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Enzymatic Oxy- and Amino-Functionalization in Biocatalytic
Cascade Synthesis: Recent Advances and Future Perspectives
Eleonora Grandi,[a] Fatma Feyza Özgen,[a] Sandy Schmidt*[a] and Gerrit J. Poelarends*[a]

[Diagram of enzymatic reactions involving oxygenation and amination processes]
Abstract: Biocatalytic cascades are a powerful tool for building complex molecules containing oxygen and nitrogen functionalities. Moreover, the combination of multiple enzymes in one pot offers the possibility to minimize downstream processing and waste production. In this Review, we illustrate various recent efforts in the development of multi-step syntheses involving C-O and C-N bond-forming enzymes to produce high value-added compounds, such as pharmaceuticals and polymer precursors. Both in vitro and in vivo examples are discussed, revealing the respective advantages and drawbacks. The use of engineered enzymes to boost the cascade outcome is also addressed and current co-substrate and cofactor recycling strategies are presented, highlighting the importance of atom economy. Finally, tools to overcome current challenges for multi-enzymatic oxy- and aminating reactions are discussed, including flow systems with immobilized biocatalysts and cascades in confined nanomaterials.

1. Introduction

Organic molecules characterized by one or more oxygen or amine functionalities are among the most used building blocks to produce pharmaceuticals, biologically active compounds, new materials, and polymers. The incorporation of oxygen and nitrogen moieties within organic molecules in traditional organic synthesis requires several chemical steps. These methods are often time-consuming, involving the isolation of intermediates and yielding a large amount of toxic waste, which is detrimental to the environment. Therefore, the development of step-economic and more sustainable enzymatic cascade approaches has gained enormous attention. In addition to their exquisite regio- and stereoselectivity, enzymes can often use renewable feedstocks as starting materials, driving chemical synthesis toward a biobased economy. Nowadays, (bio)chemists can benefit from a broad enzyme toolbox, which has greatly been enlarged through the discovery, design and laboratory evolution of new enzymes. Following the well-studied heme-dependent P450 monooxygenases (P450s) and P450-like peroxyoximes, or the flavin-dependent Baeyer-Villiger monooxygenases (BVMOs) and styrene monooxygenases (SMOs), the toolbox of oxygenating enzymes has further expanded toward a broad range of C-O bond-forming biocatalysts (Figure 1, Table 1). Some examples are the unspecific peroxyoximes (UPOs), the chloroperoxidases, and the cofactor-independent peroxyoximes, with their respective engineered variants. Other interesting groups include the O-methyltransferases (OMTs) and the FeII/epoxyglutare (2OG)-dependent oxygenases, mainly used for amino acid hydroxylation reactions. Among the aminating enzymes, some are well-known and extensively studied, for instance, the transaminases (TAs), the imine reductases (IREDS) and the phenylalanine ammonia lyases (PALs). However, new and promising C-N bond-forming enzymes emerged in recent years. Among them, the reductive aminases (RedAms), the amine dehydrogenases (AmDHs) and the C-N lyases, such as ethylenediamine-N,N-disuccinic acid (EDDS) lyase, are noteworthy (Figure 1, Table 1).
2. C-O and C-N Bond-Forming Enzymes – An Overview

2.1 C-O Bond-Forming Enzymes

Oxygenating enzymes play a crucial role in organic synthesis because of their ability to incorporate oxygen atoms into organic molecules with high chemo-, regio- and enantioselectivity. In traditional chemical synthesis, oxygenation reactions are performed with toxic oxygenating reagents or catalyzed by transition metals. Instead, C-O bond-forming enzymes can catalyze oxygenation reactions under mild reaction conditions. Among these powerful biocatalysts, the most studied thus far are the P450s and P450-like peroxyenzymes. \[53\,34\] These enzymes are especially interesting because of their ability to functionalize inert C-H bonds via hydrogenation reactions. Most P450s utilize molecular oxygen (O\(_2\)) as the oxidant. \[35\] However, O\(_2\) must first be reduced using a nicotinamide cofactor such as NAD(P)H, and an electron transport system, usually a protein partner. \[36\,37\] To simplify this complex catalytic arrangement, the use of H\(_2\)O as the oxidant instead of O\(_2\) can be a valuable alternative. However, P450s typically suffer from instability in the presence of H\(_2\)O, making their efficiency as peroxygenases rather low. \[38\,39\] More recently, research efforts have focused on tuning the catalytic activity of P450s through rational design and directed evolution beyond the simple incorporation of oxygen atoms. For instance, Arnold and coworkers focused on repurposing cytochrome P450s to catalyze nitrene- and carbene-transfer reactions as well as C-Si and C-B bond formations. \[40\,41\] In another work, a fatty acid hydroxylase from Bacillus megaterium P450, was shown to act as an artificial peroxygenase and its activity was switched to a peroxidase. \[42\] Ma et al. used P450bae5 to construct an artificial P450-H\(_2\)O system by installing a dual-function small molecule (DFSM) assisting in the H\(_2\)O\(_2\) activation. \[43\] Only a few native P450s can efficiently use H\(_2\)O\(_2\) such as P450-OleT. \[44\] These enzymes are characterized by acid-base catalytic residues that usually assist in the activation of H\(_2\)O\(_2\), while most cytochrome P450s lack this structural feature. In the system developed by Cong and coworkers, the DFSM functions as general acid-base catalyst for activation of H\(_2\)O\(_2\) and turns P450bae5 into a peroxygenase. \[45\,46\] Additionally, the engineered P450bae5-DFSM system can promote the single oxidation of guaiacol. \[47\] By combining multiple mutations of redox-sensitive residues, a variant with four combined mutations (F87A/Y160I/Y198I/M237I) was performing single oxidation of guaiacol with a total turnover number (TTN) of 8838. The authors thus showed that by rationally designing P450bae5, the peroxygenase versus peroxidase activity can be efficiently modulated. \[48\] Also, the engineering of a P450bae5-DFSM system for the direct nitration of aromatic compounds was recently achieved. \[49\] Another class of well-known C-O bond-forming enzymes is formed by flavin-dependent monoxygenases, which includes the BVMOs \[50\,51\] and SAMOs. \[52\] Among the BVMOs, cyclohexanone monoxygenases (CHMOs) are well-known representatives that catalyze the oxidation of ketones into the corresponding esters or lactones, or enol-lactones and ene-lactones. \[53\] However, BVMOs are also employed in aldehyde oxidations forming the respective carboxylic acids and alcohols, and in heteroatom oxidations, such as sulfoxidations and oxidation of amine, boron and selenium. \[54\] Despite their versatility and identified promiscuous reactivities, BVMOs typically exhibit poor stability, low expression, NADH- and oxygen-dependency and in some cases substrate inhibition, hampering their application in large-scale synthesis. \[55\,56\] Therefore, much effort has been devoted to overcome one or more of these issues affecting the biotechnological application.
of BMOs. Next to BMOs, SMOs are a highly interesting class of biocatalysts known for their ability to catalyze the stereoselective epoxidation of C=C bonds. Together with indole monooxygenases (IMOs), SMOs belong to a group of flavoproteins known as group E monooxygenases. Interestingly, like BMOs, SMOs and IMOs can also catalyze sulfoxidation reactions. Recently, the crystal structure of an IMO from Variorax paradoxus EPS (VpI ndA1) was solved in complex with FAD and 6-bromoiode, allowing valuable insights into the mechanism of VpI ndA1 and that of related group E monooxygenases. In addition, the VpI ndA1 crystal structure allowed to produce several VpI ndA1 variants with expanded substrate scope via structure-guided mutagenesis. Different from monooxygenases that use O₂ as the oxygen source, peroxygenases catalyze oxidation reactions via transferring an oxygen atom from H₂O₂ into organic molecules. UPOs and chloroperoxidases are able to introduce oxygen and halogen atoms, respectively, into a wide range of substrates with the sole co-substrate being H₂O₂. UPOs are heme-thiolate-dependent enzymes exclusively found in the fungi kingdom that catalyze the functionalization of non-activated C-H, C-C and C=C bonds. Although cytochrome P450s are able to catalyze the same reaction, especially on inert C-H bonds, they have a complex protein machinery, involving an enzyme partner for electron transfer and a reducing agent. Instead, the only oxygen donor in UPOs is H₂O₂ with no need of additional enzymes or reagents. Additionally, UPOs are characterized by their higher robustness, of which P450-like enzymes often suffer because of their frequent inactivation by H₂O₂ and oxygen uncoupling. Since the discovery of the first UPO from Agrocybe (Cyclocybe) aegerita (AeUPO) in 2004, efforts were made in improving the heterologous expression and regioselectivity of UPOs, which represent the two main issues for application on industrial scale. AeUPO was evolved in the past years to a variant, known as rAeUPO, with improved expression in heterologous organisms, like Saccharomyces cerevisiae and Pichia pastoris. The improved expression of rAeUPO paved the way for further protein engineering efforts on UPOs. For example, Alcalde and coworkers engineered rAeUPO for the efficient synthesis of 5′-hydroxypropionalanol, a human drug metabolite. Despite other limitations affecting the performance of UPOs, such as overoxidation and unwanted peroxidase activity, these enzymes continue to be highly promising biocatalysts for chemical synthesis. Although peroxygenases normally are cofactor-dependent, very recently Poelarends and coworkers evolved 4-oxobutanotenate tautomerase (4-OT) into a cofactor-independent peroxygenase that is able to accept both H₂O₂ and 1BuOOH for the asymmetric epoxidation of various cinnamaldehydes. The most promising variant, highly selective for H₂O₂, was then applied in a multi-step biocatalytic cascade for the synthesis of enantiopure epoxides and triols. Alternative biocatalysts for C-O bond-forming reactions are the OMTs and the Fell/2OG-dependent oxygenases. The former are enzymes that transfer a methyl group to an oxygen atom creating a new C-O bond. Most of the OMTs are dependent on S-adenosyl-L-methionine (SAM) as methyl donor. The main impediments for OMTs to be used on large scale are the relatively narrow substrate scope and the costly cofactor. The latter limitation has been addressed by developing cofactor-regeneration systems, while the substrate scope was expanded via protein engineering and the discovery and characterization of new O-methyltransferases. For instance, Sokolova et al. recently characterized two catechol O-methyltransferases (COMTs) from two bacterial species, Desulforomonas acetoxidans (DesAOMT) and Streptomyces avermitilis (StrAOMT). Both enzymes were biochemically characterized and their activity was investigated with a range of potential substrates, revealing that both COMTs accept catechol-like and non-catechol-like molecules. Structural analysis revealed a Mg²⁺ binding site, as well as structure similarities with other OMTs, like the characteristic SAM-binding pocket of StrAOMT, but also significant differences like the missing N-terminal helix in DesAOMT. These recently characterized OMTs open up the possibility of developing new biocatalysts via protein engineering for the O-methylation of a wide range of flavonoid-related natural products. Recently, Fell/2OG-dependent oxygenases have gained a surge in interest due to their high catalytic efficiency and remarkable turnover numbers. These enzymes are iron and α-ketoglutarate-dependent, and mainly catalyze the selective hydroxylation of non-activated C-H bonds. The hydroxylation of amino acids catalyzed by the Fell/2OG-dependent oxygenases is of high importance, leading to the formation of non-canonical amino acids (ncAA) especially in post-translational modifications of proteins. In nature, Fell/2OG-dependent oxygenases catalyze several different reactions, ranging from ring rearrangements to C-C bond formations. Because of their versatility and high efficiency, they were exploited in (chemo)enzymatic multi-step cascades, such as in the total synthesis of several complex natural products. 2.2 C-N Bond-Forming Enzymes Amide functionalities are one of the most spread functional groups present in natural products and bioactive molecules. Enzymes able to perform C-N bond formations are likewise vastly present in nature and are particularly relevant for selectively catalyzing the formation of chiral amines. Among them, TAs have successfully found application in industrial scale. TAs typically display high regio- and enantioselectivity, and no need for cofactor regeneration systems. A thorough review on the industrial application of TAs in the pharmaceutical industry has recently been published by Gilmore and coworkers, highlighting a wide variety of reactions involving α-TAs. Although TAs have successfully found their way into industrial application, several limitations still need to be addressed, for instance, the poor stability, the limited substrate scope and the challenge to produce efficient (R)-selective transaminases. Recent efforts mainly focus on finding more robust α-TAs, either by protein engineering or genome mining. IREDs form another class of biocatalysts widely studied and with a broad application to synthesize enantioenriched amines. Even though relatively newly discovered, IREDs gained attention for their ability to reduce imine groups, yielding chiral amines, with a preference for cyclic imines, and for catalyzing the kinetic resolution of chiral secondary amines. IREDs are NAD(P)H-dependent enzymes and are classified as oxidoreductases. Some IREDs can also catalyze the intermolecular reductive amination between a prochiral ketone and an amine, yet with high substrate and catalyst loading.
# Table 1. List of selected enzymes for oxy- and amino-functionalization reactions.

