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Perspective

Enzymatic epoxidation strategies for the stereoselective synthesis of chiral epoxides

Vytautas Petkevičius,[1,*] Carolin Mügge,[2,*] Dirk Tischler,[2] and Rolandas Meškys[1]
[1]Department of Molecular Microbiology and Biotechnology, Institute of Biochemistry, Life Sciences Center, Vilnius University, 7 Saulėtekio Avenue, 10257 Vilnius, Lithuania
[2]Microbial Biotechnology, Faculty of Biology and Biotechnology, Ruhr University Bochum, Universitaetsstrasse 150, 44780 Bochum, Germany
*Correspondence: vytautas.petkevicius@bchi.vu.lt (V.P.), carolin.muegge@rub.de (C.M.)
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SUMMARY

Epoxides, a class of organic compounds that feature a highly reactive three-membered oxygen-containing ring, have gathered significant attention due to their remarkable reactivity and versatility. The strained epoxide group, which can also occur naturally, renders the compound class central in many industrial processes. The beauty of nature is that it provides a manifold repertoire of enzymes allowing the formation of these epoxides with regio- and stereo-selective information as desired for certain applications, like in the food, aroma, and pharmaceutical industries. Herein, powerful biocatalytic approaches as well as recent developments are discussed to provide a perspective on potent enzymatic epoxidation as well as to direct research toward the unknown unknowns of these systems.

TOWARD THE PERFECT BIOCATALYST FOR ASYMMETRIC EPOXIDATION

The global epoxide market value is estimated to hit $78 billion in 2025, with applications spanning the production of epoxy resins, surfactants, adhesives, coatings, pharmaceuticals, and more.[1] Of particular interest are chiral epoxides, essential building blocks for stereochemically defined molecules. Given that the biological activity of chiral compounds is typically restricted to a single stereoisomer (with other isomers being inactive or even harmful), the modern development of chiral products such as drugs and active pharmaceutical ingredients (APIs) demands highly selective synthetic approaches.[2] However, only a small fraction of chiral drugs (~10%) are produced as single stereoisomers[3]; therefore, the synthesis of stereopure chiral epoxides represents both a fundamental challenge and an opportunity. There are multiple methods for synthesizing such chiral epoxides (Figure 1). The most straightforward and widely used methods include direct oxidation of alkenes (olefins), various ring-closure and condensation reactions, and kinetic resolution techniques used in organic synthesis. On the biological side, biocatalytic transformations offer promising alternatives due to their high enantio- and regioselectivity.[4] As the portfolio of natural products containing epoxide functions—in the final product or as synthetic intermediate—spans all compound classes, including terpenoids, steroids, alkaloids, polyketides, and many more, researchers have been able to identify biocatalytic processes toward oxiran ring formation based on numerous enzyme classes. These include direct epoxidation of prochiral substrates by monooxygenases or peroxidases, enantioselective hydrolysis of racemic epoxides by epoxide hydrolases, and lipase-mediated kinetic resolutions, thus paving the way toward potential new applications with enzymes converting natural or non-natural substrates.[5,6]

Despite significant progress and numerous achievements in the field, we argue that the "perfect" biocatalyst for asymmetric epoxidation remains elusive. By "perfect," we refer to an enzymatic epoxidation that fulfills several unmet or previously unattainable criteria, overcoming persistent challenges that have hindered both chemical and biological methods from advancing the synthesis of chiral epoxides. We are seeking enzymatic catalysts enabling groundbreaking transformations that facilitate a shift away from transition metal-based systems and excessive use of organic solvents toward greener, more sustainable alternatives. This hypothetical enzyme should enable the direct conversion of substrates into chiral epoxides, eliminating the need for kinetic resolution, which still requires the prior synthesis of a racemic epoxide. Therefore, oxygenases and similarly acting enzymes appear to have an advantage over other enzymes in this context. A truly versatile biocatalyst should possess a broad substrate scope, capable of converting not only natural or nature-like molecules but also structurally diverse synthetic compounds relevant to industrial applications. It should also overcome current limitations, such as the asymmetric epoxidation of unactivated, unconjugated, or terminal alkenes, challenges that remain difficult even for well-established chemical methods.[7] Preferably, the enzyme should function as a true asymmetric catalyst, meaning a collection of enzyme variants (or mutants) could be employed for enantiodivergent transformations, enabling selective synthesis of either enantiomer (*R* or *S*)

