Bio-catalytic cascades and molecular oxygen-accessing amines and nitriles

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Chapter 1
Introduction – Biocatalytic aerobic oxidation reactions

This chapter is partly based on following publication:
1.1 Introduction

In recent years, the use of biocatalysts in organic synthesis has attracted increasing attention since enzymes often work under ambient conditions (low temperatures and pressures) and possess exquisite chemo-, regio- and stereoselectivity.\(^1\) Therefore, biocatalysis represents a viable option to increase the atom-economy and to decrease the necessary energy input of a chemical reaction. Moreover, bioinformatics, enzyme engineering either by directed evolution or rational approaches along with the development of sensitive and reliable high-throughput screening methodologies open the way for biocatalysis to a growing number of possible synthetic applications.

Nature provides an arsenal of enzymes capable of performing oxidation reactions at the expense of dioxygen (preferentially in the form of air at atmospheric pressure).\(^2\) The oxidoreductase enzyme class performs oxidation reactions either using dioxygen or hydrogen peroxide or nicotine amide adenine dinucleotide cofactors (NAD(P)\(^+\)/NAD(P)H). Dioxygen and hydrogen peroxide are used both for oxidation as well as oxidative-functionalization, whereas NAD(P)\(^+\) /NAD(P)H cofactors are used for hydride abstraction or donation. During the past decade, there has been a tremendous increase of available enzymes and methodologies for performing oxygen-dependent biotransformations for the synthesis of small molecules that can be further utilized as bulk chemicals or intermediates for fine chemicals.\(^3\) Due to the enormous amount of scientific literature reporting protein sequences and applications, it can be quite difficult for synthetic chemists or process engineers to navigate the biocatalysis landscape.

This introduction aims at providing an overview on the recent progress in biocatalytic aerobic oxidation reactions, e.g., C-H functionalization, Baeyer-Villiger reaction, oxidation of alcohols and aldehydes, oxidation of amines and oxidation of alkenes. These processes are also of synthetic interest, although they assume a lower significance within the context of the present thesis. The last part of this introduction complements this overview by discussing the novel family of amine dehydrogenases, and introducing their possible application in biocatalytic cascades with other oxidase enzymes for the aerobic amination of alcohols. In fact, a crucial advantage of biocatalysis over chemocatalysis lies in the possibility to combine enzymes in cascade...
processes, which in the end permit to increase atom-efficiency and reduce costs by avoiding intermediate work-up and purification steps.\textsuperscript{4,5} Therefore, aerobic oxidation reactions that have been incorporated into cascade processes are also reported in the case that their synthetic applicability in preparative scale was demonstrated.

### 1.2 Aerobic Biocatalytic C-H Functionalization

Enzymatic oxidative C-H hydroxylation and halogenation are very attractive chemical transformations as they normally proceed with excellent regio- and stereoselectivity by consuming molecular oxygen as oxidant (Scheme 1.1a and 1.4).\textsuperscript{6,7} In Nature, these reactions are performed by the enzyme class of oxygenases, which play a pivotal role in various catabolic and anabolic processes.

#### 1.2.1 Aerobic Biocatalytic Hydroxylation

Mono-hydroxylation of unfunctionalized sp\textsuperscript{3} C-H bonds is one of the most studied enzymatic reactions. Cytochromes—in particular the cytochrome P450 from \textit{Bacillus megaterium} (P450-BM3)—are the most commonly applied biocatalysts for this type of transformation. Cytochrome P450s can catalyze the selective hydroxylation of a wide range of structurally diverse molecules (Scheme 1.1a).\textsuperscript{8} Current research in this field focuses on the maximization of the productivity of the hydroxylation reaction and on the modulation of the chemo-, stereo- and regioselectivity of the biocatalysts by protein engineering.\textsuperscript{9,10} Many cytochrome P450s possess a reductase unit that accepts electrons from NAD(P)H, and a hydroxylating unit that performs the oxygenation. An additional protein, namely a ferredoxin, can be involved in the electron transfer between the two units. A sub-family of P450s are the self-sufficient cytochromes in which a reductase and an oxygenase domain are naturally fused to give a single polypeptide chain. Therefore, self-sufficient P450s are the most applied cytochromes in chemical synthesis because of the more practical production in \textit{Escherichia coli} and the more efficient redox coupling between reductase and oxygenase domains.\textsuperscript{11} However, P450s originated from fungi have recently attracted attention since fungal genomes can offer a large variety of new genes and catalytic activities.\textsuperscript{12} For instance, a thermostable P450 CYP505A30 from \textit{Thermothelomyces thermophile} was recently discovered and
Remarkable achievement in the engineering of P450-BM3 was reported by Reetz's group. Through directed evolution, they generated P450 variants for the stereo- and regioselective hydroxylation of aromatic ketones to yield α-hydroxy ketones with TOF up to 33 min⁻¹, 99% regioselectivity and up to 99% e.e. (Scheme 1.1b). In another study, a flavin-dependent monooxygenase (HpaBC) from *Pseudomonas aeruginosa* was reported to catalyze the double mono-hydroxylation of resveratrol to yield 3,4,5,3',5'-pentahydroxy-*trans*-stilbene (1.8 g l⁻¹ in 48 h). Another human
mitochondrial CYP11B1, which was produced in E. coli along with electron transport chain proteins, was reported to hydroxylate selectively 11-deoxycortisol to cortisol in a space-time yield of 0.84 g L$^{-1}$ d$^{-1}$ (Scheme 1.2a). A 100-L scale biocatalytic synthesis of (R)-4-hydroxyisophorone was conducted using resting E. coli cells co-expressing P450-BM3 and GDH (Scheme 1.2b). The hydroxylation proceeded with 80% conversion yielding a product titer of 10 g L$^{-1}$ and a space-time yield of 1.5 g L$^{-1}$ h$^{-1}$. The same reaction was reported later in another publication, in which the hydroxylation was combined with a further oxidation to ketoisophorone catalyzed by an alcohol dehydrogenase (ADH). A total space-time yield of 1.1 g L$^{-1}$ d$^{-1}$ was obtained when both P450 and ADH were co-expressed in E. coli.