## C-O Bond-Forming Enzymes

<table>
<thead>
<tr>
<th>Enzyme Class</th>
<th>Enzymatic Function</th>
<th>Reaction Example</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heme-dependent P450 Monoxygenases</td>
<td>Monoxylogeny of organic molecules</td>
<td>( \text{R-H} + \text{O}_2 \rightarrow \text{R-OH} + \text{H}_2\text{O} )</td>
</tr>
<tr>
<td>Baeyer-Villiger Monoxygenases (BVMOs) – Cyclohexanone Monoxygenases (CHMOs)</td>
<td>Oxidation of ketones</td>
<td>( \text{CHMO} )</td>
</tr>
<tr>
<td>Styrene Monoxygenases (SMOs)</td>
<td>Styrene Epoxidation</td>
<td>( \text{O}_2 \rightarrow \text{CHMO} )</td>
</tr>
<tr>
<td>Unspecific Peroxygenases (UPOs)</td>
<td>Oxidation of non-activated C-H, C-C and C=C bonds</td>
<td>( \text{R-H} + \text{H}_2\text{O}_2 \rightarrow \text{R-OH} + \text{H}_2\text{O} )</td>
</tr>
<tr>
<td>Cofactor-independent Peroxygenases</td>
<td>Epoxidation of unsaturated aldehydes</td>
<td>( \text{OMT} )</td>
</tr>
<tr>
<td>O-methyltransferases (OMTs)</td>
<td>Methylation of hydroxyl groups</td>
<td>( \text{OX} )</td>
</tr>
<tr>
<td>Fell/2OG-dependent oxygenases</td>
<td>Hydroxylation of non-activated C-H bonds</td>
<td>( \text{Fell/2OG-dependent oxygenase} )</td>
</tr>
</tbody>
</table>

## C-N Bond-Forming Enzymes

<table>
<thead>
<tr>
<th>Enzyme Class</th>
<th>Enzymatic Function</th>
<th>Reaction Example</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transaminases (TAs)</td>
<td>Amination of aldehydes and ketones</td>
<td>( \text{NH}_3 ) + ( \text{R-C}=\text{N} \rightarrow \text{R-C}=\text{NH} )</td>
</tr>
<tr>
<td>Imine reductases (IREDS)</td>
<td>Reduction of imine</td>
<td>( \text{R-N} \rightarrow \text{R-NH} )</td>
</tr>
<tr>
<td>Reductive Aminases (RedAms)</td>
<td>Reductive amination of carbonyl groups</td>
<td>( \text{NH}_3 ) + ( \text{R-C}=\text{N} \rightarrow \text{R-C}=\text{NH} )</td>
</tr>
<tr>
<td>Amine Dehydrogenases (AmDHSs)</td>
<td>Reductive amination of carbonyl groups with ammonium salts</td>
<td>( \text{NH}_3 ) + ( \text{R-C}=\text{O} \rightarrow \text{R-C}=\text{NH} )</td>
</tr>
<tr>
<td>Phenylalanine ammonia lyase (PAL)</td>
<td>Amination of trans-cinnamic acid with ammonia</td>
<td>( \text{Ar}=\text{C}=\text{O} ) + ( \text{NH}_3 )</td>
</tr>
<tr>
<td>Ethylenediamine-(\text{N})disuccinic acid (EDDS) lyase (EDDS-lyase)</td>
<td>Degradation of (S,S)-EDDS via deamination</td>
<td>( \text{EDDS-lyase} )</td>
</tr>
</tbody>
</table>
Turner and co-workers discovered a subfamily of IREDs catalyzing the reductive amination with a 1:1 ratio between ketones and amines.\cite{20} This subclass of IREDs is known as RedAms and was firstly identified from Aspergillus oryzae.\cite{20} Low activity, modest stability, and restricted substrate specificity, limit the application of these enzymes for large scale production. To broaden the enzyme toolbox, novel IREDs were identified and characterized by Cárdenas-Fernández et al. for the reduction of dihydroisoquinolines (THIQs).\cite{20} In the context of expanding the substrate scope of IREDs acting as reductive aminases, Zheng and co-workers reported an IRED from Penicillium camemberti (PcIRED) able to accept a variety of challenging sterically-hindered amines.\cite{20} The authors also performed protein engineering via iterative saturation mutagenesis guided by an AlphaFold2 model of the PcIRED wild type. The best variant displayed an 488-fold increase in activity and improved stability. Subsequently, they screened the variants towards improved reductive amination of both ketones and aldehydes with a wide spectrum of bulky amines and performed a gram-scale synthesis of cinacalcet, a drug involved in the treatment of thyroid-related issues. Another enzyme class relevant for the synthesis of enantiopure amines are AmDHs. These enzymes catalyze the reductive amination of carbonyl compounds with ammonium salts to generate enantioenriched primary amines. Ammonium formate is both the amine and the reductant source, and it is regenerated by a formate dehydrogenase.\cite{20} Engineered AmDHs, evolved from amino acid dehydrogenases, are able to catalyze the asymmetric reductive amination of ketones.\cite{20} Recent efforts from Vergne-Vaxelaire and co-workers demonstrated the ability of native AmDHs in performing reductive amination across a broad range of ketones.\cite{20,54} Moreover, metagenomic studies keep implementing the native AmDH pool already characterized and further engineering efforts are required to expand their substrate scope towards aromatic substrates. Among ammonia lyases, a well-studied representative is PAL, which naturally catalyzes the formation of L-phenylalanine by the amination of trans-cinnamic acid with ammonia.\cite{20} Extended engineering efforts on PAL from Petroselinum crispum (PcPAL) were performed to expand its substrate scope, enabling a wider synthetic application.\cite{20,56,57} Since product inhibition has been observed for PcPAL, PALs from different organisms, such as Arabidopsis thaliana (AtPAL) or Rhodosporidium toruloides (RtPAL) have been engineered for improved catalytic efficiency.\cite{20,56} Gram-scale synthesis of several L-phenylalanine derivatives was lately achieved by a whole-cell biocatalyst comprising engineered AtPAL.\cite{20} Another emerging biocatalyst is EDDS-lyase, which catalyzes the reversible degradation of 3(S)-EDDS via two sequential deamination steps.\cite{20} Poelarends and co-workers showed the potential of EDDS-lyase, as well as 3-methylaspartate ammonia lyase, as biocatalyst for the asymmetric synthesis of various aspartic acid derivatives, showcasing its broad amine scope.\cite{20,101,108} Moreover, enantiopure substituted pyrazolidinones were synthesized using arylhydrazines as bisnucleophiles in a one-pot chemo-enzymatic cascade.\cite{20} Additionally, engineered variants of EDDS-lyase were used in the synthesis of precursors for artificial dipeptide sweeteners, with >1000-fold activity improvement for the selective hydroamination of fumarate using non-native amines.\cite{20}