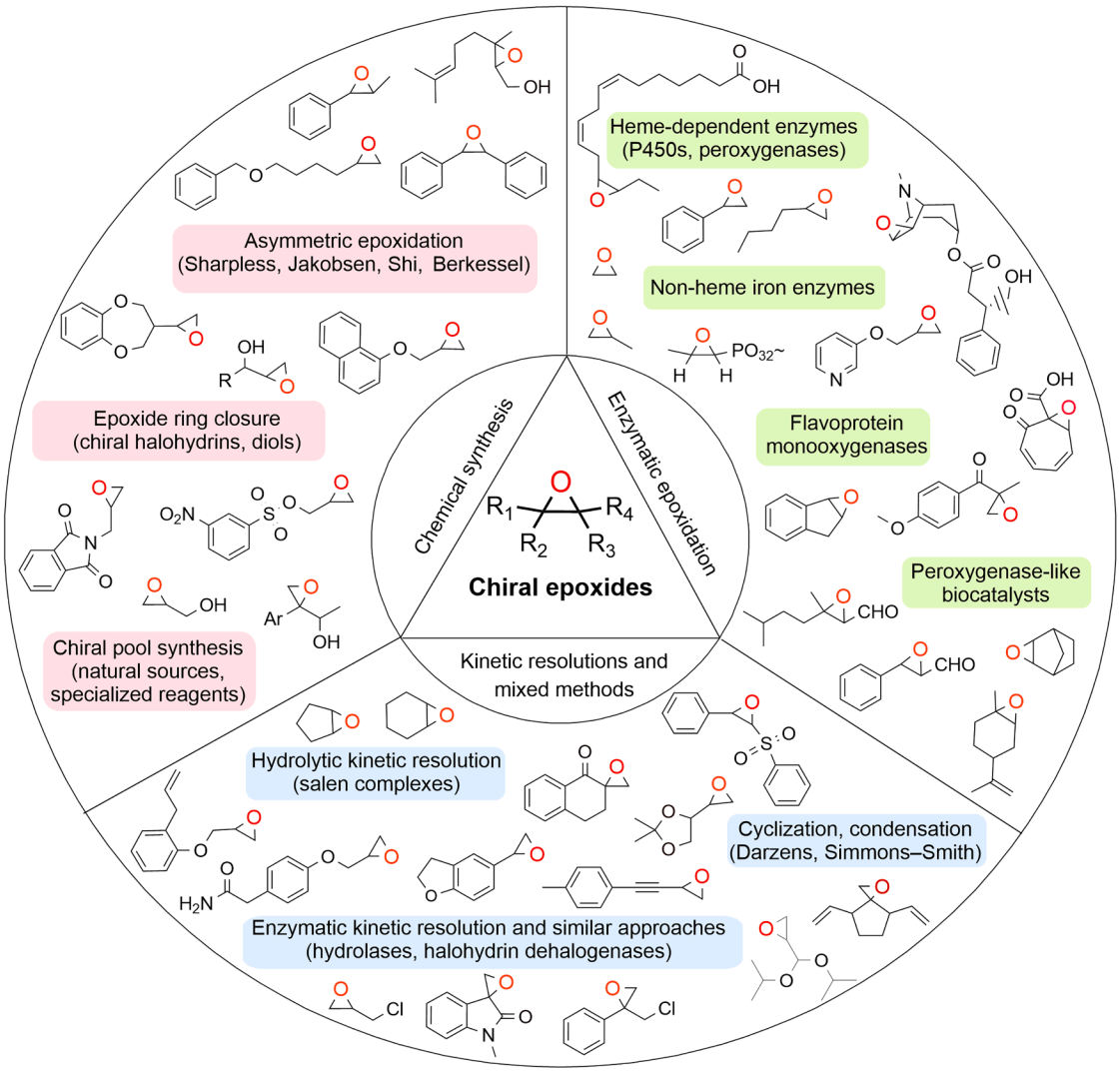

Figure 1. Routes toward chiral epoxides

The most common approaches for synthesizing stereochemically pure or enriched epoxides, along with representative examples of the resulting products.

on demand.[8] Of course, additional requirements, such as high tolerance to reaction conditions, operational stability, scalability, etc., must also be met. Here, we explore recent advances in epoxide-generating enzymes that we believe hold the most significant potential to evolve into "perfect" asymmetric epoxidation biocatalysts.

EPOXIDATION BY MEANS OF HEME PROTEINS

One of the first enzyme classes described to be capable of direct epoxidation of double bonds was cytochrome P450 (CYP) enzymes, known for their versatility in performing oxyfunctionalization reactions.[9] Their heme-thiolato unit entails a unique reactivity, relying on the redox versatility of the heme-bound iron atom. Although countless studies have contributed to elucidating the catalytic cycle of CYPs, many details are still under debate. Nevertheless, a few aspects of this highly complex redox cycle are accepted to contribute to their ability to perform epoxidation reactions. Epoxidation events frequently coincide with a degree of hydroxylation activity (which may be considered the "classical" monooxygenase activity of P450s), and the preference for one or the other reactivity is the subject of many mechanistic studies, as are efforts to enhance their reactive selectivity.[10,11]

CYPs are able to utilize molecular oxygen (monooxygenase activity) as well as hydrogen peroxide (peroxygenase activity) or even higher peroxy compounds to transfer one oxygen onto their substrate (Figure 2).[12] For epoxidation events involving H_2O_2 as oxidant, a potential $Fe^{III}(H_2O_2)$ transient intermediate has been predicted to provide a branchpoint toward the formation of the (productive) intermediates "compound (cpd) 0" or "cpd I" as well as to (unproductive) uncoupling events.[13,14] Cpd 0, an $Fe^{III}(OOH)$ hydroperoxide, can either produce the epoxide in a concerted mechanism (left outer pathway) or convert to cpd I, $[Fe^{IV}(O)]^{\cdot+}$ (inner pathway).[15] From here, an

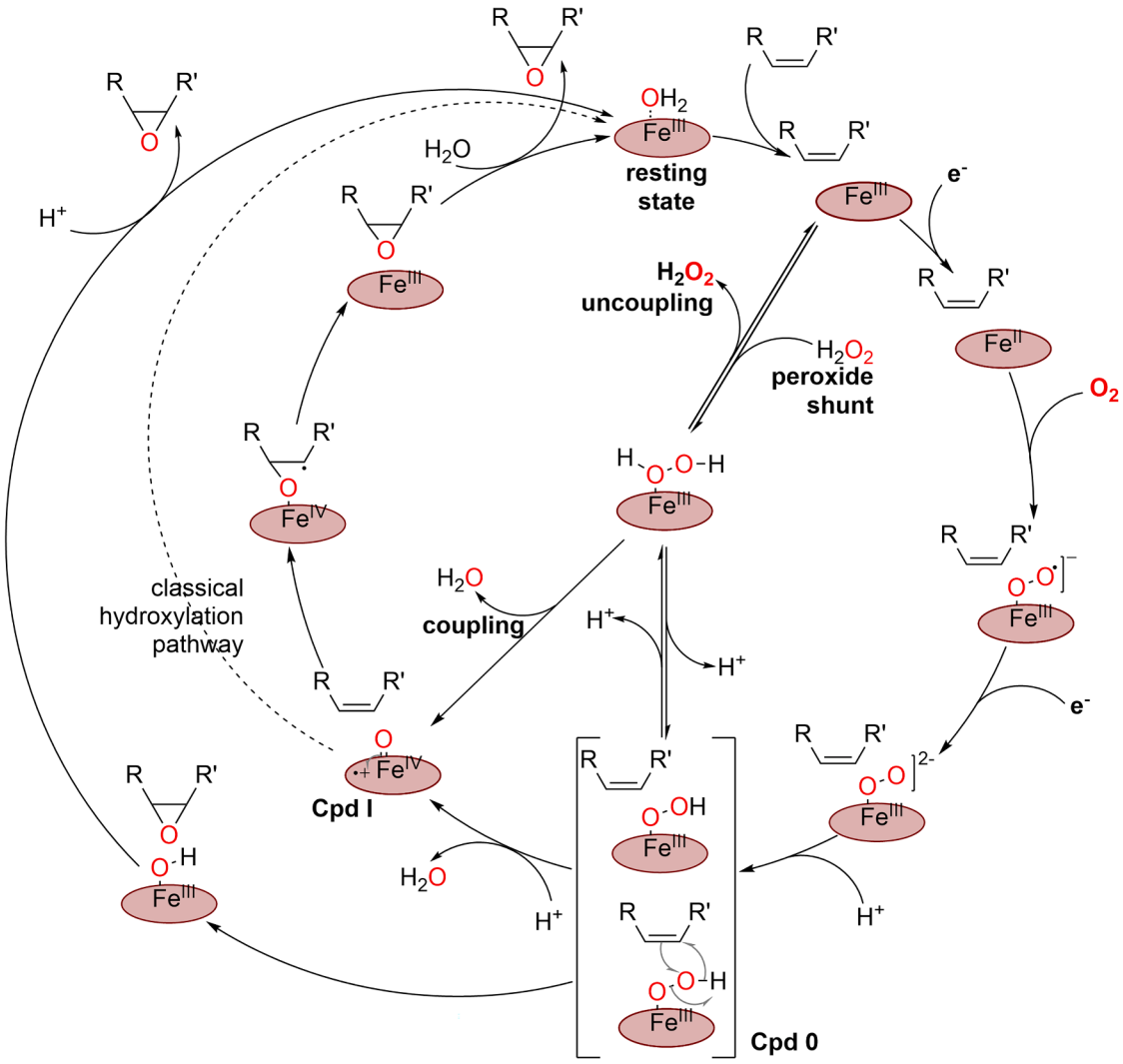

Figure 2. Catalytic cycles for epoxidation by heme-thiolato proteins

The epoxidation can be initiated from cpd 0, following a concerted mechanism (left outer cycle) or can proceed via a radical intermediate starting from cpd I. Molecular oxygen or (hydrogen) peroxides can serve as oxygen source so that either monooxygenase or peroxygenase activities of heme-thiolato proteins can be exploited for epoxidation activity.

