natural reaction: Disproportionation



^a(a) Designed biocatalytic reaction using esters as an acyl source to be transferred onto resorcinol derivatives. (b) Natural reaction of the acyltransferase represents a disproportionation.

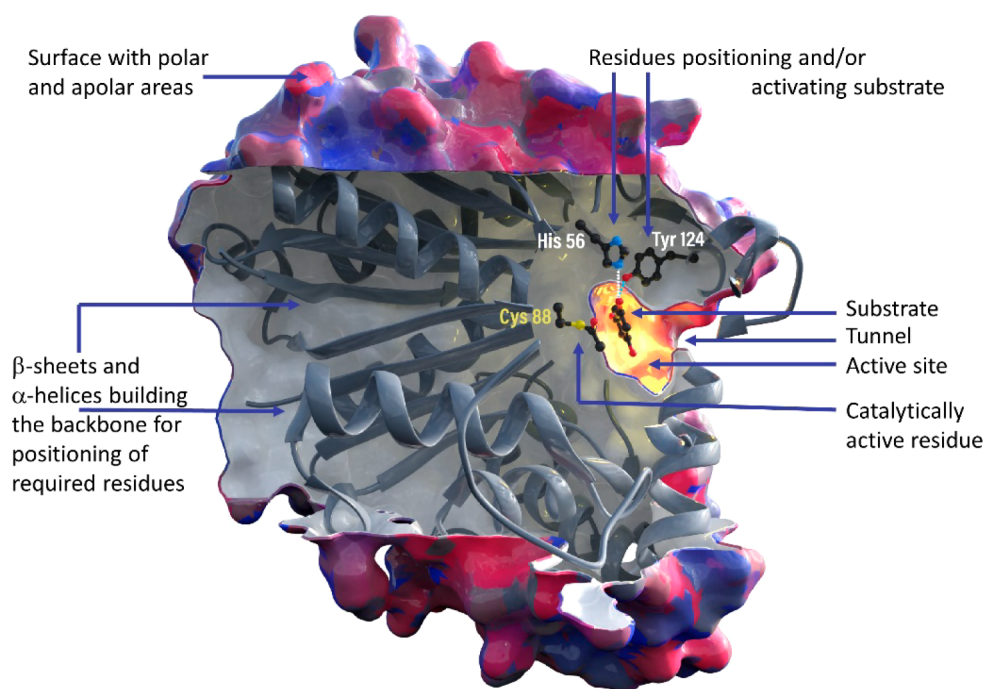


Figure 1. Representation of the features of an enzyme exemplified by an acyltransferase (PDB: 5MG5). The residues involved in catalysis, required for the covalent binding of the substrate, its positioning, or activation, are located in the active site. The latter can be accessed via a tunnel. The residues are positioned by the enzyme backbone consisting of β -sheets and α -helices. The enzyme interacts with the bulk environment via its surface comprising polar and apolar areas. (Enzyme graphic provided by Verena Resch, Luminous Lab.)

interplay of all of these different features make each enzyme a unique catalyst.

■ DEVELOPMENT OF A UNIQUE BIOCATALYST FOR A SPECIFIC REACTION

If anybody wants a catalyst that has a universal substrate scope and, at the same time, an outstanding selectivity (e.g., stereoselectivity), then one should note that this is a contradiction. The higher the selectivity, the narrower the substrate scope must be. On the contrary, the broader the substrate scope, the lower the selectivity. Considering stereoselectivity, optical purities above 99% e.e. (equals an e.r. of 99.5 to 0.5) are the ones of interest due to legal requirements in the pharma industry. Calculating the difference in transition-state energies required to differentiate between the two enantiomers, one will learn that the $\Delta\Delta G$ needed to get from 0 to 95% e.e. (e.r. of 97.5:2.5) is approximately equal to the $\Delta\Delta G$ to get from 95 to 99.9% e.e.⁷⁴ In other words, reaching an e.e. of 95% is half the effort compared with the one to get to 99% optical purity. To achieve this stereoselectivity, the interplay of all features of an enzyme mentioned above are required, which consequently leads to a specific catalyst.