3. Enzymatic Cascades for Alcohol and Amino Alcohol Synthesis

3.1. Alcohols via C-H Oxidation

UPOs can catalyze the oxynfunctionalization of a broad range of organic molecules with high regio- and stereoselectivity utilizing H$_2$O$_2$ as clean oxidant.\cite{20,103,104} However, they suffer from low stability at high concentrations of H$_2$O$_2$ in the reaction mixture. In addition, the bulk addition of H$_2$O$_2$ is difficult to control and might lead to undesired side reactions. While the controlled and portion-wise addition of H$_2$O$_2$ is in general possible, it might lead to dilution issues. As such, different concepts for the in-situ H$_2$O$_2$ generation from O$_2$ have been developed and currently represent the preferred alternative.\cite{20} Although H$_2$O$_2$ is the co-substrate, involving the in-situ generation and re-generation of H$_2$O$_2$, these reactions can be still considered as biocatalytic cascades. Specifically, they are considered as classical (chemo)enzymatic cascades as one of the oxgens deriving from H$_2$O$_2$ is eventually incorporated in the final product. Hollmann and co-workers proposed the combination of wavelength-complementary photosensitizers with in-situ H$_2$O$_2$ generation for the development of an efficient oxynfunctionalization system (Scheme 1 – a).\cite{20} In this work, formate was used as sacrificial electron donor to generate H$_2$O$_2$ in situ using formate dehydrogenase (FDH) and different photocatalysts, followed by coupling with the peroxygenase-catalyzed hydroxylation of ethylbenzene 1, yielding compound 2. Formic acid is fully oxidized to CO$_2$ by a FDH from Candida boidinii (CtFDH), resulting in the reduction of NAD$^+$ to NADH. The latter is photochemically oxidized under aerobic conditions leading to the in-situ production of H$_2$O$_2$. Several dyes with photocatalytic activity were screened for the oxidation of NADH. Among them, three were chosen (phenosafranine, methylene blue, and FMN) and combined with an evolved variant of rAaeUPO, already known for the hydroxylation of ethylbenzene.\cite{20,105,108,109} Control experiments showed that the photochemical reduction of NAD$^+$ was the rate-determining step in the conversion of 1 to 2.
Scheme 1. General scheme of enzymatic cascades catalyzed by peroxzymes with in-situ H$_2$O$_2$ production for the synthesis of aliphatic and aromatic tertiary alcohols: a) combination of enzymatic and photocatalytic H$_2$O$_2$ generation system; b) fully enzymatic H$_2$O$_2$ generation system; c) photocatalyzed H$_2$O$_2$ in situ generation utilizing SAS for the hydroxylation of ethylbenzene (c-1), halogenation of thymol (c-2) and for the synthesis of cyclohexanone 9 (c-3).

However, biocatalytic cascades employing photocatalytic systems are still far from a preparative application. For instance, in the example described above, the robustness of the FDH represents a major limitation in this system. Therefore, Hollmann and co-workers suggested an alternative strategy. Instead of adopting a photocatalytic system, a formic acid oxidase from Aspergillus oryzae (AoFOx) was applied for the direct oxidation of formic acid to CO$_2$ and H$_2$O$_2$.

Here, the photocatalytic step is not present; thus the whole system is simplified in a two-step fully enzymatic cascade. The rAaeUPO can directly use the H$_2$O$_2$ generated from formate oxidation for the oxygenation reaction (Scheme 1 - b). The optimization of the reaction conditions was directed considering some critical aspects. Firstly, the optimal ratio of 1:5 AoFOx:rAaeUPO was determined for a stable product formation. Then, the O$_2$ availability was investigated,
and it was observed that by increasing the O₂ pressure within the system the amount of final product increased by more than ten-fold. Once the optimum reaction conditions were established, the substrate scope of the whole system was investigated, and some hydroxylation and epoxidation reactions were analyzed. High turnover numbers were achieved (approx. 300,000) for the AoFOx involved in the generation of H₂O₂, highlighting the economic advantage of the system. As a proof-of-concept, the authors investigated further H₂O₂-dependent enzymes in combination with AoFOx, such as cytchrome C (CytC), known for catalyzing sulfidoxidation reactions, or the lipase CaIB for the dehydrogenation of carboxylic esters to yield either epoxides or lactones via a chemoenzymatic cascade.[113] However, the turnover numbers reached were relatively low with either CytC or CaIB. The H₂O₂ in situ generation system employing AoFOx was also used for halogenation reactions using vanadium-dependent haloperoxidases.[113] Here, when starting with 4-pentanolic acid, remarkable results were obtained, and a gram-scale reaction was achieved with 45% isolated yield for the halogenated product. However, the in situ enzymatic generation of H₂O₂ presents a major drawback: the limited range of sacrificial electron donors that can be used. Therefore, more recently, Hollmann and co-workers focused again on photocatalytic systems for the in situ production of H₂O₂.[115] To overcome photocatalysis, prevent formation of reactive oxygen species, and to access a broader sacrificial electron donor scope, the authors chose sodium anthraquinone sulfonate (SAS) as photo-catalyst. SAS is water-miscible and can promote the oxidation of methanol or other small sacrificial electron donors, reducing O₂ into H₂O₂. Furthermore, the SAS oxygen activation mechanism does not involve the formation of stable radical species, which can damage the biocatalysts used.[116] Considering all the advantages of SAS, it was used for the photocatalyzed in situ H₂O₂ formation coupled with a chloroperoxidase from Curvularia inaequalis (CN-CPO). CN-CPO is characterized by a catalytic vanadium binding site for the halogenation of thymol, with methanol as both co-solvent and sacrificial electron donor (Scheme 1 – c-2). Several parameters needed to be optimized. For instance, increasing the photocatalyst concentration after a certain threshold harmed the overall reaction outcome, leading to the accumulation of undesired side products. In contrast, changes in the biocatalyst concentration did not significantly affect the product formation. Also, other alcohols were investigated as cosolvents and electron donors, for instance 2-propanol gave similar results compared to methanol in terms of mM of compound 6 obtained, and a semi-preparative scale reaction (967 mg of 6) was achieved with an isolated product yield of 45%. Likewise, the hydroxybromination of styrene resulted in a good yield of 61% and the bromolactonization of 4-pentanolic acid yielded 80% of the final product. CN-CPO is highly stable, and the whole system itself shows great robustness with over 30 h of continuous production form. Therefore, rAsUPO, a less stable peroxozyme compared to chloroperoxidases, was coupled with SAS in the photocatalytic system. While the hydroxylation of ethylene benzene 1 resulted in only low enantioselectivity (34% ee) and the presence of overoxidized side products (Scheme 1 – c-1), the overoxidation of cyclohexane 7 to cyclohexanone 9 was desirable as it serves as the precursor for ε-caprolactone, a key intermediate of different polymers, like the nylon-6 monomer or 6-aminocaproic acid (Scheme 1 – c-3).[117-126] By adapting the ratio between 8 and 9 production in a double phase system (water phase/organic phase), 71% yield of 9 in the aqueous phase and excellent turnover numbers were achieved. This work highlights that SAS is a valid alternative to the classic heterogeneous photocatalysts as it enables high turnover numbers of the biocatalyst due to the absence of oxygen radical species in solution. However, these photocatalytic systems require further optimization, especially on the reaction concepts enabling constant illumination of the photocatalyst and enhancing the performance of the photocatalyst.

3.2 Alcohols and Amino Alcohols via Epoxidation of Alkenes

Optically pure epoxides are valuable intermediates in enzymatic cascades for synthesizing different building blocks, such as diols and amino alcohols, via enzyme-catalyzed nucleophilic epoxide ring-opening. Usually, either a monoxygenase or a peroxygenase is involved in the epoxidation step. As previously described, when H₂O₂ is the oxygen source, it is crucial to introduce an in situ H₂O₂ production system. In 2019, Yu et al. reported a three-step enzymatic cascade where an oxidase and a peroxygenase were combined (Scheme 2 - d).[121] The three-enzyme cascade was established to produce (R)-phenyl glycols 13 from 3-phenyl propanic acid or derivatives thereof (10). In the described system, two P450-like enzymes, the monoxygenase P450₉₃MD and the peroxygenase OleTₑ were coupled with an epoxide hydrolase from Aspergillus niger (AnEH) and CBFDH for cofactor recycling. The other P450-like enzyme, OleTₑ, uses the in situ formed H₂O₂ as a co-substrate for the formation of the styrene intermediate (11). With this, a mutual-benefit system is formed, and the oxidative stress brought by H₂O₂ to the P450₉₃ is overcome. A third enzymatic step was added to open the epoxide intermediate by an epoxide hydrolase, AnEH, giving the final phenyl glycol and derivatives with high conversion (up to 98%) and enantioselectivity (up to 97% ee). Epoxide intermediates are also usually present in the synthesis of either amino alcohols or amino alcohol precursors (such as vicinal azido alcohols). Paul and co-workers recently presented an attractive example of a multi-enzymatic cascade via epoxide intermediate formation.[122] On the one hand, the authors coupled a flavin-dependent styrene monoxygenase (SMO) with a halohydrin dehalogenase for the catalytic azidolysis of an epoxide intermediate, producing enantioenriched β-azido alcohols (17). On the other hand, the SMO was coupled with a chemical step by the addition of sodium azide as a nucleophile to produce α-azido alcohols (16) with high conversion (up to >99%) (Scheme 2 - e). Enantioenriched azido alcohols are interesting precursors for producing chiral aziridines and 1,2-amine alcohols, highly used as building blocks of bioactive molecules.[120] SMOs are flavoproteins composed of two subunits, StyB, the reductase component, and StyA, the oxidative component. StyB uses NADH to reduce the flavin cofactor (FAD), which StyA then utilizes for the effective oxygenation reaction. To avoid the necessity of a cofactor regeneration system, typically needed when NADH is involved, the authors chose a nicotinamide coenzyme biomimetic (NCB), 1-benzyl-1,4-di-hydroxynicotinamide (BNAH), used as the reductant by StyA. Remarkably, BNAH as the hydride donor gave comparable results to the StyB/NADH system.[120] The epoxidation step was firstly optimized by screening for the best StyA, and the StyA from Sphingopyxis fribergensis Kp5.2 (SfStyA) was selected. In addition, the enzyme and FAD concentration, and the BNAH equivalents were also optimized. After establishing the best conditions for the epoxidation step, a one-pot, two-step chemo-enzymatic cascade for production of mainly aromatic epoxides was established. Next, α-azido alcohols (16) were obtained by using sodium azide, following the ring-opening of aromatic epoxides.[124,126]
Scheme 2. Enzymatic cascades comprising monoxygenases in combination with either peroxygenases (d), halohydrin dehalogenases (e), epoxide hydrolases (f, g), or transaminases (f and g) for the synthesis of alcohols and amino alcohols via the formation of an epoxide intermediate. AP = acetophenone.