Holtmann's group presented a new strategy for the large-scale C-H hydroxylation of palmitic acid by using “surface displayed enzymes”, which are anchored in the E. coli's...
cell wall and catalyze the reaction outside of the cells. The possibility to recycle the whole cells biocatalyst resulted in TONs of over 50000.\textsuperscript{19}

Two interesting approaches to tune the regio- and stereoselectivity of cytochromes consist in the use of either directing groups or co-substrates that can partially fill the active site, thereby enabling the conversion of previously non-reacting substrates. The first methodology is exemplified by the work of Bell’s group, in which CYP101B1 was applied for the hydroxylation of diverse terpenes and their acetate derivatives with high regioselectivity (90- >99%) and up to >99% \textit{e.e.}\textsuperscript{20} An example for the second method is the use of \textit{N}-acylamino acids that act as decoy molecules in the active site of P450-BM3, thus enabling the hydroxylation of benzene (TON 40200 in 12 h; coupling efficiency 46%) without the formation of any over-oxidation by-product.\textsuperscript{21}

Notably, P450s were integrated in a number of biocatalytic cascade reactions. A previously developed cascade featuring an alcohol dehydrogenase (ADH) and a Baeyer-Villiger monooxygenase (BVMO, discussed in section 1.3) to yield lactones, was elongated by performing the initial hydroxylation of cyclic alkanes (e.g., cyclohexane, cycloheptane and cyclooctane). The cascade was accomplished by the use of \textit{E. coli} cell free extracts of P450, ADH and BVMO. A TON of 4185 was reached with respect to the P450, while the final lactone products were obtained with productivities in the range of 0.59-2.95 g L\textsuperscript{-1}.\textsuperscript{22} Immobilization of P450s is also a synthetically viable option. For example, Nidetzky’s group co-immobilized a P450-BM3 and a GDH on porous poly-methacrylate anionic resin, and performed the hydroxylation of lauric acid with TTNs of 18000, as the co-immobilized enzymes were recycled for nine times without any loss of catalytic activity. After 20 cycles, the catalytic activity was still 30%.\textsuperscript{23} Some dioxygenases are capable of catalyzing mono-hydroxylation, although they require the addition of \textit{α}-ketoglutarate as co-substrate. For instance, Hauer’s group reported a taurine dioxygenase and variants thereof that perform the asymmetric \textit{α}-hydroxylation of carboxylic acids and \textit{ω}-amino acids with excellent \textit{e.e.}'s (>96%, Scheme 1.3a).\textsuperscript{24} Later, Renata’s group performed the remote C-H hydroxylation of a set of aliphatic \textit{α}-amino acids using another Fe\textsuperscript{2+}/\textit{α}-ketoglutarate-dependent dioxygenase with a TTN exceeding 10000 (Scheme 1.3b). The elevated
selectivity of the distal C-H hydroxylation was exploited in a preparative scale chemo-enzymatic synthesis of manzacidin C (Scheme 1.3c).^{25}

Scheme 1.3. a) α-hydroxylation activity of TauD towards α-amino acids; b) GriE-catalyzed remote hydroxylation of aliphatic α-amino acids; c) Chemo-enzymatic synthesis of manzacidin C in 5 steps from L-leucine and employing GriE in the pivotal hydroxylation step.
1.2.2 Aerobic Biocatalytic Halogenation

Concerning aerobic bio-catalytic methods for C-H functionalization, halogenation must be mentioned. α-Ketoglutarate-dependent halogenases attract interest, albeit their utilization is currently complicated by the necessity of pre-activation of a substrate through a carrier protein. However, the discoveries of similar halogenases that do not require the presence of the carrier protein such as WelO5 from Hapalosiphon weltwischii (Scheme 1.4) and the more substrate promiscuous AmbO5 from Fischerella ambigua suggest the possibility for a much wider application in future.

Flavin-dependent halogenases represent a more mature class of enzymes for the aerobic C-H halogenation. These enzymes utilize their flavin cofactor (FAD → FADH$_2$) to generate a peroxo-flavin (FADH-OOH) intermediate by reaction with dioxygen in the first catalytic step. Subsequently, the peroxo-flavin cofactor interacts with a halide to generate a HOX species, which performs the final selective halogenation of the substrate. A number of reports describe the use of RebH halogenase from Lechevalieria aerocolonigenes for the preparation of halogenated compounds (Scheme 1.5a). For instance, Sewald’s group synthesized ca. 1.8 grams of 7-brominated tryptophan TFA salt in 8 days using cross-linked enzyme aggregates (CLEAs) containing RebH and two additional dehydrogenases for flavin cofactor recycling (Scheme 1.5b). Lewis’ group conducted the directed evolution of RebH to gain access to bulkier biologically active molecules as well as to change the regioselectivity of the halogenase in order to functionalize different C-H bonds at the substrate’s indole ring. Finally, Micklefield’s group carried out the structurally-guided
engineering of SttH from *Streptomyces toxytricini* which enabled to switch the chlorination of the indole ring of tryptophan from the naturally predominant 5-position to the 6-position.\(^{33}\) According with a growing interest in creating chimeric (i.e., fused) enzymes for more practical applications in chemical synthesis, halogenase RebH and flavin reductase RebF were fused to generate a self-sufficient biocatalyst. This fusion, applied as resting *E. coli* cells, permitted to increase the product titers of 7-chlorotryptophan from 44 mg L\(^{-1}\) to 120 mg L\(^{-1}\) and of 3-chloroantrahilic acid from 19 mg L\(^{-1}\) to 61 mg L\(^{-1}\).\(^{34}\) In general, a number of biocatalysts for the selective C-H halogenation at expense of dioxygen on gram-scale are available; however, major development is required in this area to overcome limitations such as low substrate loadings and a quite narrow substrate scope.

Scheme 1.5. a) General reaction scheme of flavin-dependent halogenases b) gram scale synthesis of the TFA-salt of 7-bromotryptophan.
1.3 Biocatalytic Baeyer-Villiger Oxidation

The Baeyer-Villiger oxidation is a classical chemical transformation, first reported by Baeyer and Villiger at the end of the 19th century. This reaction converts cyclic or linear ketones into lactones or esters, respectively. Baeyer-Villiger monooxygenases perform the enzymatic equivalent of this reaction through the action of a flavin cofactor. As previously mentioned for the flavin-dependent halogenases, the key step of the catalytic cycle is the reaction between the previously reduced flavin (FADH$_2$) and dioxygen to generate the catalytic peroxy-flavin intermediate (FADH-OOH). This intermediate performs the formal insertion of the oxygen atom into the substrate, hence generating the oxidized flavin cofactor (FAD). Then, the reduced form of FAD is recycled in the BVMO active site through hydride transfer from NAD(P)H. In biocatalysis, NAD(P)H is commonly recycled by an additional NAD(P)H-dependent dehydrogenase. BVMOs are widely used biocatalysts due to their high and complementary selectivity that allows chemists to perform challenging regio- and stereo-selective reactions.