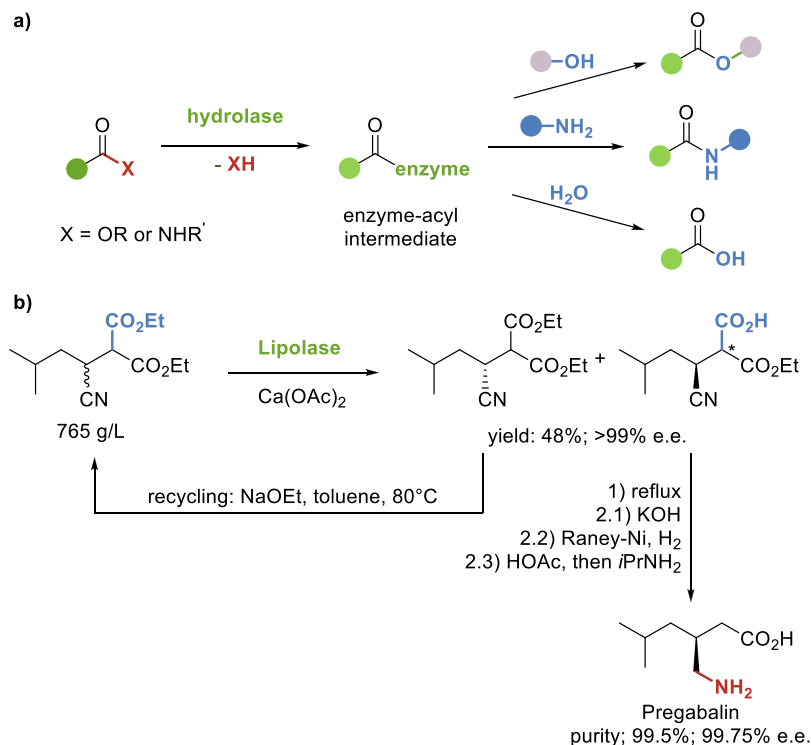
To achieve both an excellent selectivity and a broad substrate scope, modern biocatalysis relies on libraries of enzymes, similar to the catalyst libraries used in organo- and organometallic catalysis. In the case that the diversity of available libraries does not provide the desired scope, it may be required to engineer an existing biocatalyst for a specific substrate shape or even identify/evolve a new biocatalyst for a substrate for which no catalyst is available at all. The high complexity of the biocatalyst, as outlined in the previous section, makes the computational design of new variants highly challenging and has led to the development of a plethora of computational approaches for enzyme design.^{33,75}

In the early days of biocatalysis, the process/reactor was adapted to the enzyme available, which was linked to severe restrictions. Thanks to huge advances in enzyme engineering, the enzyme can now be adapted for the desired process.^{76–82} Actually, enzyme engineering even allows one to introduce new activities (Schemes 5 and 6),^{83–85} to place new catalytic machineries in the active site (Scheme 6a,e,f), or to repurpose the existing catalytic machinery (Scheme 6a,d,e).⁸⁶ Recent reviews give an overview of established biocatalytic reactions^{87,88} also used on industrial scale;^{89–96} others extend the potential of enzymes by comparing the reactions they catalyze to organic name reactions.⁹⁷

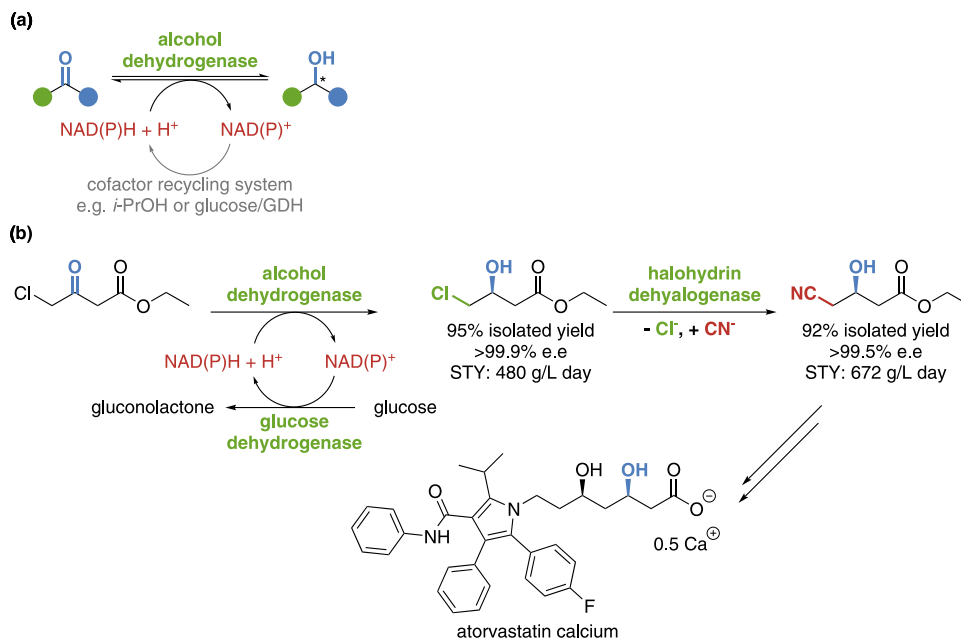
To exemplify the potential of biocatalysis in the current stage, single-step reactions are given in what follows, followed by more elaborate combinations of multiple catalysts in cascade reactions.

■ SELECTED ESTABLISHED SINGLE-STEP BIOTRANSFORMATIONS

Early biocatalytic methods for the preparation of optically pure alcohols and amines⁹⁸ mainly involved hydrolases (especially lipases and esterases) and found broad interest in synthesis on small and industrial scales.^{74,99,100} The optically enriched products were obtained via hydrolysis or the formation of esters or amides in a (dynamic) kinetic resolution of the racemic substrate.^{101–106} The latter was obtained in a classical synthetic route. In addition to the synthetically very well established hydrolases, further types of enzymes are commonly applied in synthesis, including alcohol dehydrogenases (performing the stereoselective reduction of carbonyls),^{107–112} transaminases (TAs), imine reductases (IREDs), and reductive aminases (RedAm's) (catalyzing reductive aminations).^{113–115} In contrast with hydrolases applied in resolution reactions, these enzymes allow the direct asymmetric synthesis of enantiopure

Scheme 3. Transesterification, Amide Formation, and Ester Hydrolysis Catalyzed by Lipases and Esterases^a