Good to excellent conversions were achieved (90-99% for most of the compounds tested) with high regioselectivity (up to 98:2 α-azido alcohol:β-azido alcohol ratio) and excellent ee of >99%, for all the substrates investigated. Furthermore, a one-pot, two-step enzymatic cascade was established to synthesize β-azido alcohols (17) for three selected substrates. In this case, several halohydrin dehalogenases (HHDHs) were investigated towards rac-styrene oxide and (S)-styrene oxide, with sodium azide as the nucleophile. HhE5 gave the
best results with a $\alpha:\beta$ ratio of 18:84 and overall good to excellent conversion (up to 73%) and enantio- and regioselectivity. In this work, Paul and co-workers showed an alternative use of a highly active and selective MO. S-13A, coupled with a nicotinamide cofactor biomimetic in either a chemo- or fully enzymatic one-pot two-step cascade to produce both $\alpha$- and $\beta$-aromatic azido alcohols as amino alcohol precursors. In the same year, Wu et al. adopted a similar in vitro approach by constructing two biocatalytic cascades combining an SMO with two HHDHs to synthesize both $\alpha$- and $\beta$-azido alcohols from styrene and styrene derivatives.[126]

The coupling of styrene monoxygenases with epoxide ring-opening enzymes, such as EHs or HHDHs is a useful strategy to synthesize diols and further 1,2-amino alcohols. This strategy can be applied in vitro using purified enzymes or cell-free extracts and in vivo using either resting cells or cell cultures to avoid time-consuming protein purification steps. In the past steps, several examples of whole-cell biocatalysts underlined this strategy's advantages for alcohol and amino alcohol biosynthesis via epoxide intermediate formation, either with single-cell catalysts or a multiple-cell modular approach.[127–129]

A recent work from Chang and co-workers showed the application of a one-pot four-enzyme cascade for both in vitro and in vivo catalysis involving a SMO, an EH, an alcohol dehydrogenase (ADH), and a TA.[130] With their approach, the authors transformed easily available styrenyl olefins (18) into both (S)- and (R)-$\alpha$-amino alcohols (2- amino-2-phenyl ethanol) reaching high yields (up to 64.3%) and enantioselectivities, $>99\%$ for both enantiomers (Scheme 2 - g).

Firstly, purified enzymes were used in an in vitro approach. Afterwards, a whole-cell system was constructed with E. coli cells expressing the four enzymes in two different strains. For the first step, the SMO from Pseudomonas sp. VLB120 was selected for its known extensive application.[131,132] An epoxide hydrolase from Sphingomonas sp. HXN-200 was chosen in the hydroxylation step for its high selectivity and activity towards (S)-styrene oxides (19). The three enzymes involved in the oxidation step for the formation of the $\alpha$-hydroxy ketone intermediate (23), was a (S)-selective polyl dehydrogenase (GoSCHR) from Gluconobacter oxydans. The SMO and the GoSCHR are cofactor-dependent enzymes, utilizing NADH and NAD$,^+$ respectively. Therefore, an autonomous cofactor regeneration system was created. Finally, two TAs were chosen to produce either the (S)- or the (R)-enantiomers of the $\alpha$-amino alcohols. An (R)-$\alpha$-TA (MVTA) from Mycobacterium vanbaalenii was identified by the authors as the preferred catalyst for the synthesis of (S)-$\beta$-amino alcohols (24) using (R)-$\alpha$-methylbenzylamine (MBA) as the amine donor, while an (S)-selective $\omega$-TA was needed to produce the (R)-$\beta$-amino alcohols (25).

For this, a TA from Bacillus megaterium (BmTA) was chosen with a different amine donor, L-alanine. Typically, when TAs are used in enzymatic cascade systems, an amine donor regeneration system is involved.[133] However, Chang and co-workers decided to avoid the presence of such a system to avoid complications in the complete enzymatic cascade. High conversions with up to 93.5% for the (S)-enantiomers and 97.7% for almost all the substrates investigated were achieved in the in vitro approach. The authors then established an in vitro biocatalytic cascade by constructing twelve recombinant plasmids, each containing two of the four genes needed.

Ten different recombinant E. coli pairwise combinations were proposed, and the best performing strains were further used to catalyze the asymmetric amino hydroxylation of styrene derivatives. In addition, a single tailor-made recombinant whole-cell catalyst containing all the active enzymes was constructed. Hence, nine recombinant E. coli cells co-expressing four enzymes simultaneously were prepared and investigated with the styrene substrate. Two of these nine systems were chosen, one containing MVTA, and the other BmTA, together with the first three enzymes. The presence of a cosolvent was needed to overcome the toxicity of the substrate for the cells and thus a two-liquid phase system was adopted. Interestingly, high conversions (up to $>90\%$ for both enantiomers) and enantioselectivities (up to $>99\%$) were achieved with a single-strain whole-cell biocatalyst. As a convincing proof-of-concept, for three chosen substrates, good to high isolated yields were reached (50.9–64.3%) with resting cells. The four-enzyme biocatalytic cascade presented in this work is an elegant example of an artificial biosynthetic pathway in a recombinant host. It avoids addition of the NADH cofactor and any intermediate isolation, as needed in previous work from Corrado et al.[134] This system provides a sustainable tool for enantioenriched $\beta$-amino alcohols production.

Styrene oxide (19) can also be used as an attractive starting material for the synthesis of 1,2-amino alcohols. For instance, Liu et al. coupled three enzymes for the synthesis of 1,2-amino alcohols (22 - Scheme 2 - f) in a whole cell biocatalytic system, starting from styrene oxide.[135] By combining an epoxide hydrolase (SpEH) from Sphingomonas sp., an alcohol dehydrogenase (MaADH) from Mycobacterium neoaurum, and a newly identified transaminase (PAK$\omega$-TA) from Pseudomonas aeruginosa, the authors designed a whole cell multi-enzyme system in E. coli for the synthesis of enantioenriched 1,2-amino alcohols. Once the best reaction conditions were established for the enzymes involved, the substrate scope of PAK$\omega$-TA was investigated, and the best amine donor was selected as L-glutamate (L-Glu). PAK$\omega$-TA and MaADH were cloned together in the same plasmid. However, a significant difference in specific activities of the two enzymes was observed (0.28 U mL$^{-1}$ for MaADH compared to 1.3 U mL$^{-1}$ for PAK$\omega$-TA). Consequently, the hydroxy ketone formation catalyzed by MaADH was identified as the rate-determining step. To overcome the differences observed in enzyme activities, the expression level was regulated to achieve the optimum ratio of 1.3 (PAK$\omega$-TA:MaADH). Different ribosome-binding sites were chosen to optimize the MaADH expression level, until the desired balance between the two enzymes was reached. Furthermore, a redox-self-sufficient system was added to the cascade to regenerate the NAD$^+$ cofactor and the amine donor L-Glu. Glutamate dehydrogenase (GluDH) was chosen for the recycling system utilizing 2-OG, NADH, and ammonia to form L-Glu and NAD$^+$. This strategy avoided the continuous addition of the cofactor and the co-substrate, preventing by-product accumulation and material waste. Finally, all four enzymes were cloned in two different plasmids and co-expressed in a single E. coli strain. Whole-cell biocatalyst was established for the synthesis of several 1,2-amino alcohols with considerable yields (65-96.4%) and excellent enantioselectivities ($>99\%$) starting from simple oxiranes.

### 3.3 Amino Alcohols via Carboligation and Amination

Amino alcohols are highly valuable building blocks for pharmaceuticals and form the main core of several bioactive molecules. Moreover, amino alcohols can be synthesized by coupling an oxygenation reaction with an amination reaction. However, there are interesting examples where amino alcohols and derivatives are constructed via carboligation followed by an amination reaction either via transamination or reductive amination. One example is the biocatalytic synthesis of methoxamine, a vasopressor belonging to the
class of sympathomimetic drugs, like nor(pseudo)ephedrine, which is widely used for the treatment of hypotension. The chemical synthesis of this drug provides the final molecule as a racemate and the resulting isomers are separated via column chromatography, leaving behind a high amount of waste and toxic solvents.\textsuperscript{136,137} Thus, biocatalytic cascades were exploited to achieve the asymmetric synthesis of methoxamine in a more sustainable manner (Scheme 3 – h).\textsuperscript{138}

Scheme 3. Enzymatic cascades involving carboligases in combination with either transaminase (h and i) or imine reductases for the synthesis of chiral amino alcohols (j).

Rother and co-workers established an elegant one-pot two-step enzymatic cascade for the synthesis of all four enantiomers of methoxamine, combining a pyruvate decarboxylase variant (ApPDC) from Acetobacter pasteurianus for the (S)-selective carboligation, and the acetohydroxyacid synthase (EcAHAS-I) from E. coli for the (R)-selective carboligation, with a transaminase, either from Bacillus megaterium (BmTA) or from Arthrobacter sp. (AsTA).\textsuperscript{138} Earlier work from the same research group adopted a similar approach for synthesizing the four isomers of nor(pseudo)ephedrine.\textsuperscript{139} However, in that case, for synthesizing two of the enantiomers, the transaminase was coupled with an alcohol dehydrogenase. In the methoxamine biosynthesis, the authors firstly investigated and combined the enzymes previously used for the nor(pseudo)ephedrine, but without promising results. Therefore, enzyme engineering was needed to obtain improved carboligases able to achieve high rates and conversions with the respective substrates. For the transamination step, several transaminases were screened with the desired substrates, and two of them were selected, BmTA based on
the high conversion reached for the (S)-transamination of 1-hydroxy-1,2(2,5-dimethoxyphenyl)propan-2-one (2,5-DMPAC), and AsTA as the (R)-selective transaminase with either (S)-2,5-DMPAC (28, Scheme 3 - h) or (R)-2,5-DMPAC (31, Scheme 3 - h). Three different amine donors were investigated with the chosen transaminases, and the highest conversions were achieved with isopropylamine (IPA). To obtain the pure isomer from each cascade, supercritical fluid chromatography with two chiral columns in a row was used, efficiently purifying and separating the desired methoxamine isomers. For each enantiomer, good conversions were achieved (59-80%) with high isomeric content (ic) up to 99%. Furthermore, a preparative scale reaction was established for the (1S,2R)-enantiomer, achieving a total conversion of 85% and an isolated yield of 64%, with an ic of >99%.