![Scheme 1.6. Schematic depiction of the reaction catalyzed by BVMO.](image)

In the classical chemical Baeyer-Villiger reaction, those groups that stabilize better the partial positive charge during fragmentation of the Criegee intermediate have the highest migratory tendency. Thus, if $R^1$ and $R^2$ (Scheme 1.6) are a methyl and a benzyl group, respectively, the migration of the latter will occur. In contrast, enzyme catalysis can revert this intrinsic migration tendency by stabilizing alternative intermediates in the
active site during Criegee rearrangement (Scheme 1.7a). Therefore, the “chemically unfavored” but biocatalytically accessible Baeyer-Villiger product is commonly defined as the “abnormal” product. Notably, this chemoselectivity of BVMOs can be tuned as demonstrated by studies on directed evolution of enzymes.\textsuperscript{37} Interestingly, the oxidation of (+)-trans-dihydrocarvone (Scheme 1.7a) catalyzed by wild-type BVMO produced preferentially the “abnormal” lactone, whereas the synthesis of the “normal” lactone required enzyme engineering.\textsuperscript{38} In a recent study of structure-guided saturation mutagenesis, Reetz’s group reversed the diastereoselectivity of the BVMO from \textit{Acinetobacter} sp. NCIMB 9871 (AcBVMO) for the oxidation of 4-ethylidene-cyclohexanone and 4-bromomethylene-cyclohexanone (Scheme 1.7b).\textsuperscript{39} Whilst the wild-type BVMO produced preferentially the \textit{E}-configured functionalized olefin (\textit{d.r.} from 96:4 to >99:<1), the best variant afforded the \textit{Z}-configured olefin with 4:96 \textit{d.r.}. Both \textit{E}- and \textit{Z}-configured products were synthesized on 20 mM scale (1.5 mmol, 283-560 mg), reaching 98% conversion and excellent diastereomeric ratios.

The formation of “normal” and “abnormal” products was investigated recently also for the monooxygenase from \textit{Pseudomonas putida} (OTEMO), whose wild-type enzyme produces an equal mixture of both isomers. Bornscheuer’s group was able to introduce mutations that enabled a selective formation of either the “abnormal” or the “normal” product.\textsuperscript{40} Over the past decade, many other efforts were devoted at the engineering of various catalytic properties of BVMOs. For instance, most of the available BVMOs are NADPH dependent. As the phosphorylated form of the nicotinamide adenine dinucleotide cofactor is significantly more expensive than the non-phosphorylated one, BVMO variants accepting preferentially NADH can be developed as exemplified in a recent study.\textsuperscript{41} In other cases, the stability of BVMOs was increased by protein engineering. As an example, AcBVMO was subjected to site directed mutagenesis and variants exhibiting almost 20 °C increased thermostability as well as increased tolerance to hydrogen peroxide were obtained.\textsuperscript{42} Fraaije’s group discovered a thermostable BVMO originated from \textit{Thermocrispum municipal}, which exhibits an exceptional tolerance towards acetonitrile, thus making this enzyme very attractive for chemical synthesis.\textsuperscript{43} The catalytic activity of TmCHMO was further investigated and manipulated by several
rounds of iterative saturation mutagenesis. Thus, the synthesis of several lactones from cyclic aliphatic ketones such as 3- and 4-methylcyclohexanone,

Scheme 1.7. a) Representation of normal and abnormal lactone products; b) Engineering of BVMO to afford both E- and Z- lactone; c) Kinetic resolution of bicyclic ketone using BVMO; d) Biocatalytic cascade for the synthesis of caprolactone starting from cyclohexanol.
3,5-dimethylcyclohexanone, 2-substituted cyclohexanones, 3-substituted cyclobutanones and bicyclo[4.2.0]octan-7-one proceeded with generally high chemo- and enantioselectivity. Moreover, the same enzyme was subjected to structure-guided directed evolution, thereby enabling a switch of the regioselectivity from the formation of the “normal” (99:1) to the “abnormal” lactone (2:98). The effect of the introduced mutations was rationalized, thus setting the background for further engineering.\textsuperscript{45}

The substrate scope of the BVMOs comprises various substituted cyclic ketones such as cyclohexanones, cyclopentanones,\textsuperscript{36,46} cyclobutanones,\textsuperscript{47} functionalized aliphatic ketones,\textsuperscript{36} \(\omega\)-ketocarboxylic acids,\textsuperscript{48,49} and various terpenes.\textsuperscript{36,46,50}

Due to their often excellent stereoselectivity and proven evolvability, BVMOs can also be used for kinetic resolution processes. For instance, Grogan’s group applied \textit{E. coli} cells overexpressing a BVMO from \textit{Rhodococcus jostii} (MO14) to the kinetic resolution of bicyclo[3.2.0]hept-2-en-6-one to yield (1S,5R)-2-oxa lactone in >99\% \(e.e\)., whereas the unreacted ketone enantiomer was recovered in 96\% \(e.e\.). The resolution was run for 14 h at a temperature of 16 °C and at a substrate concentration of 0.5 g L\textsuperscript{-1} (4.5 mM) (Scheme 1.7c).\textsuperscript{51}