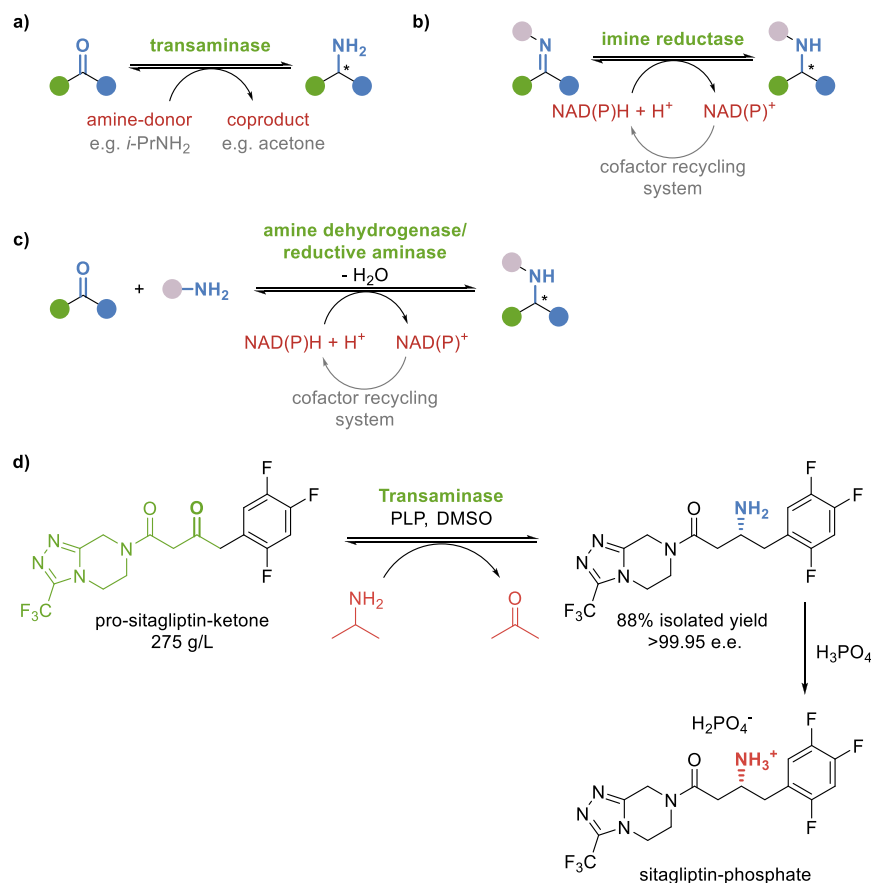
^a(a) Acyl-enzyme intermediate formed from an ester or amine can be attacked by different nucleophiles producing, for example, esters, amides or carboxylic acids. (b) Chemoenzymatic process to produce pregabalin, applying the lipase from *Thermomyces lanuginosus* (Lipolase) in a kinetic resolution.

Scheme 4. Biocatalysis with Alcohol Dehydrogenases^a

^a(a) Stereoselective reduction of ketones and aldehydes catalyzed by alcohol dehydrogenases. (b) Multienzyme process for the production of (*R*)-4-cyano-3-hydroxybutyrate via the asymmetric reduction of ethyl 4-chloro-3-oxobutanoate with an alcohol dehydrogenase in combination with a GDH (glucose/glucose dehydrogenase) recycling system, followed by a halohydrin dehalogenase-catalyzed exchange of the chlorine for a cyano group. STY = space–time yield.

alcohols and amines in one step. However, the mentioned catalysts represent just a fraction of the synthetic versatility of biocatalysis, which offers a variety of further well-developed and

synthetically characterized enzymatic transformations, including C–C bond formations,^{116–119} selective CH functionalization reactions,^{120–123} and novel chemo- and regioselective redox

Scheme 5. Biocatalytic Formation of Amines^a

^aVia (a) amino group transfer by transaminases, (b) imine reduction by imine reductases, (c) reductive amination by amine dehydrogenases/reductive aminases (both terms are used for enzymes catalyzing the same reaction; for a discussion of differentiation, refer to ref 145), and (d) biocatalytic production of sitagliptin using a transaminase. PLP = pyridoxal 5'-phosphate.

reactions.^{107,124} In the subsequent sections, a selection of representative examples of biocatalytic transformations is provided.

Transesterification and Ester Hydrolysis Catalyzed by Lipases and Esterases. The biocatalytic transesterification or hydrolysis of esters (Scheme 3a),^{74,99,100} mainly applied in kinetic resolutions, represents a standard method to produce molecules with high optical purity and is frequently used in the synthesis of active pharmaceutical ingredients (APIs).^{91,101–105,125} The applicability was demonstrated not only for molecules with central chirality but also for molecules with axial^{126,127} and planar¹²⁸ chirality. However, because the kinetic resolution of a racemic product results in a loss of 50% of the starting material, such processes are nowadays not considered to be ideal. Nevertheless, by careful planning of the synthetic route, the recycling of the undesired enantiomer can be integrated. One illustrative example is the synthesis of the anticonvulsant pregabalin (Lyrica, a blockbuster drug) by Pfizer. The first generation of the synthesis yielded the racemic API and required several rounds of recrystallization to obtain the pure enantiomer. To overcome the loss of the inactive enantiomer, various biocatalytic routes were systematically evaluated,¹²⁹ including enzymatic reductive amination¹³⁰ and enzymatic C=C reduction.^{131,132} The final route uses the lipase from *Thermomyces lanuginosus* (Lipolase, a commercial lipase).¹²⁹ A cyanodiester was chemically prepared as a racemate and transformed in a kinetic resolution to the enantiomerically

pure monoester (24 h, 48%, Scheme 3b). The resulting monoester was then converted to the final API in two additional steps. The route allowed a recycling of the undesired enantiomer and also efficiently utilized the enzyme's stereoselectivity for both the distant chiral center and the diastereotopic ester groups. The generated waste was reduced by a factor of 5, and the E-factor (environmental impact) improved from 86 to 17 compared with the first-generation process.¹²⁹