Fe^{IV}-alkoxy-radical complex, as an alternative to cpd II of the classical hydroxylation mechanism, is discussed to result in epoxide release and restoration of the resting state of the enzyme.[16,17] While there is an energetic preference for the pathway via cpd I,[18] both mechanisms are discussed to occur in different surroundings, and the orientation of the substrate within the active site is crucial for both the mechanism and the stereoselectivity of the epoxidation event: the space the substrate leaves available for the oxygen species contributes to the mode of oxygen binding and thus co-determines the nature of the oxygenation reaction and the regio- and stereoselectivity of the newly formed substrate–oxygen bond(s). For an actual biotechnological application, this implies that the precise orientation of the substrate has to be perfectly fine-tuned within the enzyme active site to achieve not only desired enantioselectivities but also significant productive turnovers.

Among CYP monooxygenases, the archetypal bacterial P450cam and P450BM3 are widely studied in epoxidation, primarily to explore their reactivity and mechanisms, also by mutagenesis,[19] and less to achieve high product titers. In this respect, P450BM3-driven styrene epoxidation has recently been investigated with the aid of decoy molecules, and it was underlined that the stabilization of heme-bound oxygen is made possible by hydrogen bonding. In addition, the available space in the heme pocket steers substrate orientation and, thus, stereoselectivity.[20] Rationalizing that cpd I is the epoxidizing species, 4-vinylbenzoic acid was recently converted to the (*S*)-epoxide with 99% enantiomeric excess (ee) by a bacterial CYP199A4 monooxygenase. Both optimal substrate binding and efficient formation of cpd I were key to productive turnovers.[21] Similar results have been reported on the same substrate with CYP199A4 mutants.[22] Interestingly, a comparison between P450cam and

chloroperoxidases revealed influencing factors of the proximal binding pocket on the selectivity toward epoxidation or hydroxylation of styrene and cyclohexene.[23]

When it comes to the actual applicability of heme-based epoxidation reactions, peroxygenases take up a special position. With great structural and sequence diversity and diverse origins, they form a not yet exhaustively investigated heme-thiolate oxidoreductase group.[16,24] By direct binding of (hydrogen) peroxide(s) instead of molecular oxygen, heme peroxygenases can act independently from both nicotinamide co-substrates ($NAD(P)^+$/NAD(P)H) and an intricate electron transport system, which renders heme peroxygenases highly attractive for biotechnological applications. Nevertheless, the supply of H_2O_2 is still a crucial factor, as too-high (local) titers may inactivate the protein. Creative H_2O_2 supply systems range from enzymatic processes via electrocatalytic to plasma-driven methods.[25,26] While typically used in cell-free settings, recent strain engineering efforts also make whole-cell or cell extract applications possible, omitting the need to isolate these proteins.[27]

The ability of CYPs to employ H_2O_2 in fatty acid conversion was shown in the late 1990s.[28] Typically driving α- or β-hydroxylation and decarboxylation reactions, the activity of fatty acid peroxygenases (FAPOs) relies on the substrates' carboxy function serving as an acid-base electron bridge, making them highly substrate specific.[29–31] Epoxidation activities of heme-peroxygenase enzymes have been reported from filamentous fungi and plants (e.g., rice, tomatoes, soybean, and oat),[32] but a detailed investigation, including their precise structure and the nature of the heme unit, will be needed to understand these enzymes. Elegant from a biotechnological point of view, FAPOs could even be applied from oat flour crude preparation in the epoxidation of fatty acids and even toward limonene, giving pure diastereomers from (*R*)- and (*S*)-limonene in the 300 mg range.[33] It remains to be elucidated how the enzymes, formally restricted to carboxy-compounds, can stabilize non-carboxy-compounds and how this could be exploited for further substrate classes.

In contrast to the rather specific substrate scope of FAPOs, UPOs (unspecific peroxygenases) are even called "dream peroxygenases" due to their independence from nicotinamide co-factors and their broad substrate spectrum.[34] UPOs of mostly fungal origin accept small to large, simple to complex, aromatic, alicyclic, and aliphatic compounds, hence the "unspecific" part of their name.[35,36] The type of oxygenation taking place largely depends on the substrate being offered, the available reaction sites within the molecule, and how it is allocated within the substrate binding site with respect to distance and angle toward the heme-Fe plane. Interestingly, UPO enzymes have recently been used in fatty acid epoxidation.[37] Enlargement of the substrate channel accessing the heme active site in *Cvi*UPO from *Collariella virescens* had significant effects on activity and selectivity (such as epoxidation vs. hydroxylation or multi-epoxidation), with the wider substrate channels allowing for higher conversion and multiple epoxidation at the cost of selectivity.[38,39] Different *Mro*UPO variants from *Marasmius rotula* demonstrated the importance of substrate access and an ideal distance between the heme-bound oxygen of cpd I and the available double bond for epoxidation-hydroxylation selectivity, which is directed from the available space in the heme-substrate pocket.[40] This rationale has promoted access to selective epoxidation catalysts toward specific double bonds in polyunsaturated C18 fatty acid substrates, longer representatives, and complex mixtures.[37,41,42] Substrate accommodation in UPOs does not rely on a carboxylic function or another functional group but seems to mainly rely on available space, as the epoxidation of long-chain terminal olefins (C12:1 to C20:1) is equally possible for a panel of UPOs. Enzyme-specific turnover and selectivity could partly be fine-tuned by reaction engineering (e.g., oxygen source, pH, or 60% acetone as co-solvent), which demonstrates the importance of both having an optimal enzyme as well as optimized reaction conditions to achieve productive epoxidation reactions.[43]