BVMOs were also integrated in various multi-enzymatic processes. In 1991, Willets’ group presented the first biocatalytic “hydrogen-borrowing” cascade through the combination of an ADH with a BVMO.\textsuperscript{52} This concept was recently applied in the conversion of cyclohexanol to \(\varepsilon\)-caprolactone.\textsuperscript{53} A second generation process also implemented a lipase (CAL-A) in order to directly synthesize caprolactone oligomers; thus, >99\% and 43\% conversions were reached after 24 h for reactions at 200 mM and 500 mM scale, respectively (Scheme 1.7d).\textsuperscript{54} A further improvement in the biocatalytic synthesis of \(\varepsilon\)-caprolactone was the generation of a chimeric enzyme by genetically fusing a BVMO (TmCHMO) with an ADH (TbADH) in a single polypeptide chain. The combination of the fused TmCHMO-TbADH with a lipase enabled quantitative conversion at 200 mM substrate concentration, equal to a TON of 20000. The biocatalytic cascade was performed with a continuous feeding of substrate as the TmCHMO was affected by substrate inhibition at substrate concentrations above 10 mM. Product inhibition was also circumvented as the \(\varepsilon\)-caprolactone intermediate was removed in situ through the further polymerization catalyzed by the lipase CAL-
A. Alternative ADH-BVMO aerobic oxidation systems were also developed by using "double-smart co-substrates" in a bi-enzymatic convergent cascade. In another independent study, Kroutil’s group integrated an AcCHMO mutant into a multi-enzymatic cascade to convert cyclohexanol to ω-aminocaproic acid (i.e., the hydrolyzed form of ε-caprolactam). The application of BVMOs in aerobic catalytic processes is expected to increase because new enzymes, especially from fungi, are continuously discovered. These BVMOs such as the one from Aspergillus flavus display high catalytic activity also at higher substrate concentrations (30 mM).
1.4 Aerobic Biocatalytic Oxidation of Alcohols, Aldehydes and Carboxylic Acids

Biocatalytic oxidation of alcohols and aldehydes can be catalyzed by two families of oxidoreductases, namely oxidases and dehydrogenases. In order to catalyze the substrate oxidation, oxidases utilize in their oxidized (resting) state mainly a flavin or a copper center as cofactor that is bound to the enzyme. Abstraction of a hydride from the substrate generates the reduced form of the cofactor, which can directly interact with molecular oxygen for the regeneration of the oxidized resting state. In this process, hydrogen peroxide is generated as by-product. Hence, in biocatalysis it is very common to add a catalase for the disproportionation of the formed hydrogen peroxide. Biocatalytic oxidation of alcohols and aldehydes is also possible using dehydrogenases, which utilize NAD$^+$ or NADP$^+$ as cofactors. In contrast to oxidases, dehydrogenases are incapable of direct activation of dioxygen. Therefore, the oxidized form of the cofactor (NAD$^+$) is regenerated by another enzyme or chemical process. As chapter 1 of this thesis is mainly focused on catalytic aerobic oxidations, we will discuss only those examples in which the NAD-dependent substrate oxidation was combined with an aerobic regeneration of the oxidized NAD$^+$. Apart from the oxidation of alcohols and aldehydes, this section will also discuss oxidative decarboxylation.

1.4.1 Aerobic Oxidation of Primary Alcohols

Primary alcohols can selectively be oxidized to aldehydes or a further oxidation to carboxylic acids can also occur. Aerobic oxidation of primary alcohols to aldehydes can be catalyzed either by alcohol oxidases (AOxs), or laccases plus mediator or alcohol dehydrogenases (ADHs) plus NAD$^+$ regeneration system. In general, strict chemoselectivity can be achieved with ADHs.

1.4.1.1 Aerobic Oxidation of Primary Alcohols Using Alcohol Oxidases or Laccases

Alcohol oxidases (AOxs) have been considered as the first choice for the oxidation of primary alcohols to aldehydes as they require only dioxygen as additional reagent.
However, potential over-oxidation to carboxylic acids and the not particularly broad substrate scope of the available AOxs has limited their application in chemical synthesis until to date. As mentioned above, most of AOxs use a flavin or copper cofactor to catalyze the reaction (AOx, Scheme 1.8). As elevated concentrations of the by-product hydrogen peroxide can be detrimental for enzyme stability and activity, catalase is frequently added as disproportionation catalyst.

A number of robust AOxs are currently available and their applicability was demonstrated in large scale biotransformations. In 2002, Arnold’s group enhanced the thermostability of a copper-dependent galactose oxidase (GOx) from Fusarium species by directed evolution. In 2008, Turner’s group further engineered this GOx variant in order to accept aromatic secondary alcohols. Among the created variants, GOxM3-5 exhibited the broadest substrate scope for a panel of aromatic secondary alcohols.

GOxM3-5 also showed the ability to oxidize benzyl alcohol to benzaldehyde and this catalytic activity was implemented in a number of multi-enzymatic cascades aimed at the synthesis of diverse products. For instance, Kroutil’s group combined GOxM3-5 with ω-transaminases (ωTA) to produce various substituted benzyl amines. Turner’s
group reported the combination of GOx (M3-5 and F2 variants) either with xanthine dehydrogenase or with chemical oxidants to produce carboxylic acids and amides from substituted benzaldehydes or aliphatic ω-amino primary alcohols (Scheme 1.9). GoX_{M3-5} was paired with periplasmic aldehyde oxidase from *E. coli* to yield carboxylic acids, among which the most important one was furandicarboxylic acid. The reaction proceeded with quantitative conversion at 50 mM scale in a one-pot sequential process.

Turner and Woodley’s groups investigated the optimization of the catalytic performance of GOx for large scale processes. In particular, the addition of horseradish peroxidase or catalase along with potassium ferricyanide was important for maintaining the catalytic activity of GOx during the course of the reaction. Finally, the authors demonstrated that the cell free extract of GOx is the most practical and economically profitable form for the utilization of this biocatalyst. The biocatalytic oxidation of benzylic alcohol to benzaldehyde was conducted in a 250-mL aerated reactor in aqueous buffer at pH 7.4 and at 50 mM substrate concentration. Quantitative conversion was reached in 6 hours (Scheme 1.10).

Very recently, the ability of GOx to promiscuously oxidize aldehydes to carboxylic acids and nitriles were reported. However, these catalytic properties will be discussed further in their corresponding sections.