The broad synthetic potential of hydrolases/lipases goes beyond their remarkable stereoselectivity, as in some cases, enzymatic esterifications are more efficient than the established chemical protocols.¹³³ The toolbox of available enzymes of the class is readily growing, with the most recent addition being acyltransferases that are able to catalyze transesterifications even in an aqueous environment.^{134–136}

Stereoselective Reduction of Ketones to Alcohols Catalyzed by Alcohol Dehydrogenases. Biocatalysts belonging to the family of alcohol dehydrogenases (ADHs) catalyze the reduction of ketones or aldehydes to the corresponding (chiral) alcohols (Scheme 4a).^{107–112} Stereo-complementary biocatalysts allowing access to the (*R*)- as well as the (*S*)-enantiomer have been identified in many cases.^{108,137} Hydride equivalents are supplied by a nicotinamide cofactor (NADH or NADPH), which is used in catalytic amounts and recycled *in situ*, for instance, by using an excess of a sacrificial alcohol, for example, 2-propanol, as a hydride donor, thereby exploiting the reversibility of the ADH-catalyzed reaction.¹³⁸

Table 2. Selection of Well-Characterized Biocatalytic Transformations with Potential for Synthesis

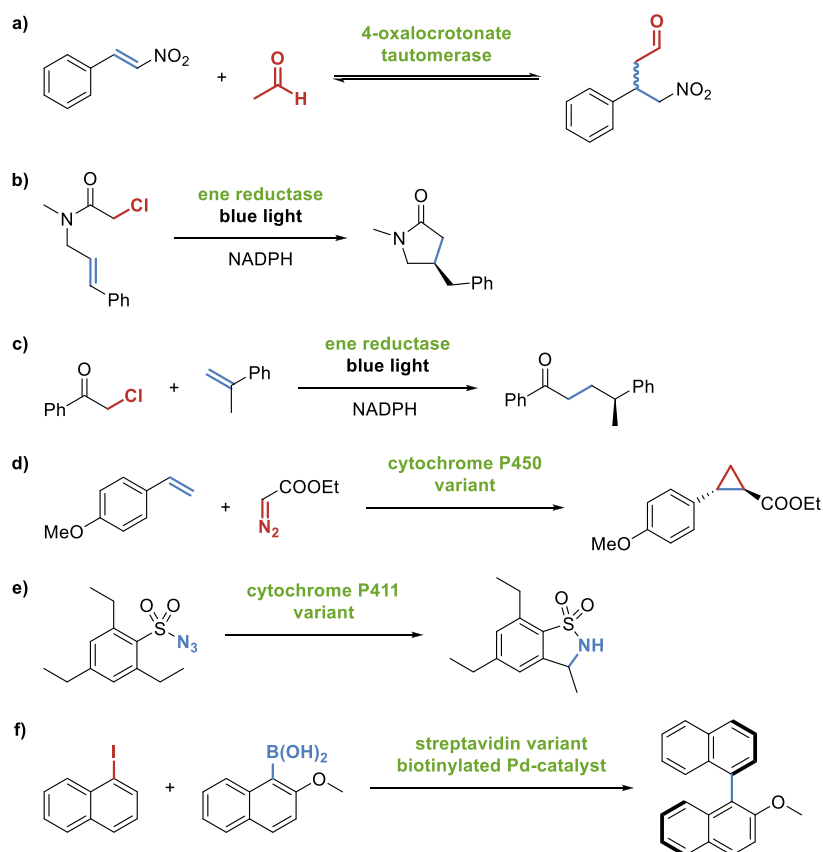
reaction and enzyme family	comments and references
Ester and Amide Formation and Hydrolysis	
hydrolases carboxylic acid reductases ^a	good substrate range and excellent stereoselectivity ^{74,98–105} fatty acids and aromatic carboxylic acids; require ATP ^{148–150}
Reduction of Ketones and Aldehydes to Alcohols	
alcohol dehydrogenases	good substrate range; stereocomplementary enzymes available; require NADH or NADPH; equilibrium ^{107–112}
Reduction of Carboxylic Acids to Aldehydes	
carboxylic acid reductases	fatty acids and aromatic carboxylic acids; require NADPH and ATP ^{149,150}
Reduction of CC Double and Triple Bonds	
ene-reductases	substrate requires an electron-withdrawing group (carbonyl, nitrile, or nitro); require NADH or NADPH ^{151–153}
Reductive Amination of Ketones and Aldehydes	
imine reductases and reductive aminases	stereocomplementary enzymes available; require NADPH; equilibrium ^{114,143,144}
transaminases	stereocomplementary enzymes available; require amine as amino-donor; equilibrium ^{113,141}
amino acid dehydrogenases and amine dehydrogenases	require NADH or NADPH; equilibrium ^{114,143,144}
Oxidation of Alcohols to Ketones/Aldehydes (and Carboxylic Acids)	
alcohol dehydrogenases oxidases	good substrate range; stereocomplementary enzymes available; require NAD ⁺ or NADP ⁺ ; equilibrium ^{107–112} require O ₂ and form H ₂ O ₂ as byproduct ¹²⁴
Hydroxylation of C–H Bonds	
P450 monooxygenases	require O ₂ and NADPH; upscaling might be challenging; vast variety of enzymes available ^{120,124,154–158}
flavin-dependent monooxygenases	phenolic substrates; require O ₂ and NADPH ¹⁵⁹
dioxygenases	dihydroxylation of aromatics; require O ₂ ¹⁶⁰
(unspecific) peroxygenases	broad substrate scope, require H ₂ O ₂ ; good scalability; moderate regioselectivity ^{161,162}
C–O Bond Formation and Cleavage	
cobalamin-dependent methyltransferases	aromatic methyl ether formation and cleavage; equilibrium; require anaerobic conditions ¹⁶³
SAM-dependent methyltransferases	aromatic and aliphatic ether formation; require SAM cofactor; troublesome upscaling ¹⁶⁴
Baeyer–Villiger Oxidation	
Baeyer–Villiger monooxygenases	require O ₂ and NADPH
C–C Bond Formation	
aldolases	broad variety of enzymes known; equilibrium ¹⁶⁵
(de)carboxylases	carboxylation of aromatic molecules or styrenes; require bicarbonate, equilibrium ^{118,166}
hydroxynitrile lyases	stereocomplementary enzymes available; require HCN; spontaneous background reaction; equilibrium ^{167,168}
SAM-dependent methyltransferases	require SAM cofactor; troublesome upscaling ¹⁶⁴
acyl transferases	Friedel–Crafts acylation of resorcinols; require esters as acyldonors ^{116,165}
CC Bond Cleavage: Decarboxylation	
(de)carboxylases	aromatic carboxylic acids or cinnamic acids as substrates, equilibrium ^{118,166}
P450 monooxygenases	(functionalized) fatty acids to terminal alkenes ^{120,162}
Halogenation	
flavin-dependent halogenases	aromatic substrates (tryptophan derivatives); require O ₂ and NADH ¹⁶⁹