Having many-fold potential in applications from pharmaceuticals to polymers and a multimillion-ton global market,[44] enantiopure styrene oxides and derivatives are of interest to many stakeholders. Making use of the rather robust UPOs to produce styrene oxides is thus a logical consequence, even though the performance (selectivity and activity) of styrene (derivative) epoxidation by UPOs is still exceeded by other, more specialized enzymes (e.g., styrene monooxygenase [SMO], cf. below), leaving room for biotechnological optimization. For *Agrocybe aegerita* UPO (*AaeUPO*), poor stereoselectivity (7% ee) was found for styrene, but *cis*-methylstyrene was epoxidized with <99% ee (1*R*,2*S*-(+)).[45] By reaction engineering as an integral part of optimizing biotransformation reactions and using a broadened substrate panel, *Aae*UPO could be applied at high turnovers (>50,000), but the stereoselectivity for terminal double bonds remained mediocre. Only (1*R*,2*S*)-β-methylstyrene oxide, a building block for (pseudo)ephedrine, was produced in >99% ee and at appreciable product titers.[46,47] Other UPOs were discovered and described with similar activity and stereoselectivity toward styrene, e.g., *Hsp*UPO from *Hypoxylon* sp. *EC*38, with stability in organic solvents (e.g., 5 vol % acetonitrile) and at biotechnologically relevant hydrogen peroxide concentrations (up to ca. 4 mM).[48] The panel of UPOs with reported activity toward styrene derivatives has been significantly expanded,[49] and the commercial availability of UPOs will surely give rise to new epoxidation possibilities—ideally with improved stereoselectivities and increased activities—in the near future. Still, the main challenge of UPOs remains the trade-off of stability, substrate promiscuity, and high activity at the price of only marginal (stereo)selectivity, which is owed to their wide substrate binding channel and active site. These "dream peroxygenases" could, however, become "perfect catalysts" for epoxidation reactions by the targeted design of the substrate binding site toward specific substrates—hence trading back promiscuity for selectivity.

In search of effective and enantioselective epoxidation reactions aside from the UPO hype, a recent approach is to mimic peroxygenase activity within a classical CYP scaffold with the aid of so-called dual-functional small molecules (DFSMs). P450BM3 variants were supplemented with *N*-(ω-imidazolyl fatty acyl)-*l*-amino acids, where the acyl amino acid group anchors within the protein active site, allowing for the imidazole unit to act as acid-base catalyst and to activate H_2O_2.[50] By this, the production of (*R*)-styrene oxide was made possible with ee values up to 91% in initial studies and raised to 99% with further

improved variants of P450BM3.[51] Importantly, this result was unmatched by the same variants operating under classical NADPH/O_2-driven conditions. This concept was validated and broadened to the use of ^{t}BuOOH as an oxygen donor.[52] Furthermore, halogen-substituted styrenes could be used as substrates with high ee values and TON (turnover number) beyond 2,000.[53] Mechanistically, it is discussed that the DFSM enables the creation of a proton channel, connecting the DFSM-imidazolyl group to the nearby hydrogen atom of H_2O_2. This channel facilitates heterolytic O-O cleavage and the formation of cpd I, similar to the proposed activation of H_2O_2 in natural peroxygenases. Without the DFSM, the formation of cpd I through this mechanism is ineffective. Future optimization could make use of this concept and incorporate, e.g., non-canonical amino acids to equally activate H_2O_2 and stabilize cpd I, potentially easing the workflow of enzyme production.

Turning archetypical monooxygenases into peroxygenases is an elegant approach to make use of different selectivities.[54] Notably, CYP238A1 from *Pseudomonas putida* KT2440, initially described as a monooxygenase, has recently been exploited for peroxygenase activity. Epoxy-norbornane (EPO-NBE), a building block for biologically active heterocyclic compounds, has been produced at a 30 L scale, yielding 77.6 $g \cdot L^{-1}$ EPO-NBE by an engineered variant of CYP238A1.[55] Dictated by the substrate, selective production of the *exo* product is achieved, which is the same as that produced by chemical epoxidation with, e.g., 3-chloroperbenzoic acid (*m*-CPBA). This example shows that many CYP-based enzymes might have more than their initially ascribed reactivity and that there are still hidden gems in the heme-based epoxidation field that are yet to be uncovered.

EPOXIDATION UTILIZING NON-HEME METALLOPROTEINS

Nature's ability to orchestrate complex oxidative transformations through non-heme iron enzymes has captivated scientists for decades. Although non-heme iron systems, both mononuclear and dinuclear, constitute a substantial portion of known metalloenzymes, they are still only rarely associated with selective alkene epoxidation. Mononuclear non-heme iron oxygenases are well appreciated for catalyzing a wide range of reactions, including desaturation, hydroxylation, cyclization, dehydrogenation, and, in some cases, epoxidation.[56] These oxygenases are predominantly involved in biosynthetic pathways, particularly the formation of secondary metabolites and natural products such as alkaloids, terpenoids, and polyketides.[57] To activate molecular oxygen, they utilize a common structural motif known as the "2-His-1-carboxylate facial triad," consisting of a mononuclear iron center coordinated by two histidine residues and one carboxylate (glutamate or aspartate) residue.[58] An additional organic cofactor (α-ketoglutarate or tetrahydrobiopterin) is required in some enzymes.[59] High-spin oxo-iron species are often implicated in the mechanisms of non-heme iron oxygenases, though the specific catalytic mechanisms and intermediates may vary depending on the particular enzyme. Typically, the epoxidation pathway for a saturated C–H bond proceeds via a ferryl (Fe(IV)-oxo) species (Figure 3A), which initiates hydrogen atom transfer (HAT), forming an Fe(III)-OH complex (Figure 3C), followed by rebound desaturation and epoxidation of a substrate compound as seen in AsqJ oxygenase, HppE non-heme oxygenase, and similar enzymes.[60–62] When considering the use of such enzymes as epoxide producers for broader biotechnological applications, it is important to note that epoxidation activity in mononuclear non-heme iron enzymes is relatively rare. Moreover, it is typically associated with a complex reaction mechanism that often involves a preceding desaturation, dehydrogenation, or elimination step before the epoxide is formed. In some cases, these processes even compete against each other (depending on the specific substrate), diminishing possible epoxide synthesis. Additionally, enzymes from biosynthetic pathways tend to exhibit relatively narrow substrate specificity, and methods to enhance their substrate promiscuity to non-natural substrates remain unclear, as there have been few successful efforts in this area. More promisingly, recent reports are providing deeper mechanistic insights, gradually revealing previously unknown aspects of these enzymes. Key residues have been identified that are involved in substrate positioning and reactivity, such as in taurine α-ketoglutarate dioxygenase (TauD).[63] The ability to influence reaction outcomes (epoxidation vs. hydroxylation), as seen in hyoscyamine 6β-hydroxylase (H6H) from the scopolamine biosynthetic pathway,[64] has also been demonstrated. On a more positive note, the relatively simple structure and flexible iron center of these enzymes make them attractive candidates for engineering, allowing advanced quantum chemical calculations and *in silico* modeling,[65] which can then be validated through experimental methods. Indeed, these oxygenases are already being engineered with non-canonical amino acids as axial ligands of the iron center to modify oxofunctionalities for desired applications,[66] potentially enabling access and generation of specialized epoxidases. Another notable advantage for future biosynthetic applications is their reliance on abundant, small, and relatively inexpensive metabolites (e.g., α-ketoglutarate or pterin) as sacrificial reductants rather than requiring complex redox partners like heme- and flavin-dependent enzymes. Since several non-heme iron oxygenases have already entered the industrial scale,[67] the effective epoxide-forming enzymes (natural or genetically improved) would likely be much appreciated.