In summary, engineered variants of GOxs are biocatalysts possessing a demonstrated applicability on a large scale as well as a compatibility with other biocatalysts and chemical reagents. It is expected that genome mining and further enzyme engineering will lead to the discovery and creation of new copper-dependent AOxs possessing

![Scheme 1.10. Oxidation of benzyl alcohol catalyzed by GOx_{M3-5} in a bio-reactor.](image-url)
different substrate specificities. An example is the recently discovered AOx from *Colletotrichum* species, which exhibited catalytic activity towards aliphatic substrates.\textsuperscript{68} Flavin-dependent AOxs were also applied in biocatalysis and their substrate scope was extensively reviewed elsewhere.\textsuperscript{69,70} In this section, we present a selection of recent research that illustrates their possible synthetic applications. For instance, aryl alcohol oxidases (AAOxs), and in particular the sub-family of vanillyl alcohol oxidases (VAOs), act on aromatic substrates such as eugenol and vanillin.\textsuperscript{71} However, they can also accept a broader range of substrates. Hollmann’s group reported the large scale oxidation of trans-2-hexen-1-ol at 500 mM concentration catalyzed by AAO from *Pleurotus eryngii* (PeAAOx) using either a two-liquid-phase aqueous buffer/dodecane system (also other solvents were tolerated) or neat substrate, reaching TONs of

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\text{Scheme 1.11. Oxidation of alcohols using flavin-dependent alcohol oxidases on preparative scale: a) PeAAOx as biocatalysts; b) AcCO6 as biocatalyst.}
\]
650000 and 2.2 million, respectively (Scheme 1.11a).\textsuperscript{72} Another promising AOx is the choline oxidase from \textit{Arthrobacter cholorphenolicus} (AcCO) that was engineered to accept saturated, unsaturated, aliphatic, cyclic, aromatic, heteroaromatic and functionalized alcohols.\textsuperscript{73} The final AcCO6 variant also exhibited increased thermostability (e.g., Tm increased from 32 °C to 52 °C) and enhanced tolerance towards various organic solvents that were applied in bi-phasic reactions. Conversely, the wild-type enzyme was inactive with most of the tested organic solvents. A representative 100 mg scale bio-oxidation converted >99% of 1-hexanol (72% isolated yield; Scheme 1.11b). In a follow-up study, AcCO6 was applied in a cascade process with reductive aminases to give access to N-alkylated secondary amines at 100-150 milligram-scale with 25 mM substrate loading; isolated yields varied from 49% to 74%.\textsuperscript{74}

Laccases are copper-dependent enzymes that catalyze the aerobic multi-step, one-electron oxidation of phenols, thus leading to the formation of phenolic radicals which can further polymerize.\textsuperscript{75} These enzymes have various applications in biotechnology ranging from biomass conversion, bioremediation and biosensing.\textsuperscript{76} The ability to aerobically degrade lignin is of particular interest.\textsuperscript{77} Laccases are routinely used for the oxidation of primary and secondary alcohols and aldehydes. However, in these reactions, they oxidize mediator compounds, which in turn catalyze the oxidation. (Scheme 1.12) An early demonstration for the applicability of laccases was the oxidation of several substituted benzyl alcohols to their corresponding benzaldehydes using ABTS as mediator. A laccase from \textit{Trametes versicolor} was used in this study.\textsuperscript{78}
However, the requirement for more efficient mediators was evident. Therefore, the use of 2,2',6,6'-tetramethylpiperidine-N-oxyl (TEMPO) was introduced in 2001, when authors demonstrated its usefulness in the oxidation of various aliphatic and aromatic alcohols, amines and ethers to yield the corresponding aldehydes.\(^7\) Sheldon’s group performed further studies, thus revealing that TEMPO and derivatives thereof are indeed the most active and suitable mediators for laccase-catalyzed oxidations.\(^8\)

As laccase/mediator system is most commonly applied for the non-enantioselective oxidation of a mixture of racemic secondary alcohols, other detailed application will be given in section 1.4.2.1.

### 1.4.1.2 Aerobic Oxidation of Primary Alcohols Using Alcohol Dehydrogenases

Traditionally, alcohol dehydrogenases (ADHs) have been applied mainly for the asymmetric reduction of prochiral ketones as they often display an excellent stereoselectivity. Since recently, ADHs are also applied for the oxidation of alcohols and many companies supply panels of these enzymes.\(^8\) ADHs utilize NAD(P)H as cofactors, whose high costs (especially the phosphorylated form) make their use in stoichiometric amounts economically unviable. Therefore, NAD(P)H is used in catalytic amounts in the presence of a regeneration system. An “aerobic regeneration system” for the oxidation of NAD(P)H to NAD(P)\(^+\) is catalyzed by flavin enzymes called nicotinamide adenine dinucleotide oxidases (NOxs, Scheme 1.13).\(^8\)

![Scheme 1.13. Oxidation of primary alcohols using ADH and NOx.](image)

Interestingly, the use of the ADH/NOx system for the aerobic oxidation of primary alcohols has not been frequently applied, albeit it is recently gaining popularity.\(^8\) One
reason may be that most of the commonly applied ADHs in biocatalysis operate preferentially on secondary alcohols rather than on primary alcohols. Furthermore, as aldehydes are usually regarded as intermediates for the synthesis of more complex products, the biocatalytic aerobic oxidation is frequently integrated into cascade processes. For instance, Otha’s group reported the oxidation of arylaliphatic primary alcohols to aldehydes catalyzed by 2-phenylethanol dehydrogenase, followed by a further oxidation to carboxylic acids catalyzed by phenylacetaldehyde dehydrogenase. The oxidized form of the NAD cofactor was recycled by NOx and dioxygen in both steps. Mutti’s group applied an engineered ADH from Thermoanaerobacter ethanolicus (TeS-ADH I86A W110A) in combination with NOx for the oxidation of aliphatic alcohols to aldehydes. The ADH/NOx system was integrated in an orthogonal network for the amination of alcohols.

1.4.2 Aerobic Oxidation of Secondary Alcohols

From the synthetic point of view, oxidation of secondary alcohols can serve two main purposes: (i) kinetic resolution of racemic alcohols or (ii) synthesis of ketones. The latter application is often implemented in linear biocatalytic cascades. AOx and ADH/NOx systems are used for the oxidation of secondary alcohols, although achieving quantitative oxidation often represents a challenge as these enzymes commonly prefer to oxidize one enantiomer of a racemic mixture. Therefore, literature reports many examples for the kinetic resolution of racemic secondary alcohols and a limited number of cases of non-enantioselective oxidation. Indeed, the non-enantioselective oxidation of racemic alcohols is most commonly performed with the laccase/mediator system.