^aCarboxylic acid reductases can also be used for amide or ester formation by letting the thioester intermediate react with an amine or alcohol instead of a hydride [NAD(P)H] as the nucleophile.

Alternatively, an additional biocatalyst for NAD(P)H recycling may be employed that consumes a cheap donor molecule such as glucose or formate, giving as a coproduct gluconic acid and CO₂, respectively. The thermodynamic driving force of these auxiliary reactions ensures that the equilibrium of the overall process is shifted to the product side. ADHs have been applied at substrate concentrations of >100 g L^{−1}, such as, for example, in the

production of the chiral intermediate of the antiasthmatic API montelukast.¹³⁹

An impressive example developed by Codexis is the synthesis of (*R*)-4-cyano-3-hydroxybutyrate, the chiral intermediate of the cholesterol-lowering drug atorvastatin (Scheme 4b).¹⁴⁰ The process utilizes three enzymes, with the key step being the enantioselective reduction of ethyl 4-chloro-3-oxobutanoate to (*S*)-ethyl 4-chloro-3-hydroxybutanoate by an ADH. The cofactor

Scheme 6. Enzyme-Catalyzed Reactions That Are New to Nature^a

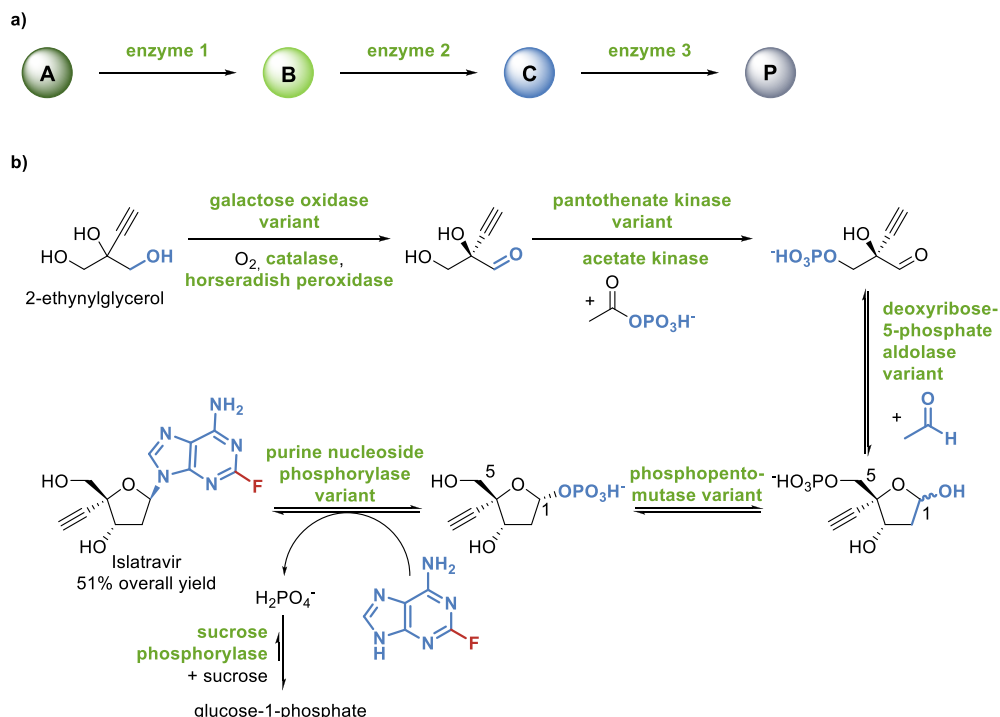
^a(a) 4-Oxalocrotonate tautomerase-catalyzed conjugate addition. (b) Photobiocatalytic cyclization catalyzed by an ene-reductase. (c) Photobiocatalytic intermolecular C–C bond formation catalyzed by an ene-reductase. (d) Biocatalytic cyclopropanation catalyzed by a cytochrome P450 variant, developed via directed evolution. (e) Biocatalytic C–H amination catalyzed by a cytochrome P411 variant, developed via directed evolution. (f) Biocatalytic Suzuki reaction catalyzed by a hybrid catalyst utilizing the streptavidin-biotin method.