Recently, an interesting building block for the synthesis of the antimicrobial florfenicol was produced in a similar way by Lin and co-workers.[140] The authors developed a biocatalytic two-step cascade combining a transketolase (TK) for the carboligation step and a α-transaminase (α-TA) for the amination step, achieving the amino alcohol 36 as key intermediate for the synthesis (Scheme 3 – i). Here, alanine was used as the α-TA co-substrate while an NADH regeneration system was coupled with a lactose dehydrogenase (LDH) for pyruvate side product removal. Different TKs were considered suitable catalysts for the first step. Based on docking studies of the desired substrate bound in the active site, an engineered variant of TK (EcTK) from E. coli was selected.[141,142] Indeed, one of the mutations in the EcTK variant could properly orient the substrate in the active site. For the second step, six α-TAs were screened, and one of them, ATAI17, a homolog of Arthrobacter sp. TA, gave the best results. To obtain the desired (R)-α-hydroxy ketone 35, structure-guided engineering was conducted on the (S)-selective EcTK variant to invert its enantioselectivity towards the (R)-enantiomer. Two evolved variants were obtained, one exhibiting the highest enantioselectivity affording the product with 95.2% ee, EcTK_YYH, and the other one, EcTK_YYF, showing slightly lower enantioselectivity (92% ee), but higher activity. Also, two rounds of directed evolution were performed on ATAI17, achieving increased (R)-enantioselectivity and improved ketone/aldehyde substrate selectivity, with reduced benzylamine side product accumulation.

To further decrease benzylamine side product formation and to achieve highest yield of the final amino alcohol, EcTK_YYF was selected. In a continuous reaction mode, 51% overall yield and an excellent ee of >99% were reached. Lin and co-workers further investigated a sequential one-pot mode, where only the components of the first step were added at the beginning, followed by the addition of the engineered ATAI17_ACHH variant to overcome the side reaction issue. In addition, the TK with higher enantioselectivity (EcTK_YYH) was chosen. Here, a maximum of 73% yield and very high de (96%) and ee (>99%) were achieved. Finally, a preparative-scale synthesis was established resulting in an isolated yield of 65% and retention of de and ee. The cascade’s broad substrate scope was demonstrated by investigating other p-substituted benzaldehydes. For instance, p-苯甲酰苯甲醇 was obtained in 80% yield and excellent enantiopurity (>99% ee). This work shows how two powerful biocatalysts, optimized by site-directed saturation mutagenesis, can be used for the sustainable cascade synthesis of a key pharmaceutical precursor.

N-substituted amino alcohols are also fundamental building blocks in preparing natural products and pharmaceuticals. Recently, a biocatalytic route towards N-substituted 1,2-amino alcohols was proposed by Ma and co-workers (Scheme 3 - j).[143] These authors used an IRED for the reductive amination of α-hydroxymethyl ketones to provide N-substituted amino alcohols. Thiamine diphosphate (ThDP)-dependent enzymes can produce α-hydroxymethyl ketones via the carboligation of different substituted aldehydes with formaldehyde. Therefore, a one-pot two-step enzymatic cascade was envisioned, involving benzaldehyde lyase (BAL) as the ThDP-dependent enzyme coupled with an IRED to produce chiral vicinal N-substituted amino alcohols. Firstly, the authors focused on optimizing the reductive amination step. Several IREDS were investigated towards three α-hydroxymethyl ketones and cyclopropylamine in the presence of NADPH. Ten IREDS were selected to further investigate the amine substrate scope with aliphatic and aromatic α-hydroxymethyl ketones. For several substrates, a preparative-scale reaction was performed resulting in excellent conversions and stereoselectivities (>99%) and good to high isolated yields (50-80%). Then, Ma and co-workers optimized the first step of the cascade with six different ThDP-dependent enzymes. PaBAL from Polimorphobacter arashiensis was chosen, which exhibited the highest catalytic activity with a preference for aryl- and aliphatic aldehydes. High conversions (91-99%) were achieved in the preparative scale reactions for the carboligation step. A one-pot two-step biocatalytic cascade was then established for four selected aldehydes using cyclopropylamine as the amine donor. The authors could prove the applicability of their methodology by the preparative-scale synthesis of (R)-4-(2-bromophenyl)-2-(methylamino)-butan-1-ol, a precursor to anti-malarial and cytotoxic drugs. This work nicely demonstrates the usefulness of multi-step biocatalytic cascade synthesis for the environmentally friendly production of complex molecules.

### 4. Enzymatic Cascades for the Synthesis of Amines, N-Heterocycles, and Amino Acid Derivatives

#### 4.1 Linear Amines and Diamines

Chemical strategies, such as N-alkylation and reductive amination, have thus far mainly been applied for synthesizing primary and secondary amines. However, these approaches involve the use of toxic alkylating reagents, the formation of unwanted overalkylation products, and an excess of amine. In addition, transition-metal catalysts and harsh conditions are usually involved.[2,144] Although aldehydes are used as starting materials, they are often not easy to handle and quickly oxidizable. Therefore, alcohols, carboxylic acids, or ketones can be valuable substitutes as starting materials. To overcome the above-mentioned challenges in the chemocatalytic strategies, more sustainable biocatalytic approaches are required. In recent work, Chen et al. proposed the application of a (R)-selective α-TA for the selective synthesis of chiral linear amines using ketones as starting materials in combination with an atom-economic amine donor supply system (Scheme 4 - k).[145] In the past decade, several examples of (R)-selective α-TAs already proved their biocatalytic potential.[146–148] However, typically cost-intensive and atom-inefficient amine donors such as D-alanine or isopropylamine (IPA) are used.[149,150]
Scheme 4. Synthesis of linear amines via biocatalytic cascades either involving transaminases (k and m) or reductive aminases (l) in vitro or transaminases as whole-cell biocatalysts (n and o).
**REVIEW**

Chen et al. proposed an inexpensive and easy-to-handle recycling system involving ammonium formate as both the amine donor and the reducing agent. The high atom economy of the whole process is specially brought by the release of water and CO\(_2\) as the only wastes. By coupling the (R)-selective \(\omega\)-TA (Am\(\omega\)-TA) form *Aspergillus terreus* with an engineered AmDH and the formate dehydrogenase (CbFDH) from *Candida bombinii*, a one-pot, tri-enzymatic cascade was established. Ammonium formate is the substrate of both AmDH and CbFDH. On the one hand, the ammonium ion is converted into the desired amine donor (44) and water via the reductive amination of an auxiliary ketone (43). On the other hand, the formate ion is involved in the nicotinamide cofactor recycling, being converted to CO\(_2\) by CbFDH in the presence of NAD\(^+\). The *in situ* formed amine donor (44) undergoes transamination with a target ketone (41) catalyzed by the chosen (R)-selective \(\omega\)-TA. Excellent conversion of \(>99\%\) was reached within 24 hours by investigating different auxiliary ketones (43 and target ketones (41) as the substrates for AmDH and Am\(\omega\)-TA, respectively. The same \(\omega\)-TA used by Merck & Co. for the biocatalytic cascade synthesis of sitagliptin, ATA117-Mut11,[151] was also investigated by Chen et al. in their newly developed cascade, giving an alternative route for sitagliptin synthesis from prositagliptin ketone, achieving high conversion of 97%. The advantage of the proposed pathway is the use of ammonium formate as the sole driving force instead of using IPA, which requires the removal of the byproduct aceton and an excess of the amino donor itself. Finally, to prove the applicability of their system, a preparative scale synthesis was performed for one selected target ketone, reaching \(>99\%\) conversion, excellent enantioselectivity (\(>99\%\) ee) and good isolated yield (71%). The so-called ‘chimocatalytic hydrogen borrowing’ approach was shown as a valid alternative when alcohols are used as starting materials. Here, the alcohol is converted into the corresponding carbonyl compound, which then undergoes reductive amination.[144,152–158] One of the first examples of hydrogen borrowing biocatalytic cascades was developed by Turner and co-workers in 2015, combining an alcohol dehydrogenase and a transaminase in a one-pot, two-step cascade, obtaining an extensive range of chiral amines with high conversion (up to \(96\%\)) and excellent enantioselectivity (up to \(99\%\) ee).[159] In a more recent work from Turner’s group, the authors established a two-step cascade starting with alcohols (45) or carboxylic acids (46) for synthesizing secondary amines (48) via reductive amination (Scheme 4 - I). Precisely, a reductive amine (AspRedAm) from *Aspergillus oryzae* is coupled with either an alcohol oxidase (AO) or a carboxylic acid reductase (CAR). In a previous work involving a hydrogen borrowing cascade, the AspRedAm was coupled with different alcohol dehydrogenases (ADHs) starting from primary and secondary alcohols.[160] One of the central issues of this previous work was the reversibility of the step involving the ADH. This problem was solved by coupling the AspRedAm with an irreversible step, either applying an engineered choline oxidase variant (AcCO\(_3\)) or the wild-type CAR (CARsr) from *Segniliparus rugosus*, allowing the *in situ* formation of the aldehyde intermediate.[161] Using AcCO\(_3\) and AspRedAm, a wide range of substrates was accepted by both enzymes, and conversions up to \(99\%\) were achieved, showing the diverse applicability of this system. In the case of the AspRedAm coupled with the CARsr, two cofactor recycling systems were needed: the glucose/glucose dehydrogenase (GDH) for the NAD\(^+\)-cofactor and one for ATP. Although only a small range of amines and carboxylic acids were investigated, in most cases high conversions (\(>96\%\)) were reached. Preparative scale reactions were established with good conversions (84-99%) and isolated yields (49-74%). This work is an intriguing example where the catalytic power of AspRedAm is exploited in enzymatic cascade synthesis as a valid replacement of many chemical methods, avoiding substrates overalkylation and harsh conditions.