Unlike their mononuclear counterparts, non-heme dinuclear iron enzymes are primarily found in bacterial catabolic pathways, where they play a crucial role in the assimilation of various hydrocarbons, including methane, ethane, butane, ethene, propene, tetrahydrofuran, phenol, and toluene, as carbon sources.[68] These enzymes are typically multicomponent systems, where the active site, a carboxylate-bridged dinuclear iron center, is located deep within a four-helix bundle in the catalytic subunit.[69] This site is capable of generating a Fe(IV) "diamond core" structure, known as compound Q, which is considered the most powerful oxidant found in nature (Figure 3B).[70] As a result, these enzymes can catalyze the highly challenging C–H bond cleavage of methane, ethane, propane, and butane—a feature no other enzyme has been reported to achieve. Additionally, these enzymes can utilize a high-spin Fe(III) peroxo-bridged intermediate, designated as H_{peroxo} (Figure 3D). It has been suggested that H_{peroxo} is a more electrophilic oxidant than Q, favoring two-electron oxidation pathways (e.g., epoxidation of

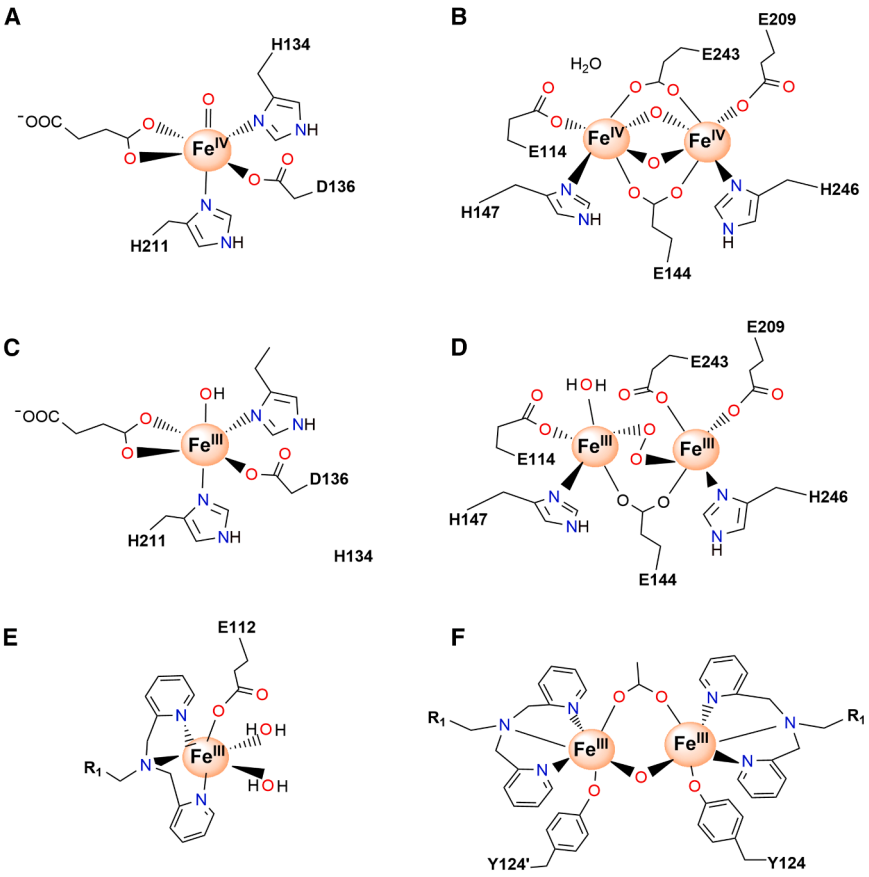

Figure 3. Structural features of non-heme iron centers

(A and C) Ferryl (Fe(IV)-oxo) species (A) and Fe(III)-OH complex (C) from AsqJ oxygenase (PDB: 6K0E), representing catalytic centers of mononuclear non-heme iron enzymes in various states. (B and D) Fe(IV) diamond-core structure, compound Q (B), and Fe(III) peroxo-bridged structure, H_{peroxo} (D), from sMMOs (PDB: 6D7K) showcase the main active forms of dinuclear non-heme iron enzymes.

(E and F) Artificial non-heme iron centers are displayed, both capitalizing on biotin-streptavidin technology and Biot-et-DPA (R_1) ligands, mononuclear (E, PDB: 6UIY) and dinuclear (F, PDB: 6VO9).

olefins). In contrast, Q predominantly facilitates one-electron oxidation (e.g., methane-to-methanol conversion).[68] Therefore, in addition to their natural role in hydrocarbon hydroxylation, enzymes such as soluble methane monooxygenases (sMMOs) were exploited to convert ethene, propene, 1-butene, and certain halogenated analogs into their corresponding epoxides.[71] Bulkier substrates, such as 1-pentene, 2-pentene, 1-hexene, 1,7-octadiene, 1-octene, and some aryl alkenes, can be converted to epoxides using alkane monooxygenase (AlkB) and various toluene monooxygenases (ToMOs).[72,73] Most recently, the phenol monooxygenase-type PmlABCDEF (Pml) enzyme has been demonstrated to catalyze epoxidation even with a broader range of vinyl and allyl arenes.[74] This enzyme also enables the selective oxidation of *O*-alkenyl-substituted pyridines, primarily producing the corresponding epoxides over potential *N*-oxide products while maintaining decent stereoselectivity.[75] Thus, their synthetic utility is evident, as dozens of different substrates have been successfully converted into epoxides. However, this substrate promiscuity comes at a cost, often leading to overoxidation or selectivity issues. In some cases, particularly with sMMOs and AlkB, the desired epoxides were accompanied or even dominated by alcohol byproducts resulting from the C–H oxidation instead of the C=C double bond.[71,72] Stereoselectivity also varies: small olefins and styrene derivatives yield epoxides with ee values barely exceeding 50%, requiring the application of directed evolution methods for improvement.[76] In contrast, some enzymes can produce longer-chain epoxide and aryl glycidyl ethers with moderate to excellent yields (75%–100% ee).[74,77] Another hurdle is the heterologous expression of non-heme diiron monooxygenase genes in convenient, fast-growing hosts like *E. coli*, which is often challenging, sometimes for reasons that are not clear. It is likely a combination of the following factors: the phylogenetic distance between hosts, physiological stress caused by misfolded proteins, the multisubunit nature of the enzyme complex, and stress from reactive oxygen species (ROS) generation.[78] Therefore, we can speculate that the ideal epoxidase, based on non-heme diiron proteins, should be stripped of "unnecessary" subunits and exist as a single-peptide protein, facilitating expression as a recombinant protein. This putative enzyme should also feature direct electron transfer and preferentially utilize H_2O_2 rather than requiring reduced electron carriers, such as NAD(P)H or FADH. This might have seemed overambitious decades ago, but it is now gradually becoming a reality as we witness the development of catalytically active miniature forms of sMMO (mini-sMMO) and the functional expression of sMMOs in *E. coli*.[79,80] Since non-heme diiron enzymes have already been subjected to commercialization, and research on modifying their enzymatic activity has been successful, simpler enzymatic systems hold transformative potential beyond just epoxide synthesis.[69,81]