NADPH was recycled using a second enzyme (glucose dehydrogenase (GDH)). The substrate loading, space–time yield, and catalyst yield of the reduction were tremendously enhanced by improving the biocatalysts via directed evolution to finally form (S)-ethyl 4-chloro-3-hydroxybutanoate in 95% isolated yield with >99.9% e.e. and a space–time yield of 480 g product/L per day. The intermediate was then further converted to (R)-4-cyano-3-hydroxybutyrate using engineered halohydrin dehalogenase.¹⁴⁰ The process combines several strengths of biocatalysis, including a high stereoselectivity, straightforward catalyst engineering with directed evolution techniques, and the compatibility of biocatalysts to work concurrently in one pot.

Reductive Amination Catalyzed by Transaminases, Imine Reductases, Reductive Aminases, and Amine Dehydrogenases. Because nature provides a great diversity of chiral amines, ranging from amino acids to alkaloids, it is not surprising that a multitude of amine-forming enzymes have been identified.^{115,141,142} The classes that currently have the strongest synthetic potential are TAs,^{113,141} IREDs, RedAm's, and amine dehydrogenases (AmDHs) (Scheme 5a–c).^{114,143,144} IREDs, RedAm's, and AmDHs all enable the stereoselective reduction of imines, however, with complementary substrate scope. Because the reactions of all four classes are reversible, different methods to shift the equilibrium toward the desired product are applied. In the case of TAs, the required amine donor (e.g., 2-propylamine or 1-phenylethylamine) may be used in excess to

push the reaction. Often, the donor-oxo product (e.g., pyruvate, in case alanine is used as amine donor) is further transformed by an additional enzyme (e.g., reduction to lactic acid) or recycled (e.g., conversion of pyruvate with alanine dehydrogenase).¹¹³ For the nicotinamide-dependent IREDs, AmDHs, and RedAm's, a cofactor recycling system is crucial to push the equilibrium.

The synthetic route toward sitagliptin, the API of Januvia, used to treat diabetes mellitus type 2, showcases not only the synthetic feasibility of biocatalytic aminations but also the efficient methods for the development and fine-tuning of potent biocatalysts. The initial synthesis of the API proceeded via a rhodium(I)–Josiphos-catalyzed asymmetric hydrogenation followed by a sophisticated purification from contaminations with rhodium and the undesired product enantiomer. Consequently, a TA-catalyzed amination of pro-sitagliptin ketone was developed.¹⁴⁶ Because wild-type TAs transformed only a mimic molecule (Scheme 5d, highlighted in green) rather than the bulky target substrate, the catalyst was improved via a “substrate walking” approach. In this method, the catalyst's activity is initially improved for a (small) mimic molecule by directed evolution. This is followed by optimization for a slightly larger version of the substrate and is repeated until the final target can be converted under process conditions. After a total of 11 rounds of directed evolution, 27 amino acids of the initial TA were mutated, yielding a catalyst that was able to convert 275 g L^{−1} pro-sitagliptin-ketone in an isolated yield of 88% with an e.e.

Scheme 7. Multienzyme Cascade Reactions^a

^a(a) General scheme of a three-enzyme, three-step cascade reaction: Starting material A is converted via intermediates B and C to product P. (b) Process for the production of islatravir in a multienzyme cascade sequence.

of >99.95%. The process demonstrated a 10–13% higher yield and a 56% higher productivity and produced 19% less waste compared with the previous chemical route.¹⁴⁷

Biocatalytic Single-Step Transformations with Synthetic Potential. The examples mentioned above are complemented by a variety of additional biocatalytic transformations that may be directly applied for synthetic purposes. Table 2 provides a personal selection of synthetically applicable and well-characterized enzyme families according to the reactions they catalyze. More detailed overviews of specific biocatalytic reactions can be found in the associated references.