1.4.2.1 Aerobic Oxidation of Secondary Alcohols Using Alcohol Oxidases and Laccases

Oxidation of secondary alcohols by AOxs is attractive, albeit it is still underdeveloped. Although a large number of AOxs were identified, obtaining non-enantioselective secondary alcohol oxidases (SAOs) that possess a broad substrate scope remains still
a quite elusive goal. The catalytic activity of a number of SAOs from various microbial strains was reported. However, unfortunately, neither the amino acid sequence of these enzymes nor the encoding genes were identified. Therefore, the most applied A0xs for the oxidation of secondary alcohols in biocatalysis are again the variants of the galactose oxidase (GOx) from Fusarium sp. The GOx variants were applied in the kinetic resolution of secondary alcohols and the highest (i.e., synthetically applicable) activities were revealed with 1-phenylethanol and derivatives thereof (Scheme 1.14). In combination with horseradish peroxidase for the disproportion of the hydrogen peroxide byproduct, GOxM3-5 was capable of performing the kinetic resolution of these substrates in 3 h with excellent enantioselectivity (50% conversion and >99% e.e.) at a 50 mM racemic substrate concentration.

Laccase/TEMPO is a versatile system for the oxidation of racemic secondary alcohols as exemplified by a number of studies by Gotor-Fernández’ group. The system was used to catalyze the selective oxidation of aliphatic diols yielding lactones with high regioselectivity; in this study, seven substrates were fully converted at a 30 mM scale within 2.5-7 hours. Similarly, the laccase/TEMPO system was utilized for the oxidation of α,ω-diols to yield lactones (Scheme 1.15a) and primary alcohols to yield aldehydes.
or carboxylic acids. The selectivity of the oxidation to give aldehydes or carboxylic acids could be tuned by adjusting the amount of TEMPO in the reaction as well as by modifying the reaction time. The oxidation of unprotected amino-alcohols resulted in the formation of lactones (Scheme 1.15b), which was caused by the oxidation of the amine to the imine, followed by a hydrolysis of the imine to the aldehyde and a final lactone formation. The oxidation of the amine moiety could be prevented by the utilization of a protecting group. However, the expected lactam was not the only final product produced, as a competitive reaction pathway occurred which yielded almost exclusively the corresponding enamine when longer reaction times and harsher conditions were applied.\(^8\)

One of the advantages of the laccase/TEMPO system is that the oxidation of secondary alcohols proceeds in a fully non-enantioselective manner, which is not the case with the commonly applied AOxs and ADHs. Therefore, the laccase/TEMPO system was integrated in other cascade processes, in which racemic secondary alcohols were first oxidized to the ketone and then converted further into, for example, amines. This two-step sequential cascade employed \(\omega\)-transaminases in the second amination step (Scheme 1.16).\(^8\)

![Scheme 1.15. Utilization of the laccase/TEMPO system for the synthesis of: a) lactones from diols; b) lactones, lactams or enamines.](image-url)
Laccase/TEMPO was also applied for the preparation of enantiopure secondary alcohols, when an ADH was used to stereo-selectively reduce the generated ketone intermediate. The search for even better mediators than TEMPO is still a very active research field. One example is the use of 2-azaadamantane-N-oxyl (AZADO) for the oxidation of primary and secondary alcohols. By optimizing the reaction conditions, the use of AZADO enabled a 4-fold lower loading of mediator compared to TEMPO, while affording a more than 10-fold higher conversion of the model substrate in presence of PhCF₃ as co-solvent. The use of AZADO was particularly beneficial for the oxidation of more sterically hindered secondary alcohols. In general, AZADO performed in 5-10 mol%, whereas TEMPO is routinely added at higher loading (20 mol%, in this study). Finally, Monti’s group exploited the laccase/TEMPO system for a peculiar [1,3]-oxidative transposition of tertiary allylic alcohols. The methodology

Scheme 1.16. Application of laccase/TEMPO for the stereoselective amination of secondary alcohols.
operated efficiently both in water and in acetonitrile, thereby enabling the chemo-enzymatic synthesis of fragrances such as trans- and dehydro-Magnolione® (Scheme 1.17). In this synthesis, the first step was performed by the laccase/TEMPOBF₄⁻ system at a ca. 200 mg scale.⁹⁰

### 1.4.2.2 Aerobic Oxidation of Secondary Alcohols Using Alcohol Dehydrogenases

Due to the scarcity of synthetically applicable and characterized AOxs acting on racemic secondary alcohols, ADHs have been frequently applied for these oxidations. In particular, secondary ADHs in combination with NOx were applied either for the deracemization via stereo-inversion of secondary alcohols⁹¹ or for a further utilization of the ketone intermediate in cascade processes.⁸⁵,⁹² Notably, the oxidation of racemic

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Scheme 1.17. Oxidative transposition of tertiary allylic alcohols using the laccase/TEMPOBF₄⁻ system.
secondary alcohols was almost always accomplished by combining two stereo-complementary ADHs. Examples of non-selective ADHs are rare. In independent studies, Musa’s and Mutti’s groups generated and characterized non-enantioselective ADH variants from *Thermoanaerobacter ethanolicus* and applied them either for deracemization\(^9\),\(^\text{93,94}\) or alcohol oxidation.\(^\text{85}\) Fraaije’s group engineered enzymatic fusion proteins between a selection of three ADHs (LbADH, TbADH and ADH-A) and a NADPH-dependent NOx. These fusion enzymes were applied for the aerobic kinetic resolution of rac-1-phenylethanol, which exhibited a TON of up to 10000 in presence of minimal amounts of the NAD cofactor (100 μM) at a 50 mM substrate concentration.\(^\text{95}\) Pietruszka’s group reported the re-purposing of cytochrome P450-BM3—which performs the aerobic hydroxylation of myristic acid—for the regeneration of NAD\(^+\) in oxidative kinetic resolution of secondary alcohols. However, they also discovered the ability of the cytochrome to act as “truly” NOx and performed enzyme engineering to enhance this activity. Subsequently, engineered P450 was used to recycle the NAD cofactor in the ADH-catalyzed kinetic resolution of rac-1-phenylethanol affording either the (R)- or (S)-configured product in a high yield ((S)- 86 mg; (R)- 95 mg) and e.e. ((S)-98%; (R)- 95%) on hundred-milligram scale. (Scheme 1.18). Finally, the oxidation of vanillyl alcohol to vanillin was also performed.\(^\text{96}\)

### 1.4.3 Aerobic Oxidation of Aldehydes

Biocatalytic oxidation of aldehydes at the expense of dioxygen can be performed with either aldehyde oxidases (AlOxs), promiscuous alcohol oxidases (AOxs), laccases, or aldehyde dehydrogenases (ALDHs) in combination with NOx.
1.4.3.1 Aerobic Oxidation of Aldehydes Using Aldehyde Oxidases, Promiscuous Alcohol Oxidases or Laccases

Although there are not many reports on the AlOx-catalyzed oxidation of aldehydes, some of these enzymes exhibit the ability of over-oxidizing alcohols to carboxylic acids. In 2018, Turner’s group reported that GOxM3-5 can perform a second oxidation step from aldehyde to carboxylic acid upon the in situ formation of the hydrate form of the aldehyde (i.e., gem-diol). In this work, 5-methyl-2-pyrazinemethanol, 2-quinolinecarboxaldehyde and 4-pyridinemethanol (25 mM) were oxidized quantitatively in 10 ml-scale reactions at 0.25 mmol substrate concentration (ca. 40 mg) within 24 h (Scheme 1.19). This reaction is synthetically useful since GOxM3-5 accepts N-heteroaromatic benzylic alcohols, which are common structures in pharmaceutically relevant molecules.