Drawing inspiration from the role of non-heme iron proteins, artificial metalloprotein (ArM) systems represent a cutting-edge frontier in bioinorganic chemistry, with the potential to overcome the challenges associated with the natural non-heme iron enzymes described previously while offering additional benefits such as enhanced reactivity, the ability to catalyze reactions absent in nature, sustainability, and tunability. For mononuclear iron ArMs, various protein scaffolds have demonstrated their ability to accommodate iron centers, which can be incorporated naturally through metal ion coordination or supplemented as complexes with polydentate ligands.[82] Coordination is facilitated

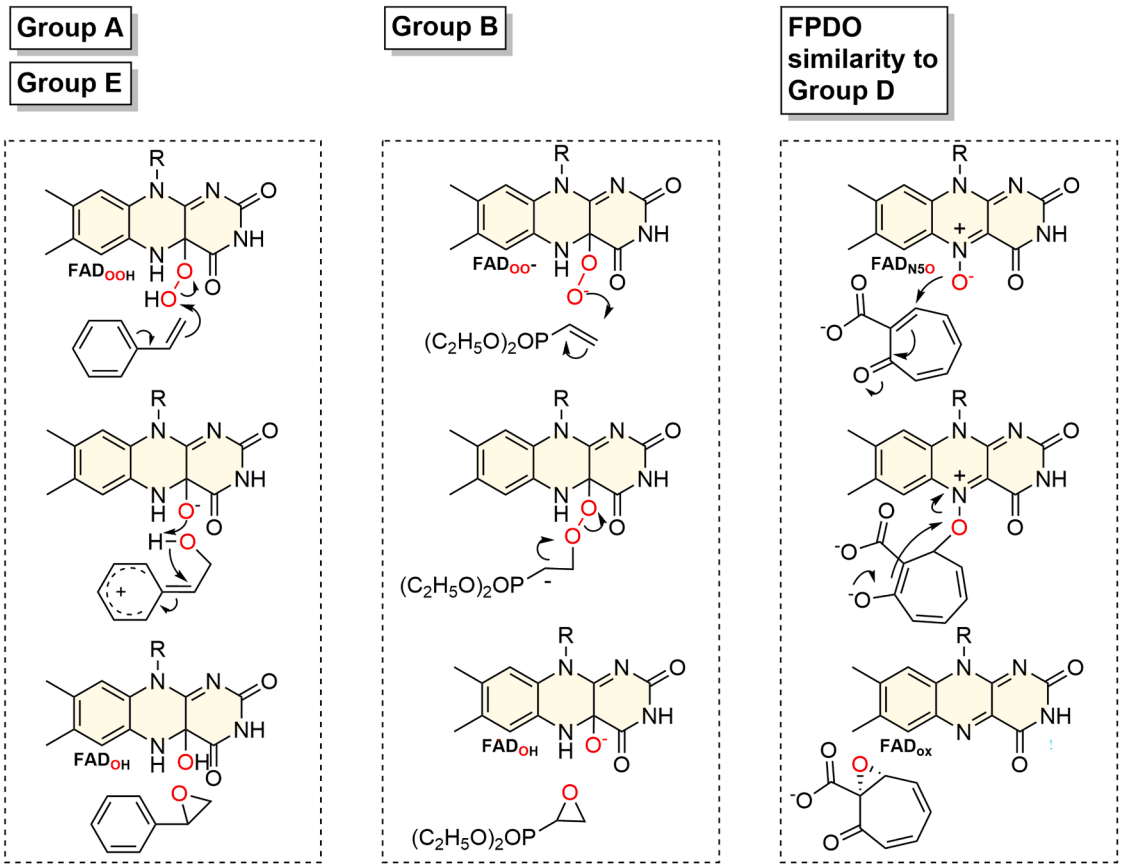

Figure 4. Flavoproteins harboring reduced flavins in the active site can activate molecular oxygen toward different species and allow substrate epoxidation

Here, only the (proposed) substrate attack leading to the epoxide is shown. Especially, case group B (CHMO) awaits mechanistic verification, and epoxidation was only observed for substrates bearing a strong electron-withdrawing group.

by either natural or non-natural amino acids (Figure 3E). In contrast, incorporating diiron active sites into ArMs remains challenging due to the complexity of tailoring a binding environment for two metal centers (Figure 3F).[83] While non-heme iron ArMs have demonstrated some oxidative catalytic activity, their research and development are still in the early stages; therefore, their ability to catalyze epoxidation remains largely unexplored. Significant time and research will likely be required before artificial epoxidases of this nature are successfully developed. A major focus is ongoing advancements in enzyme engineering and modeling tools, particularly with the aid of artificial intelligence (AI), which could accelerate the creation of functional epoxide-generating non-heme iron molecules. Whether natural but tailored enzymes or bioinspired ArMs will emerge as the superior approach in this field remains speculative.

EPOXIDATION BY MEANS OF FLAVOPROTEINS

Flavoprotein monooxygenases (FPMOs) employ flavin mononucleotide (FMN) or flavin adenine dinucleotide (FAD) cofactors to activate molecular oxygen (O_2) toward a flavin-peroxy-intermediate to facilitate substrate oxygenation (Figure 4).[84] We distinguish eight FPMO groups that catalyze a plethora of reactions, such as hydroxylation, heteroatom oxygenation, Baeyer-Villiger oxidation, light emission, and epoxidations.[85] Many occur naturally, but some are artificial in the sense that the reactive flavin intermediate facilitates the oxygenation of non-natural substrates.