REACTIONS NEW TO NATURE, CATALYZED BY ENZYMES

Novel methods to utilize enzymes for transformations unprecedented in nature have been developed to broaden the synthetic applicability of biocatalysis even further.^{170–173}

Reaction Promiscuity of Natural Enzymes. The idea that enzymes are limited to their natural substrate is a common misconception about enzymes that has been proven to be unambiguously wrong.^{95,174} In fact, enzymes are promiscuous not only concerning their substrate but also regarding the type of reaction they catalyze. This should not be surprising considering that an enzyme is also just a chemical catalyst and certain catalytic machineries will allow different types of reactions depending on the amino acid residues present and the substrates and reaction conditions provided. An example is the broad reactivity demonstrated for 4-oxalocrotonate tautomerase,¹⁷³ which is a rather short peptide (62 amino acids) and possesses an N-terminal proline that can be exploited by analogy to proline-based organocatalysts.^{175,176} Although its native reaction is the name-giving tautomerization of 4-oxalocrotonate to its enol, it also catalyzes conjugate additions (Scheme 6a), aldol

reactions, and asymmetric epoxidations with high enantiomeric excess.^{173,177–181}

Providing special conditions like the illumination of biocatalysts with visible light is a method to unlock novel reactivities with established enzymes for specific substrates.^{170,182,183} For instance, whereas the native reaction of the flavin-dependent ene-reductases is the asymmetric reduction of activated alkenes,^{151–153} excitation with blue light allows the active-site flavin to perform single-electron transformations that trigger, for instance, radical dehalogenations followed by C–C bond formations via the radical intermediate (Scheme 6b,c).^{184–186}

Novel Reactions Catalyzed by Variants. Wild-type enzymes already often demonstrate low activities for reactions other than their natural transformations. The systematic generation of enzyme variants, either by design based on an enzyme structure (rational design) or by random mutagenesis (directed evolution), proved to be an efficient tool to elevate such side activities to a synthetically useful rate.⁸⁵ One of the enzyme classes demonstrating broad reaction promiscuity is the cytochrome P450 family. These biocatalysts are well known for regioselective C–H oxidation leading to hydroxylation, but they also catalyze a variety of other reactions including decarboxylation, epoxidation, reductive dehalogenation, amine/oxygen dealkylation, and sulfoxidation.^{120,154–158} Promiscuous activities may be enhanced or even newly introduced via enzyme engineering, as demonstrated by many research groups over the past decades. Of particular prominence in this context is the work of Frances Arnold, who was awarded the 2018 Nobel Prize in Chemistry “for the directed evolution of enzymes”.^{187,188} This technique was used to engineer variant cytochrome P450 enzymes (and also other heme-dependent enzymes) to catalyze carbene-transfer reactions, forming cyclopropanes using diazo-

esters as carbene precursors with high diastereo- and enantioselectivity (Scheme 6d).^{189,190} In another example, substituting the conserved heme-binding cysteine of cytochrome P450 enzymes for a serine led to a new class of biocatalysts termed P411 enzymes, as the characteristic peak in the CO-titration absorbance spectrum shifted from 450 to 411 nm. One of the outstanding reactivities of P411 variants (among other P450 variants) is their ability to aminate C–H bonds via a nitrene transfer from aryl sulfonyl azides as nitrene precursors with up to 89% e.e. (Scheme 6e).^{191,192}

Using the Protein Backbone as a Chiral Carrier for Abiotic Catalysts. As previously stated, enzymes might be considered as huge chiral ligands for any catalytically active molecule in the active site, be it an organocatalyst (e.g., coordinated cofactors such as flavins, pyridoxal 5'-phosphate, or thiamine pyrophosphate) or a metal catalyst (e.g., iron in hemes, copper, or zinc). Consequently, biohybrid catalysts have been developed whereby the protein backbone binds a chemical catalyst within its chiral environment, enabling new-to-nature reactions with protein-based stereocontrol.^{172,173,193} One of the most broadly applied systems in this context utilizes the high affinity of streptavidin toward biotin derivatives ("biotinylated catalysts").¹⁹³ This concept has been exploited to enable a variety of transition-metal-catalyzed reactions like the "Suzukiase", which uses a biotinylated monophosphine palladium complex bound to a streptavidin variant to catalyze asymmetric Suzuki reactions with up to 90% e.e. (Scheme 6f).¹⁹⁴

MULTIENZYME REACTIONS IN ONE POT (BIOCATALYTIC CASCADES)

Because biocatalysts originate from organisms that, in general, live under comparable conditions in an aqueous environment, the operational parameters of different enzymes usually significantly overlap. This intrinsic compatibility makes combining biocatalysts in one pot to perform several reactions concurrently in a cascade a highly appealing option. This approach saves tedious workup steps and reagents for the isolation of intermediates, and it may help to shift reaction equilibria and to reduce overall costs and time (Scheme 7a).^{111,195–197} An illustrative example utilizing three enzymes to perform a sequence of two reactions in one pot is the synthesis of atorvastatin, as already discussed in Scheme 4.¹⁴⁰ A biocatalytic cascade with high impact was recently developed for the synthesis of the anti-HIV drug islatravir by teams of Merck and Codexis.¹⁹⁸ Faced with problems in the chemical synthesis, a biocatalytic route was devised based on the bacterial nucleoside salvage pathway. The most active enzymes for the individual steps were selected from screenings and optimized using directed evolution methods to catalyze the desired reaction. The reaction sequence starts with a desymmetrization via the oxidation of 2-ethynylglycerol by a galactose oxidase variant, supported by a catalase and a horseradish peroxidase for the decomposition of reactive oxygen species. This is followed by phosphorylation employing a pantothenate kinase variant, supported by an acetate kinase for ATP recycling, to yield a phosphorylated aldehyde intermediate (Scheme 7b). All following steps are performed simultaneously to shift unfavorable equilibria. The phosphorylated aldehyde is coupled to acetaldehyde in an aldol reaction, catalyzed by a deoxyribose 5-phosphate aldolase, forming the non-natural sugar scaffold. After shifting a phosphate group from position 5 to 1 by a phosphate transferase, the target API islatravir is finally assembled by a purine nucleoside phosphorylase. In the last step, a sucrose