The need for sustainable routes in the synthesis of amine-based building blocks is also crucial for producing bioplastics and polymers. Biocatalytic multi-enzymatic cascades emerged as preferred strategy to synthesize amine polymer precursors in a sustainable way, providing access to the use of natural feedstocks as renewable sources instead of starting with petroleum-based chemicals. In this context, an example reported by Fedorchuk et al. demonstrates the biocatalytic conversion of adipic acid (49, Scheme 4 - m) to 1,6-hexamethylenediamine 53, driven by two CARs, the wild type (wt) and a specifically designed variant, and two \(\omega\)-TAs in a one-pot four-enzymes cascade.[160] In the presence of the selected cofactor and amine donor (either L-alanine or L-glutamine) recycling systems, the CAR wt in combination with a \(\omega\)-TA showed high activity towards adipic acid 49, giving 95% substrate conversion to 6-aminoacapric acid 51. However, the same CAR enzyme combined with a second \(\omega\)-TA gave a low conversion from 51 to 53. Therefore, the authors adopted a rational protein engineering strategy based on the CAR wt crystal structure in complex with AMP and succinate. A single variant of a CAR from *M. chelonae* was found to display the highest activity for compound 51. The final cascade setup involved the CAR wt, the CAR variant (L342E), and two \(\omega\)-TAs from two different organisms, achieving an overall conversion of 30% to the final compound 53 and 70% of the intermediate 51. Overall, this is an interesting example of protein engineering aiming at improving enzymatic activity towards the cascade’s intermediate, achieving enhanced conversion into the desired final product. This cascade provides a powerful tool to enable biocatalytic transformation of renewable sources to amine-based polymer building blocks.

Another strategy to generate aryl or alkyl amines and biopolymer monomers with amine functionalities is using whole-cell biocatalysts.[163] Enzymatic cascades in whole cells present the main advantage of being less cost-intensive (expensive cofactors avoided) and time-consuming (protein purification not needed) than their cell-free counterparts. Yun and co-workers developed a parallel anti-cascade reaction involving an \(\omega\)-TA in combination with an aldehyde reductase (AHAR), starting from \(\omega\)-hydroxy fatty acids (\(\omega\)-HFAs) (54) or \(\alpha\)-, \(\omega\)-diols (60) to produce \(\omega\)-amino fatty acids (\(\omega\)-AFAs) (56) and \(\alpha\), \(\omega\)-diamines (64), respectively (Scheme 4 - n).[162] Here, the amine donor chosen for the \(\omega\)-TA is benzylamine 57, which is converted by the \(\omega\)-TA to benzaldehyde 58 in the presence of pyridoxal phosphate (PLP). The 58 is further converted to benzyl alcohol 59 by the AHAR. A single-cell system expressing both enzymes from two different plasmids was preferred as it avoids mass transfer issues. Excellent conversions were achieved for \(\omega\)-AFAs (56) and \(\alpha\), \(\omega\)-diamines (64) (up to \(99\%\) in the case of \(\alpha\), \(\omega\)-diamines), with different carbon chain lengths, demonstrating that single whole-cell biocatalysts can be suitable tools for the production of bioplastic polymers from renewable feedstocks. In this context, Yoo and co-workers recently reported a modular cell-based approach involving three *E. coli* cell-based modules (Scheme 4 - o).[163] A modular approach was also adopted by Wu et al. in 2016 for synthesizing different hydroxylated and aminated compounds, like diols and amino alcohols[164] and more recently by Wang et al. for the synthesis of \(\alpha\), \(\omega\)-dicarboxylic acids as polymer building blocks.[165] In the in vivo modular methodology, each cell module, with specific functions and expressed enzymes, is optimized individually. Furthermore, combinatorial optimization is needed to efficiently produce the desired...
final product. In the work from Yoo et al., the first module (Scheme 4 – o. cell H) is characterized by the hydroxylation of fatty acid methyl esters (FAMES) (67) by a non-heme alkane monoxygenase system composed of three enzymes (AlkB cloned in a separate plasmid and AlkG/AlkT redox partners cloned within the same plasmid) and an endogenous hydrolase (lipase or esterase). In the second module (Scheme 4 – o. cell R), a CAR from Mycobacterium marinum and a phosphopantetheinyl transferase (Sfp) from B. subtilis work in a concerted way to provide carboxylic acid reduction and subsequent conversion of the formed aldehyde intermediate (not shown in Scheme 4) into the respective alcohol. The third and final module (Scheme 4 – o. cell A) was constructed by cloning an AHR and a \( \alpha \)-TA in two plasmids in a single E. coli cell, converting either o-HFAs or \( \alpha, \omega \)-diols (67) into the corresponding aminated product (68). The three modules were combined to finally produce \( \alpha, \omega \)-diamines (68) from renewable fatty acid esters. As in the case of purified enzymes, also whole-cell biocatalysts can be combined in a sequential stepwise or continuous mode. The authors explored both alternatives and observed comparable results in both cases after an overall reaction time of 48 h. However, for the preparative-scale reaction, the sequential step-wise mode was preferred, resulting in higher product accumulation than in the continuous mode. In addition, a higher final product amount was obtained by running the upscaled reactions in a pH-controlled manner (avoiding pH fluctuations) instead of using simple shake flasks. Some improvements are still needed since a significant amount of aminol side product, where only one of the two hydroxyl functionalities is converted to the amine, was observed. Most likely, this is due to the higher affinity of the transaminase for the 1,12-diol compared to the 12-amino intermediate.\(^{162}\) Metabolic pathway engineering and synthetic biology techniques are frequently limited by redox constraints, enzyme activity optimization and the selection of a suitable host.\(^{162}\) Here, the de novo design of in vivo microbial consortia-mediated cascades gives an intriguing alternative. Furthermore, modular in vivo biocatalysts have the advantage of avoiding possible limitations related to protein expression as well as redox balance issues often occurring when expressing multiple enzymes in a single microorganism. Thus, whole-cell biocatalysts have great potential for synthesizing a broad range of amine-based polymers in an environmentally benign fashion.

4.2 N-Heterocycles

4.2.1 Tetrahydroisoquinoline (THIQ) Derivatives

An important class of heterocyclic amines are tetrahydroisoquinoline (THIQ) scaffolds, present in various natural products and synthetic biologically active molecules, among which benzylisoquinoline alkaloids (BIAs) are a significant group of plant secondary metabolites. The extraction of these compounds from plant sources is usually mediated by a tyrosinase fraction of the two. Therefore, only some of these BIAs were developed.\(^{169}\) C\(\text{c}\)-\(\text{C}\) group of the THIQ core and C1 aryl moiety. Furthermore, a different approach proposed by Yang et al. combines IREDs with the coenzyme N-methyltransferase (CNMT) for the biocatalytic synthesis of several (S)-norcoclaurine derivatives (74, \( R^1 = \text{OH}, \ R^2 = \text{H} \)) and similar compounds as starting materials (Scheme 5 – p). The concept of a ‘triangular cascade’ was used to synthesize several BIAs by incorporating up to four enzymes. In other cases, the final compounds were obtained by combining only two or three enzymes. In the first step, an ortho-hydroxylation was performed by a tyrosinase from Candidatus nitrosopumilis salaria BD31 (CnTyr) to produce L-DOPA (70, \( R^1 = \text{OH}, \ R^2 = \text{H} \)) from L-tyrosine (69, \( R^1 = \text{OH}, \ R^2 = \text{H} \)) and dopamine (72, \( R^1 = \text{OH}, \ R^2 = \text{H} \)) from tyramine (71, \( R^1 = \text{OH}, \ R^2 = \text{H} \)). A tyrosine decarboxylase homolog (EftYrDC) from Enterococcus faecalis DC32 gave the best results for the formation of dopamine, via the decarboxylation of L-DOPA, and tyramine derivatives, from L-tyrosine derivatives. CnTyr and EftYrDC showed promising activities and a relatively broad substrate scope. Once the reaction conditions for the hydroxylation and the decarboxylation were established, CnTyr and EftYrDC were combined with a versatile transaminase (CvTam) from Chromobacterium violaceum and a norcoclaurine synthase (\( \Delta 29, \text{N} \)) to produce L-baccatin 3.\(^{176,177}\) In the first two steps, the order of CnTyr and EftYrDC was changed depending on the derivative produced and the presence of CvTam was not necessary in all the cases. For some compounds, the aldehyde needed for the Pictet-Spengler reaction was added and not produced in situ. Therefore, only some of these BIAs were obtained via a ‘triangular cascade’. Eventually, in this work, a ‘mix and match’ strategy was successfully used to generate natural and non-natural BIAs with high yield (35-99%) and enantioselectivity (75-99%).

Hailes and co-workers recently extended this work, adding different O- and N-methyltransferases (MTs) to obtain several THIQ methylated derivatives (Scheme 5 – q).\(^{172}\) The methyltransferases chosen were SAM dependent. However, SAM is a cost-intensive cofactor, and it limits the applicability of MT-catalyzed reactions on large scale. This problem was overcome by introducing a SAM supplying system involving two additional components, methionine adenosyltransferase (MAT) and methythioadenosine nucleoside (MTAN). Like this, BIA methylated derivatives and THIQs with an aliphatic chain or cyclic moiety in position C1 were obtained with good yields (up to 56%) and excellent regioselectivity, with a regioisomeric ratio of up to 95:5 (6-OMe:7-OMe). Besides (S)-norcoclaurine, other BIA derivatives such as (S)-norlaudanosoline (74, \( R^1 = \text{OH}, \ R^2 = \text{H} \), aryl = catechol moiety) have high biological relevance. Here, O-methylated derivatives of (S)-norlaudanosoline were produced with different regioselectivity, depending on the SAM equivalents and the type of OMTs used. Different OMTs showed opposite regioselectivity on the different OH-groups on the THIQ core and C1 aryl moiety. Noteworthy is the ability of two of the OMTs selected, RnCOMT and MfSalC, to methylate selectively different catechol moieties within the same molecule. Furthermore, RnCOMT was further coupled with an N-methyltransferase (GCNMT) to obtain another important THIQ alkaid, (S)-orientaline (80). A different approach proposed by Yang et al. combines IREDs with the coenzyme N-methyltransferase (CNMT) for the biocatalytic synthesis of THIQ derivatives (Scheme 5 – r).\(^{173}\) The authors used this combination to construct a whole-cell biocatalyst to produce phenylisoquinoline alkaloids (PIAs, \( n = 0 \), Scheme 5 - r) and BIAs (\( n = 1 \), Scheme 5 – r). Starting with chemically synthesized dihydroisoquinolines (DHIQs) (83) and using engineered IREDs to accept sterically hindered DHIQs at the C1 position, a valuable and
efficient system for synthesis of intermediates 84 was established. Based on docking studies of an (S)-selective IRED (IR45)\(^\text{\ref{174}}\) in complex with a PIA with a bulky substituent on the C1, the authors identified two target residues to be engineered, and two selected double mutants of IR45 showed increased activity and substrate tolerance towards hindered PIAs. These mutants were then used in a whole-cell enzymatic cascade in combination with a CNMT with broad substrate scope to obtain five methylated THIQ derivatives (85) in high isolated yield (up to 98%) and with exquisite enantioselectivity. A combined cell-free and whole-cell biocatalytic system was established to overcome the bottleneck of membrane permeability of two of the DHIQ precursors. For the imine reduction of chemically synthesized DHIQs, IRED/GDH containing cell-free extract was added directly to the E. coli culture expressing the N-methyltransferase. This approach provided complete conversion and high isolated yields for the desired compounds. This two-enzyme whole-cell system is one of the first examples of efficient whole-cell biocatalysis for synthesizing complex PIAs and BIAs.