Fraaije and Mattevi’s groups engineered a 5-(hydroxymethyl)furfural oxidase (HMFO), which is capable of performing the three step oxidation of 5-(hydroxymethyl)furfural to 2,5-furandicarboxylic acid. The product is of interest for the production of biodegradable plastics from renewable feedstocks.

Figure 1.19. Over-oxidation of aromatic primary alcohols to carboxylic acids catalyzed by a GOx variant.
As described in sections 1.4.1.1 and 1.4.2.1, aldehydes can be oxidized to carboxylic acids, lactones, etc. by the laccase/mediator system. The reaction is commonly performed starting from alcohols. The reaction can be stopped at the aldehyde level or run further to the carboxylic acid depending on the reaction conditions.\textsuperscript{86}\textsuperscript{98}

### 1.4.3.2 Aerobic Oxidation of Aldehydes Using Aldehyde Dehydrogenases

Mutti’s group reported the oxidation of a panel of sixty-one aliphatic, aryl-aliphatic, benzylic, hetero-aromatic and bicyclic aldehydes to the related carboxylic acids catalyzed by three recombinant ALDHs.\textsuperscript{99} Among the investigated substrates, fifty were converted with elevated yield (up to >99%). The exceptions were a few ortho-substituted benzaldehydes, bicyclic heteroaromatic aldehydes and 2-phenylpropanal. In all cases, the expected carboxylic acid was shown to be the only product (>99% chemo-selectivity). Other oxidizable functionalities within the same molecule (e.g. hydroxyl, alkene, and heteroaromatic nitrogen or sulfur atoms) remained untouched. The ALDHs were applied as isolated enzymes or whole cell biocatalysts in combination or in absence of NOx as the aerobic cellular metabolism could re-oxidize NAD cofactor in some cases. The synthetic applicability of the method was exemplified in the two-gram scale synthesis of 5-(hydroxymethyl)furoic acid from 5-(hydroxymethyl)furfural, affording perfect chemo-selectivity and 61% of isolated yield (Scheme 1.20). Ohta’s group applied one ALDH in an aerobic cascade for the oxidation of seven aromatic and aliphatic alcohols to carboxylic acids.\textsuperscript{84} Hollmann’s group utilized ADHs in combination with NOx for the regeneration of NAD\textsuperscript{+} in the oxidative dynamic kinetic resolution of profen aldehydes to yield optically pure carboxylic acids. However, the reported mismatch in the operating reaction conditions between ADH and NOx limited significantly the performance of the system. Finally, the optimal conditions were found
by abandoning the NOx system and using acetone instead in a “co-substrate approach”.100

1.4.4 Aerobic Oxidative Decarboxylation

Biocatalytic oxidative decarboxylation yields terminal alkenes. Currently available chemical (i.e., homogeneous and heterogeneous catalysis) as well as enzymatic processes require major development to allow industrial applicability.101 A decarboxylating heme-containing enzyme was described from Jeotgallicoccus sp. (OleT) in 2011. This decarboxylase was initially reported to utilize H₂O₂ instead of dioxygen, thus acting as a peroxygenase.102 However, in 2014, Li’s group
demonstrated the ability of OleT to catalyze the oxidative decarboxylation of C12-C20 fatty acids using dioxygen and NADPH, thus acting as a cytochrome P450 (Scheme 1.21a). Faber’s group investigated further the possibility of a biocatalytic application and could successfully form C3-C21 alkenes (albeit at 10 mM loading of the substrates and only up to 3.9 mM product formation). However, OleT could also be applied for the synthesis of terminal dienes from dicarboxylic acids. Notably, OleT was also reported to accept various non-linear carboxylic acids on a hundreds of milligrams scale.

Wang’s group created a chimeric enzyme by fusing OleT with the reductase domain of P450-BM3. The chimera could efficiently utilize dioxygen and NADPH for the production of several mono- and di-olefins from the corresponding carboxylic acids.

Scheme 1.21. Oxidative decarboxylation catalyzed by OleT affording terminal alkenes: a) general scheme; b) preparative scale production of alkenes using OleT-BM3R fusion.
This enzymatic reaction proceeded with product titers of up to 2.51 g L\(^{-1}\) and volumetric productivities of up to 209.2 mg L\(^{-1}\) h\(^{-1}\) at low catalyst loadings (\(\sim\)0.02 mol %). A 1.5 gram-scale decarboxylation of stearic acid led to 60% isolated yield of 1-heptadecene (Scheme 1.21b). It is expected that in future approaches, the biocatalytic decarboxylation to olefins will be largely exploited as it provides an interesting alternative to current chemical methods.

A non-heme Fe-dependent oxidase from *Pseudomonas putida* capable of forming 1-alkenes from fatty acid was recently discovered and biochemically characterized.\textsuperscript{108}

### 1.5 Biocatalytic Aerobic Oxidation of Amines and Imines

Principally, oxidative transformations of amine and imine substrates can yield aldehydes, ketones, nitriles and amides (Scheme 1.22). All of the above-mentioned products are important for many applications. Amines, amides and nitriles are used as intermediates or are final products for bulk chemicals, pharmaceuticals, agrochemicals, flavors and fragrances.\textsuperscript{109-111} Green synthesis of optically pure amines constitutes one of the key challenges for the pharmaceutical industry. Current research efforts in the field of biocatalytic synthesis of primary, secondary and tertiary amines focus on the reductive amination of carbonyl compounds utilizing several classes of enzymes (mainly dehydrogenases and transferases).\textsuperscript{112} The biocatalytic oxidative aerobic preparation of amines is typically performed by deracemization processes, in which an
amine racemate is converted into an optically pure product. Other analogous applications concern the synthesis of α-keto acids from their corresponding amino acids. Finally, the biocatalytic oxidations of imines to nitriles or amides were very recently added to the biocatalytic reactions’ toolbox.