phosphorylase helps to shift the reaction equilibrium toward the nucleoside product by removing the liberated phosphate. Overall, the reaction sequence involves nine enzymes, five of which were engineered in up to 12 rounds of directed evolution. The route produces a single enantiomer of islatravir in 51% overall yield without intermediary workup, showcasing the enormous synthetic potential of enzymatic cascade processes.¹⁹⁸

THE FUTURE STARTS NOW

The synthetic application of biocatalysts is still a relatively young field of research, and enzymes are complex molecules, so it is fair to admit that there are still many challenges that need to be addressed. One issue is definitely the stability of the catalyst and thus its lifetime and consequently the space–time yields in processes.¹⁹⁹ Research to elucidate what makes a protein stable under process conditions is a hot topic and needs to be expanded to make the stabilization of enzymes more straightforward. Further challenges are the costs and the time required for developing a suitable catalyst in the lab, which need to be reduced to allow the fast implementation of enzyme-catalyzed processes in industry.²⁰⁰ Another important aspect is that biocatalysis needs to be incorporated as part of chemistry curricula at universities to train the chemists of tomorrow in its application and to give them a broader methodological horizon. Future synthetic chemists should be made aware that biocatalysts can be used in single-step transformations or in cascades comprising several biocatalysts or, if suitable, can be incorporated in metabolic pathways of living organisms.²⁰¹ For a sustainable future, we also have to appreciate that proteins are made up of renewables and are biodegradable, contributing to a much-desired circular economy.

Biocatalysis needs to be incorporated as part of chemistry curricula at universities to train the chemists of tomorrow in its application and to give them a broader methodological horizon.

Although a substantial number of biocatalysts are already described for several synthetically useful reactions (cf. the selection in Table 2), even more extensive libraries of stable, highly active enzymes need to be and will be developed in the future to cover the theoretically possible substrate scope of each reaction. Depending especially on the size but also on the functional groups present in the substrate, the possibly suitable biocatalysts may then be easily identified by comparison with previous results obtained for a specific enzyme. To give everybody easy access to biocatalysts, libraries of well-described enzymes with known sequences may be distributed via institutions, or plasmids encoding these biocatalysts may be deposited and made available in repositories (first examples are available^{202,203}) that distribute them for a small fee and with the only requirement of citing the original work.

Following the current publications, the scope of reactions performed by biocatalysts is also constantly expanding, and much progress can be expected for years to come. In addition to some emerging biocatalysts listed in Table 2 (performing, e.g., halogenation or amide formation reactions), catalysts for various types of C–C bond formation²⁰⁴ are needed, with oxidative C–C coupling probably posing the biggest challenge.^{205,206}

In general, the demands of modern synthesis will require the continued development of novel catalytic methods. Tight reaction control in a confined space has been identified as a key factor in this context,⁴¹ and proteins arguably provide extensive opportunities to achieve this control. Additionally, synergistic catalysis is a key feature of enzymes.

The future will see both a diversification of available enzymes and the development of novel hybrid catalysts with new-to-nature activities.

Therefore, when it comes to reactions requiring high chemo-, regio-, or stereoselectivity, biocatalysts offer a high chance for success. Because of the infinite number of theoretical possibilities for a protein backbone (e.g., for a protein containing 300 amino acids, 20³⁰⁰ permutations exist, which is a number bigger than the number of atoms in the universe), the question often is not whether a suitable catalyst is possible but whether it can be identified. The screening of libraries of variants has proven to be a viable approach for discovering new enzymes and improving known ones, but would it not be preferable if we were able to design them? Whether machine learning will become a key technology in this context will be seen in the future,^{207–209} but for sure, computational design requires various approaches and methods.^{33,75} Huge efforts have been spent on designing enzymes, and basic research in the years to come will provide new approaches to create highly efficient, stable biocatalysts that are also easy to produce.

In the future, a biodegradable biocatalyst may be developed/ designed for any specific transformation desired and thereby shortcut organic synthetic routes to produce the target molecule in a minimum number of steps ideally requiring only environmentally benign reagents.

The past has already indicated the high potential of biocatalysts in organic synthesis, and the present has paved an avenue for exploiting biocatalysts in synthetic routes, tailoring and evolving wild-type enzymes, and creating hybrid catalysts, for example, by incorporating low-molecular-weight catalysts in a protein environment.¹⁷³ The future will see both a diversification of available enzymes and the development of novel hybrid catalysts with new-to-nature activities. Consequently, in the future, a biodegradable biocatalyst may be developed/ designed for any specific transformation desired and thereby shortcut organic synthetic routes to produce the target molecule in a minimum number of steps ideally requiring only environmentally benign reagents.

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Notes

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