Scheme 5. (Chemo)enzymatic cascades for the synthesis of tetrahydroisoquinolines (THIQs) both in vitro (p and q) and in vivo (r).

4.2.2 Cyclic Amines
Among N-heterocycles, piperidines and pyrrolidines are some of the most widely spread and used building blocks to produce complex natural products and pharmaceuticals.\(^\text{\[175-178\]}\) However, most procedures involved in their stereoselective synthesis are based on harsh chemical conditions and involve costly transition metal catalysts.\(^\text{\[176-178\]}\) In this context, biocatalysis can be a sustainable alternative for their selective synthesis. As previously mentioned, building biocatalytic routes in whole cells has several advantages, and using whole-cell biocatalysts expressing multiple enzymes in either single-cell or multiple-cell modules is particularly useful to avoid the addition of cost-intensive cofactors. Two interesting approaches have been reported by the Turner group, in which a CAR (MCAR) from Mycobacterium marinum, two commercially available \(\omega\)-TAs and two stereocomplementary IREDs were combined to access both enantiomers of the desired amine products 90 (Scheme 6 – s and t)\(^\text{\[179,180\]}\). In the first approach, a hybrid system was established in which the CAR and the IRED were used as two separate whole-cell biocatalysts, while the \(\omega\)-TA was employed as lyophilized cell lysate (Scheme 6 – s). Like this, an efficient one-pot enzymatic cascade was constructed for the regio- and stereoselective synthesis of mono- and disubstituted piperidines and pyrrolidines.\(^\text{\[179\]}\)
Starting from keto acids of different lengths (86), 2,4- and 2,6-disubstituted piperidines were produced with high conversions (>98%) and remarkable enantioselectivities (>98% ee), even with bulkier substituents. In the case of 2,5-disubstituted pyrrolidines, slightly lower conversions were achieved (up to 95%) with comparable enantioselectivities (>98% ee). Turner and co-workers provided one of the first examples of highly efficient artificial enzymatic cascades using whole-cell biocatalysts for the stereo-controlled production of complex heterocyclic amines. As follow-up work, the same biocatalytic cascade was investigated as a single whole-cell system expressing four enzymes (CAR, TA, IRED, and Sfp) cloned within the same plasmid (Scheme 6 – t). Ten plasmid systems containing four to five genes expressing the desired enzymes were designed and investigated with different keto acids as starting materials. After several optimizations of the reaction conditions and plasmid design, five piperidine derivatives were obtained with up to 93% conversion and enantiopurity of up to >98 ee. Two piperidine derivatives were successfully produced on milligram scale with up to 59% isolated yield and >98% ee demonstrating the biocatalytic applicability of this methodology. Although keto acids have been mainly used for the biocatalytic synthesis of N-heterocycles, as described with the examples from the Turner group,[179,180] diamines can also be considered a valid alternative as starting materials. As linear keto acids, biogenic terminal diamines are easily available from biomass and can be used as renewable feedstocks.[181] In this context, Al-Shameri et al. proposed a three-step two-enzyme cascade synthesis of N-heterocycles starting from diamines of different lengths (91), involving a putrescine oxidase (PuO) and an IRED (Scheme 6 – u).[182] In addition, a cofactor regeneration system, including an oxygen-tolerant hydrogenase (SH) from *Ralstonia eutropha* H16, and H2 as the only reductant, was chosen as a valid alternative to the classical GDH/glucose system, providing high atom economy.[183] This innovative cofactor recycling system avoids side reactions due to the reactivity of primary amines with aldehydes, including the open chain of glucose-6-phosphate generated from glucose reduction by GDH. Those side reactions can be reversible but can be an issue for the isolation and purification of the desired heterocycle 94. After a directed evolution campaign, the substrate acceptance of the PuO was expanded, improving the overall cascade outcome. This alternative approach produced five pyrrolidine and piperidine derivatives with good product formation and enantioselectivity, especially with chiral piperidine variants. A similar approach was adopted by Ford et al., where Cbz-protected L-3-aminopiperidine 98 and L-3-aminoazepane 99 were synthesized starting from the respective N-Cbz-protected amino alcohols 95 in a one-pot two-step cascade, combining a glucose oxidase (GOase)/horseradish peroxidase (HRP) system with an IRED/GDH/glucose system for the reduction of the spontaneously formed imine intermediate.[184] Here, the alcohol moiety of the starting material is oxidized to the respective aldehyde, which spontaneously undergoes intramolecular cyclization to form the imine intermediate. The addition of catalase was needed for H2O2 decomposition. The imine intermediate is then reduced by...
where an in vitro approach was used, Yu and co-workers chose an in vivo system consisting of a three-cell modular cascade, finally yielding ε-caprolactam (113). Specifically, the three modules consisted of an oxidation module (Scheme 7 – w-3, Module 1) involving an alcohol dehydrogenase (ADH), a CHMO, and a LA to yield the 6-hydroxyhexanoic acid intermediate (110), an amination module where an AH and a TA produced 6-AmAH (Scheme 7 – w-3, Module 2) and a third module converting 6-AmAH into ε-caprolactam, using either a CAR or a cyclase (Scheme 7 – w-3, Module 3). Optimizing every single cell module was needed to gain the best expression conditions of each enzyme in different E. coli cell strains. The final combination of enzymes was: ADH and CHMO co-expressed in two plasmids in the same cell, the LA, and a fused version of the TA and the AH expressed in two separate plasmids in another cell, and a third cell expressing the cyclase for the last step. Although a moderate final yield was achieved (2.8 mM of ε-caprolactam from 10 mM of starting material), this modular cascade approach provides an elegant strategy with a high chance to enhance productivity by improving the final cyclase step.

4.3 Amino Acid Derivatives

Canonical and non-canonical α-amino acids are essential precursors of numerous biologically active molecules and pharmaceuticals. Established chemical synthesis of such building blocks involves several steps, toxic reagents, and racemic mixture production, requiring additional late-stage chiral resolution steps. Methods involving multi-step enzymatic routes provide an attractive alternative in terms of low environmental impact, atom, time and cost efficiency, and sustainability. Amino acids such as L-phenylalanine and L-tyrosine and derivatives, including the D-isomers, were produced in different enzymatic cascades involving enzymes such as PAL, P450BM3, monoxygenase and several C-C lyases. More recently, γ-hydroxy-α-amino acid derivatives were produced in a one-pot two-step biocatalytic cascade by coupling a pyruvate hydratase-aldolase (HBPA) with a TA (Scheme 8 – x). Directed evolution of the HBPA was necessary to improve its activity towards substrates bearing a phenyl moiety. The first step involving either HBPA wt or HBPA variant showed moderate to excellent enantioselectivities (80-96% ee) toward most of the substrates. Three strategies were employed for the amination step: two involving a transaminase (TA) and two different amine donors, either L-alanine or benzylamine, and a third involving a branched-chain α-amino acid aminotransferase (BCAT) with L-glutamate as amine donor.

The first strategy involving L-alanine as the amine donor with a pyruvate recycling system was successful only for two substrates, achieving up to 47% conversion. A second strategy was then investigated, changing the amine donor from L-alanine to benzylamine. The benzaldehyde formed was further converted to the corresponding benzoin (119) by a benzaldehyde lyase (BAL) to shift the equilibrium of the transamination step. Although product 117 was obtained for most of the substrates with decent conversions, the low amount obtained for aromatic substrates encouraged the authors to follow another strategy, involving BCAT and a recycling system for L-glutamate and the removal of 2-OG by an aspartateaminotransferase (AsAP). Finally, a third chemical step was added to produce different Cbz-N’-γ-butyrolactones (118) as fundamental building blocks for biologically active molecules.

Review

the IRED/GDH/glucose system to give the final desired Cbz-protected N-heterocycle of interest with high enantiom purity. Protein engineering was also performed on the GOase, based on docking studies with the starting material 91 (n = 1 or 2) within the active site. The best variant was then used in the biocatalytic cascade, with up to 54% isolated yield for the protected L-3-aminoazepane. With this, Ford et al. demonstrated the applicability of enzyme-catalyzed cyclic amine formation in a one-pot streamlined cascade, producing two products which can be directly incorporated into chemical processes to synthesize more complex molecules.

4.2.3 Lactams

Developing sustainable synthesis strategies toward nylon-6 and polyamide polymers has become another target in biocatalysis. In this context, Yun and co-workers recently published a one-pot biocatalytic cascade approach for the synthesis of nylon precursors (Scheme 7 – w-1 and w-3). Usually, the nylon-6 polymer is produced by ε-caprolactam ring-opening and subsequent polymerization. Therefore, ε-caprolactam is a fundamental building block for producing nylon monomers, and it is usually obtained either from cyclohexanone or 6-aminohexanoic acid (6-AmHA). In section 2.1 of this review, we briefly described that 6-aminohexanoic acid can be obtained from cyclohexanol in biocatalytic cascades. In this example, Krouti et al. showed the combination of two cofactor self-sufficient biocatalytic cascade modules starting from cyclohexanol for synthesizing 6-AmAH (Scheme 7 – w-1). However, the multi-step biocatalytic production of lactams is still under investigation, and there are only a few examples reported thus far. Hence, the work reported by Yun’s group is rather comprehensive and could pave the way for developing sustainable routes to ε-caprolactam and similar compounds. In a first attempt, Yun and co-workers suggested a novel biosynthetic approach for synthesizing lactams of different lengths (102) starting from the corresponding cycloalkylamine (100, Scheme 7 – w-1, green).