In natural product biosynthesis, the bacterial formation of monensin and lasalocid A includes crucial regioselective and stereoselective epoxidations toward the final bioactive products.[86] PhqK is a fungal group A FPMO involved in alkaloid conversion derived from *P. simplicissimum*.[87] This enzyme is a single-component FPMO that epoxidizes the indole alkaloid paraherquamide K or L. The reaction takes place at the indole moiety and triggers a semi-pinacol rearrangement toward either paraherquamide M or N. Other single-component FPMOs also perform epoxidations, which are of importance for metabolism in eukaryotes. One example is cholesterol synthesis, in which squalene epoxidase (SQLE) transforms squalene to (3*S*)-2,3-oxidosqualene.[88,89] In plants, the stepwise synthesis of violaxanthin from zeaxanthin is achieved by means of zeaxanthine epoxidase (ZEP). Both SQLE and ZEP are localized in membranes and use FAD as a cofactor to activate molecular oxygen. The reducing equivalents are provided from electron-transfer

complexes located in membranes as well. More structural and mechanistic details are needed to properly classify the enzymes, as both SQLE and ZEP are structurally close to group A FPMOs, but some representatives utilize additional reductases, making them two-component systems like group E FPMOs.

Cyclohexanone monooxygenase (CHMO) is one of the best-studied type I BVMOs (group B of FPMOs) and is known for its substrate promiscuity.[90] It is a single-component FPMO and harbors a tightly bound FAD cofactor, while NADPH serves as a reductant. Importantly, the nicotinamide co-substrate remains bound throughout the catalytic cycle. The prototype substrate is a keto-compound, such as cyclohexanone, that undergoes Baeyer-Villiger oxidation. Besides its natural role, CHMO can perform various artificial oxygenations, including asymmetric epoxidations, which result in short-lived intermediates. In the case of BVMOs, a C4a-peroxy-FAD (anionic flavin species) is the reactive intermediate that attacks substrates as a nucleophile. CHMO could only perform epoxidation of a narrow type of substrate structure, such as vinyl phosphonates, carrying a strong electron-withdrawing group. As soon as this electron-withdrawing effect is lowered by other substituents at the vinyl moiety, no epoxidation is observed anymore. Nevertheless, the observed reactions were highly enantioselective (>98% ee) and stipulated to be promising toward the synthesis of fosfomycin [(−)(1*R*,2*S*)-1,2-epoxypropylphosphonic acid], which is a potent antibiotic compound. Unfortunately, the respective precursor, diethyl-1-propenyl phosphonate, was not converted. This might resemble a potential target for protein engineering to improve BVMOs to accept other vinyl phosphonates for asymmetric epoxidations.

The majority of FPMOs are two-component systems, and among them, several systems can perform selective epoxidations. Those are often naturally involved in degradative processes, e.g., in the degradation of plant-based materials and of xenobiotics, which can all be accepted as carbon sources for specialized organisms. Auxins, especially indole-3-acetic acid (IAA, or heteroauxin), are very prominent plant hormones. Related compounds arise from bacterial metabolism of tryptophan or can be found in plants and even mammalian organisms, making them abundant in various environments. As a logical consequence, some bacteria were found to utilize those compounds as carbon sources. Recently, the metabolic pathway of IAA degradation in *Caballeronia glathei* DSM50014 showed that a group D FPMO (designated as IacA/G) initiates the route via epoxidation of IAA. The formed 2,3-epoxy-IAA is rather unstable, and water is rapidly incorporated to yield a respective dihydrodiol. This two-component system obtains reducing power likely from NAD(P)H via a flavin reductase IacG. The detailed mechanism of IacA/G and the mode of action within the pathway await elucidation. From a phylogenetic point of view, this system forms a new subgroup among the group D FPMOs with potential for application, as multiple IAA derivatives are converted in a regioselective fashion.

Two very prominent FPMO systems for regio- and enantioselective epoxidation are SMOs and the related indole monooxygenases (IMOs). Both belong to the two-component group E FPMOs, where the reduced FAD is generated by a reductase and can either be directly transferred or diffuse toward the monooxygenase subunit.[91–93] This opens a plethora of options to drive these systems for selective oxygenations. Making use of the natural reductase or related enzymes in natural and artificial fusion enzymes and of chemical or electrochemical reduction, even hydrogen-driven cascades have been studied.[94,95] All approaches share the goal of maximizing the efficient conversion of reducing power into epoxidation activity (coupling). However, these enzymes are prone to uncoupling, and a lot of the energy provided to reduce FAD, which is needed to activate the molecular oxygen in the monooxygenase, is wasted, as the formed peroxy-FAD decomposes rapidly to oxidized FAD and H_2O_2 before the substrate can be epoxidized. More effort is needed to make the enzymes efficient. With excellent enantioselectivity, SMO and IMO convert styrene-like compounds predominantly into the *S*-styrene epoxide. Interestingly, another set of enzymes belonging to this FPMO group was found to be *R*-selective, giving access to both enantiomers of styrene oxide and related molecules.[96] With the stereoselectivity and how it can be modulated uncovered, powerful FPMOs for stereoselective epoxidation can be designed.[97] Still, the natural substrate awaits to be elucidated, and their catalytic power needs to be improved.

Very recently, a novel type of FPMO was described as an archetypal flavin-dependent dioxygenase.[98] It was designated TdaE and is involved in the bacterial tropone natural product biosynthesis. These compounds have various roles in nature due to their chemical structure, which results in signaling, antibiotic, and virulence factor activities, among others. TdaE uses a flavin to activate molecular oxygen and introduces the two O atoms into the substrate in a consecutive manner. Respectively, its classification as dioxygenase or monooxygenase is controversial. Considering the substrate and final product, one can see this as dioxygenase with a complex reaction mechanism performing two consecutive monooxygenation reactions. Structure-wise, the enzyme has similarities with monooxygenases containing an acyl-coenzyme A (CoA) dehydrogenase fold. It is most interesting that this special activity is achieved by an uncommon oxygen activation mechanism, which occurs via flavin-N5-peroxide and flavin-N5-oxide species, respectively. To initiate the catalytic cycle, the flavin needs to be reduced by substrate dehydration; NADH or NADPH does not allow enzyme reduction. Thereafter, the reduced flavin activates molecular oxygen. These two reactive species are needed for the consecutive CoA-ester cleavage and epoxidation in tropone biosynthesis.

FPMOs are powerful epoxidases, and some epoxides are short-lived, as mentioned, due to their chemical nature and subsequent rearrangements or autocatalytic reactions. Hence, more potential epoxidation reactions by this type of catalyst are to be expected and await to be uncovered from nature's toolbox.