The cycloalkylamine can serve as the amine donor of a α-TA, and its deaminated ketone (103) is further converted by a cyclohexanone monoxygenase (CHMO) into the corresponding lactone (104). Then a lactonase (LA) catalyzes the ring-opening of the lactone intermediate to obtain the corresponding hydroxy fatty acid (105), further transformed into its oxidized form (106) by an aldehyde reductase (AHR). The aldehyde formed serves as a substrate for the ω-TA in the synthesis of 6-AmHA (101), which undergoes intramolecular cyclization in the presence of a cyclase with water as the sole waste. Thus, an in vitro cofactor/coproduct recycling system was established, achieving high conversions (>99%) on analytical scale and good isolated yields (62-85%) for the intermediate 6-AmAH (101) in an upscaled reaction. Based on these promising results, the authors coupled this four-enzyme system to the final lactamization catalyzed by an engineered diyclase, reaching up to 44% conversion. A multi-cell system was also investigated, where the purified enzymes used in the in vitro approach were utilized as whole-cell biocatalysts (Scheme 7 – w-1, purple). In this case, a two-cell system was developed, reaching up to 80% conversion to the desired 6-AmAH intermediate. A three-cell system was then adopted to gain the final lactam derivatives with conversions of up to 74%.

In a follow-up work, the same authors suggested an alternative way to synthesize ε-caprolactam starting from the inexpensive cyclohexanol (107) compared to cycloalkylamines (Scheme 7 – w-3). In contrast to the work of Krouti’s group (Scheme 7 – w-2), a Scheme 7
Scheme 7. Biocatalytic cascade synthesis of ε-caprolactam and derivatives (w-1, w-2 and w-3) starting from either cycloalkylamines (w-1) or cyclohexanol (w-2 and w-3), where cascade w-3 is an evolution of cascade w-2.
Scheme 8. Enzymatic cascades to produce amino acid derivatives in vitro (x) and in vivo (y and z).

Whole-cell biocatalysts can also be employed in amino acid synthesis, which is more cost-effective than their in vitro counterparts, especially when expensive cofactors and cofactor recycling systems are involved. Tert-L-leucine (127), a building block for synthesizing relevant antiviral and antitumor drugs, was produced simultaneously with another chiral intermediate of steroidal drugs, 7,12-
dioxilithocholic acid (125), in a two-enzymatic whole-cell cascade (Scheme 8 – y).

The first step involved double oxidation of cholic acid (124) in positions 7 and 12 catalyzed by two specific dehydrogenases (7α-HSDH and 12α-HSDH). Both dehydrogenases need NAD\(^+\) as a cofactor. In this in vivo system, a cost-effective self-sufficient hydride-shuttling cascade was constructed. The cofactor is regenerated by a leucine dehydrogenase (LeuDH), producing the other valuable compound tert-L-leucine, using ammonium as the co-substrate and producing water as the only waste. After several optimizations of the overall system in vitro, an E. coli single-cell biocatalyst was developed, using two plasmids expressing 12α-HSDH and 7α-HSDH/LeuDH, respectively, and achieving a yield of 94% for 7,12-dioxilithocholic acid (125). Besides optimizing the enzyme expression, maximized using different vectors, other factors needed to be considered and optimized, such as the substrate concentration and the addition of NAD\(^+\). The optimum substrate concentration was established at 100 mM, and 98% conversion was reached with no need for external supplementation of NAD\(^+\), benefiting the cost-effectiveness of the system. A preparative-scale reaction produced 7,12-dioxilithocholic acid and tert-L-leucine in 80% and 65% isolated yield, respectively, proving the applicability of the whole-cell biocatalyst at gram-scale.

An important active pharmaceutical ingredient on the market is L-DOPA, used as treatment of neurodegenerative diseases. Recently, Galman et al. proposed a biocatalytic in vivo cascade for the synthesis of the L-DOPA precursor L-3,4-dihydroxyphenylalanine (L-DOPA) by combining an OMT and an engineered PAL (Scheme 8 – x).[109] As previously mentioned, OMTs need SAM as a cofactor to perform catalysis. Therefore, the authors used an engineered E. coli BL21 (DE3) strain to regenerate SAM in vivo, thereby reducing the overall process costs. Moreover, the substrate chosen was ferulic acid (128), which can be easily derived from biomass degradation, making their approach even more sustainable. The enzymatic methylation of the -OH group in the para position in ferulic acid is challenging, and there are only a few OMTs known that are able to selectively transfer a methyl group onto para-substituted catechols.[70,194,195] Thus, Galman et al. first characterized an underexplored putative 4-O-methyltransferase (EOMT) from Erichia japonica, which was further engineered to enlarge substrate acceptance and increase the para-regioselectivity.

A triple mutant showed remarkable improvement in activity for the majority of substrates, reaching up to >98% conversion. Next, an engineered system was developed to regulate the in vivo methyl cycle in E. coli via upregulation and downregulation of the genes involved in the production of SAM from homocysteine. This was achieved by introducing one mutated version of the MetK gene (metK), expressing an enzyme involved in the conversion of the methionine intermediate to SAM. The engineered strain was firstly used in an in vitro cascade as cell lysate in combination with the purified EOMT triple mutant with the addition of ATP and methionine to stimulate the production of SAM in situ with ferulic acid as the substrate. Since promising results were obtained (>98% conversion), the in vivo methylation of ferulic acid was attempted where the EOMT variant plasmid and the plasmid containing the SAM regeneration system genes were transformed in the same E. coli cell. Again, >98% conversion of ferulic acid into the corresponding methylated product was reached after 48 hours. Moreover, to demonstrate the synthetic application of this system, resting cells expressing a PAL variant were added in a stepwise mode once full methylation of ferulic acid was completed in a one-pot, two-step whole-cell cascade. The addition of the PAL variant gave access to L-3,4-dihydroxyphenylalanine (L-DOPA) with an isolated yield of 60% and excellent enantiopurity (>99% ee).

5. Conclusions and Future Perspectives

The above-described examples highlight the remarkable usefulness of combining multiple enzymes, especially C-O and C-N bond-forming enzymes, in one pot to construct biocatalytic cascades for production of complex molecules from simple precursors under environmentally benign conditions. Nonetheless, there are still challenges to be overcome. A recent perspective by Burgener et al. lists some of the main drawbacks enzymatic cascades still face.[196] One challenge relates to the often poor enzyme stability, which frequently occurs when the cascade conditions do not correspond to the optimum operating conditions of the enzymes involved. Directed evolution can boost enzyme robustness under certain conditions, for instance toward high concentrations of H\(_2\)O\(_2\) as in the case of peroxidases. In addition, enzyme immobilization and flow biocatalysis are highly interesting for increasing operational efficiency of enzymatic cascades.[197] For instance, Turner et al. recently reported a flow system in which immobilized transaminases and imine reductases were coupled with either an engineered choline oxidase or a galactose oxidase to achieve continuous flow synthesis of linear amines from alcohols.[198] Another example reported by Paradisi and co-workers shows a flow system for producing 6-aminoacaprylic acid from c-6-capro lactone, coupling an immobilized lipase with a co-immobilized multi-enzyme system consisting of a transaminase, an alcohol dehydrogenase, and an NADH oxidase in packed-bed reactors.[199] The use of immobilized enzymes and flow systems also allows to overcome issues related to product separation from the biocatalyst and reduces mass transfer limitations. Moreover, flow reactions enable recycling of the immobilized enzymes involved, thus significantly reducing the amount of waste produced. The complexity of cascades as well as frequently encountered cross-reactivities are further challenges that must be addressed to pave the way towards a general application of the concept. Here, promising solutions applying tight regulation for controlling the activity of each enzyme to overcome cross-reactivities in cascade reactions are currently under investigation.[200]

Another challenge is the need for efficient and inexpensive cofactor recycling systems. Improving the use of cofactor-free enzymes and their exploitation in biocatalytic cascades could dramatically reduce the costs of the overall synthesis. Some examples of whole-cell biocatalysts give another solution to this issue, utilizing the cellular NAD(P)\(^+\)H and ATP source or tuning the natural cofactor production in vivo to enhance the overall cascade outcome.[199] Albeit whole-cell biocatalysts are less cost-prohibitive and time-consuming than their in vitro counterparts, other systems were developed in the past years mimicking cellular factories via cell-free compartmentalized biocatalysis.[201] Various methodologies have been developed in the recent years to construct artificial cells via enzymes encapsulation by water-in-oil droplets, transfer to liposomes for the synthesis of ethanol from lactate,[202] using metal-organic framework nanoparticles as nanoreactors, mainly based on zeolitic imidazolate frameworks,[202,203] or adopting DNA origami structures (1D, 2D or 3D) to spatially separate the enzymes,[204] improving substrate channelling and the overall cascade rate and stability. The further exploitation and implementation of these technologies for the multi-enzyme synthesis of complex molecules containing oxygen and nitrogen functionalities

REVIEWS

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is expected to mature, overcoming the difficulties associated with cascades and paving the way towards general applicability of the concept.

Finally, despite recent advances in expanding the toolbox of C-O and C-N bond-forming enzymes, the synthetic scope of these enzymes is rather restricted to a limited number of starting materials and activity and selectivity issues with nonnative starting materials remain mostly unresolved. In particular, controlling regioselectivity in the presence of chemically equivalent positions or achieving selective oxy- or amino-functionalization of less-activated positions, including nonactivated C–H bonds, still represent major challenges that need to be addressed. Hence, the discovery and engineering of efficient and selective enzymes via genome mining, directed evolution and computational design will certainly be necessary to further implement oxy- and amino-functionalization steps in enzymatic cascades. This will ultimately result in new tailored artificial biosyntheses of high value-added compounds, such as pharmaceuticals and polymer precursors, enabling a more sustainable future.

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Oxygenation and amination reactions are widely spread in synthetic chemistry to produce valuable compounds. Nowadays, the importance of sustainable strategies to introduce oxygen and amino functionalities into organic molecules is increasing. This review discusses recent examples of multi-step biocatalytic cascades involving oxy- and amino-functionalization reactions to produce value-added compounds such as pharmaceuticals and polymer precursors.

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