OTHER ENZYMATIC EPOXIDATION APPROACHES

In recent years, alternative enzymatic epoxidation systems have also been developed. The general idea is to leverage enzymatic frameworks for the direct or indirect use of ROSs (usually H_2O_2, t-BuOOH, or *in-situ*-generated peracids) to facilitate epoxidation. This strategy has led to the emergence of several novel peroxygenase-like biocatalysts. A prominent example

Table 1. A comparison of different enzymes applicable for epoxidation reactions

Biocatalyst	Oxidant	Substrate scope	Regioselectivity	Enantioselectivity	Cofactor recycling	Scale	Room for improvement	Reference
Cytochrome P450 monooxygenases	O_2	moderate	moderate to excellent	moderate to excellent	required or whole-cell conversion	10 mg–1 kg	catalytic efficiency	Yan et al.[55]; Kubo et al.[107]
Heme peroxygenases	H_2O_2/ tBuOOH	narrow (FAPO) to very wide (UPO)	moderate to low for UPOs	low-moderate	none required	10–100 g	enantio- and regiospecificity, eliminating unwanted reactions	Kluge et al.[45]
Non-heme metalloproteins	O_2	very wide	moderate to excellent	moderate to excellent	whole-cell conversion	>1 g	development of an *in vitro* system, minimized enzymatic complex	Petkevičius et al.[108]
Artificial metalloproteins	O_2/ H_2O_2/ AcOOH	narrow-moderate	moderate	moderate	none required	mg	catalytic efficiency	Sauer et al.[109]
FPMOs	O_2	moderate	moderate to excellent	moderate to excellent	required or whole-cell conversion	g–kg	unwanted reactions, reducing uncoupling	Colonna et al.[90]; Tischler et al.[110]
Lipases	H_2O_2	wide	low	low	none required	g–kg	enantio- and regiospecificity	Tannert et al.[111]
DERA or 4-OT	H_2O_2	narrow	good	moderate to excellent	none	>1 g	catalytic efficiency	Sigmund et al.[100]; Zhou et al.[101]

is 4-oxalocrotonate tautomerase (4-OT), which has been successfully engineered to catalyze the oxidation of a wide range of α,β-unsaturated aldehyde substrates.[99] This system demonstrates high conversion rates (up to 98%), excellent enantioselectivity (up to 98% ee), and good product yields (50%–80%). Notably, it operates independently of cofactors, is adaptable to enzymatic engineering, and has been successfully applied at the gram scale—making it a promising starting point for the development of environmentally friendly, large-scale biocatalytic processes for the synthesis of enantiopure epoxides.[100] Interestingly, this α,β-unsaturated aldehyde framework was also tested with another non-natural peroxygenase, this time based on 2-deoxy-D-ribose-5-phosphate aldolase (DERA).[101] Notably, both anti- and syn-selective epoxidations were achieved, exhibiting moderate to high diastereoselectivity and excellent enantioselectivity. Despite these promising results, the α,β-unsaturated aldehyde framework also presents limitations, as its presence appears to be a prerequisite for substrate conversion. Hydrolases can be used not only in the traditional role of catalyzing the kinetic resolution of racemic mixtures but also in direct epoxidation processes. One such approach utilizes lipases to generate peroxycarboxylic acids *in situ* using a combination of an organic solvent, a carboxylic acid, and aqueous hydrogen peroxide (H_2O_2); the resulting peroxycarboxylic acids can then be applied to epoxidize alkenes.[102] These systems are currently applied to the conversion of terpenes, with ongoing studies investigating a range of lipases, highlighting the potential for broader application to synthetically valuable transformations.[103,104] Moving forward, we can expect this technology to exploit the advantages inherent in the use of hydrolases (simplicity, the absence of a need for coenzymes and their regeneration, and relatively easy scale-up); however, a notable limitation of this method is the lack of stereoselective control, as epoxides are produced as racemic mixtures.[105,106]

CONCLUSIONS AND FUTURE PERSPECTIVES

It can be pointed out that, in general and formally, independent of the enzyme class, there are "oxygen-philic" and "peroxide-philic" reactions (Table 1). Based on the intrinsic properties of individual oxygenases, a dichotomy in the application of enzymes for epoxidation reactions is forming. Hence, oxygen-philic biocatalysts seem to be better suited for whole-cell conversions, including engineering complex metabolic pathways for biosynthesis of fine chemicals or for *in vitro* conversion when, in addition to the target double bond, the substrate harbors other peroxide-sensitive groups or scaffolds. In contrast, peroxide-philic reactions are potentially more easily scalable; therefore, *in vitro* conversions at large commodity levels could be feasible.

In any case, multiple challenges have to be solved to make a potential industrial process involving a biocatalytic epoxidation economically attractive.

(1) A reliable prediction of substrate scope, especially enantioselectivity, for any target enzyme in a proper time course is still unavailable, notwithstanding a fast progression in protein modeling. Such a shortage in *in silico* methods profoundly hampers a high-throughput screening of the desired variants of biocatalysts.

(2) An efficient production of soluble and active recombinant epoxidases, particularly consisting of multiple hetero-subunits or components, still requires an enormous empirical effort and input.
(3) Organic solvents and high substrate load-resistant enzymes are highly desired to achieve process intensity of industrial relevance. Especially for the oxygen-philic enzymes, this is still difficult to achieve.
(4) The intrinsic reactivity of epoxides, especially at high concentrations, could affect their integrity and thus the efficiency of the biocatalytic system. Reaction engineering needs to address the requirements of the enzyme as well as of the substrate/product pair.
(5) Life cycle assessment is an extremely valuable tool for the evaluation of the developed synthesis pathways and, specifically, to identify critical bottlenecks to improve the sustainability of the overall process. To date, appropriate studies on the valuation of chemical epoxidation vs. biocatalytic ones are scarce, if not unique.[111]

To summarize, we cannot point at one or another group of biocatalysts and announce it as a frontrunner to evolve for asymmetric epoxidation on an industrial level, as real-world examples remain limited, and many relevant studies are still in progress. For example, ongoing efforts to engineer P450 BM3 for the synthesis of a key precursor to the anti-tuberculosis drug delamanid not only streamline existing synthetic routes but also open new avenues for designing sustainable, selective, and cost-effective processes in the development of both new and existing pharmaceuticals.[112] This is likely to become a key focus area for biocatalytic epoxidation as enzymatic methods continue to evolve and are poised to play a central role in the future of selective and affordable drug manufacturing. Moreover, a growing body of experimental and theoretical data, as well as an underexplored potential of deep learning and synthetic biology approaches, is creating an explosive mixture of knowledge that clearly is going to be converted into the development of powerful biocatalysts, including ones acting, for example, against the conventional rules of Prilezhaev's epoxidation.

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AUTHOR CONTRIBUTIONS

V.P., C.M., D.T., and R.M. wrote the initial draft and edited the manuscript.

DECLARATION OF INTERESTS

V.P. and R.M. declare potential conflicts of interest since Vilnius University has filed EPO patent application EP24158908.4.

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