1.5.1 Aerobic Kinetic Resolution and Deracemization of Amines and Amino Acids

1.5.1.1 Aerobic Kinetic Resolution and Deracemization of Amines Using Dehydrogenases

The biocatalytic synthesis of α-chiral amines can be accomplished by the reductive amination using amine dehydrogenases (AmDHs) or imine reductases (IREDs). Since the reaction is reversible, these enzymes can be employed in kinetic resolution or deracemization processes. Thereby, the (de)aminating enzyme is again coupled with a nicotinamide oxidase (NOx) for the recycling of the NAD⁺ cofactor. However, the kinetic resolution is limited by a maximum of 50% of the theoretical yield. If the oxidative kinetic resolution is coupled with a non-selective reduction step (such as borane reduction in this case), the process can run iteratively, thus leading theoretically up to quantitative conversion into the desired enantiomer. Such a process is called Scheme 1.23. Deracemization of amines using RedAms.
deracemization. However, neither AmDHs\textsuperscript{115} nor IREDs\textsuperscript{116} operate efficiently in the kinetic resolution or deracemization as a considerably high enzyme loading is required until to date. In general, IREDs were used for the deracemization of 1-methyl-1,2,3,4-tetrahydroisoquinoline and N-ethynyl-2,3-dihydro-1H-inden-1-amine on a 100 mg scale (Scheme 1.23) with moderate success.\textsuperscript{116}

1.5.1.2 Aerobic Kinetic Resolution and Deracemization of Amines Using Monoamine Oxidases

Monoamine oxidases (MAOs) are flavin-dependent enzymes that perform the enantioselective oxidation of amines to imines at the expense of dioxygen and forming hydrogen peroxide as byproduct (Scheme 1.24).

MAOs are highly stereoselective enzymes, thus they were extensively applied in deracemization processes.\textsuperscript{112} The most studied MAO is the enzyme from \textit{Aspergillus niger} (MAO-N), first reported by Legge’s group almost 30 years ago.\textsuperscript{117} The substrate scope of wild type MAO-N is limited to smaller aliphatic substrates; therefore, many efforts were devoted to the engineering of variants capable of accepting a wider range of substrates. Turner’s group created the first variant, MAO-N D1, that accepted bulkier substrates such as substituted α-methylbenzylamine and 1-(1-naphtyl)ethylamine.\textsuperscript{118} Afterwards, a library of MAO-N variants with complementary substrate scope was created by directed evolution. For instance, MAO-N D3 acting on cyclic secondary amines was immobilized on Eupergit\textsuperscript{®} C and was employed, in combination with ammonium borane, for the deracemization of 2-phenylpyrrolidine (14.7 g l\textsuperscript{-1}) on a 50 ml scale, giving 80% yield and 98% e.e. after 48 hours.\textsuperscript{119} Since MAOs are in general (S)-selective, an (R)-selective amine oxidase was engineered starting from 6-hydroxy-D-nicotine oxidase from \textit{Arthrobacter nicotinovorans}, which naturally displays (R)-selectivity.\textsuperscript{120} The best variant was active on nineteen out of the thirty-four tested cyclic 

\[ \text{NH}_2 \xrightarrow{\text{MAO}} \text{NH} \]

\[ \text{O}_2 \xrightarrow{\text{H}_2\text{O}_2} \text{H}_2\text{O} \]
and bicyclic aliphatic and aromatic racemic amine substrates. Furthermore, 500 mL scale reactions at 5 mM substrate concentration afforded 55-93% yields with e.e.’s of 84->99%. Lau’s group reported a promiscuous cyclohexylamine oxidase (CHAO) from Brevibacterium oxydans. CHAO provided access to the opposite enantiomer of α-methylbenzylamine compared with the wild-type MAO-N. Therefore, the enzyme was very recently further engineered for the deracemization of 2-substituted 1,2,3,4-tetrahydroisoquinolinones (THQ). Six 2-substituted-THQs derivatives (5 mM concentration) were deracemized at 100 mL scale with 91->99% e.e. and isolated yields in the range of 58%-92%. For detailed information on available MAO enzymes, we refer the reader to a couple of recent reviews.

Scheme 1.25. Deracemization of alkaloids using MAO-N variants.
Turner’s group evolved a MAO-N variant that exhibited activity towards aminodiphenylmethane (benzhydrylamine) and 1-phenyltetrahydroisoquinoline templates. The final engineered MAO-N biocatalyst (D11) was applied in the chemo-enzymatic synthesis of APIs such as Solifenacin and Levocetirizine as well as the natural products (R)-coniine, (R)-eleagnine, (R)-leptaflorine and (R)-harmicine (Scheme 1.25).\textsuperscript{124}

The pivotal deracemization step was performed in 48 h at 0.5-1 g-scale (15 mM substrate concentration) with isolated yields ranging from 45-90% and e.e.s ranging from 97-98%. Castagnolo’s group discovered the aromatization activity of MAO-N variants towards 1,2,5,6-tetrahydropyridines (THP). The aerobic aromatization of 4-phenyl-1,2,3,6-tetrahydropyridine was performed at 500 mg scale (10 mM substrate concentration, Scheme 1.26) affording chemically pure 4-phenyl-pyridine in 71% isolated yield.\textsuperscript{125}

Most of the MAO-catalyzed deracemization processes utilize non-stereoselective chemical reducing agents. However, the atom-economy of the deracemization can be significantly improved if the MAO-catalyzed kinetic resolution is combined with the IRED-catalyzed reductive amination of the formed imine intermediates.\textsuperscript{126} The main requirement, apart from mutual compatibility between MAO and IRED, is that the enzymes display opposite enantioselectivity (e.g., the MAO oxidizes aerobically the (S)-amine to the imine and the IRED reduces the imine to the (R)-amine). By switching
the selectivity of both enzymes, the other enantiomer is also accessible. The reactions were performed at 1.55 mmol scale (10 mM substrate concentration). A current limitation of this methodology seems to be the compatibility of the substrate scope between MAOs and IREDs. However, with the wide-spread use of genome mining and continuous progress in enzyme engineering, it is expected that these limitations will be addressed in the near future (Scheme 1.27). Reetz’s group reported an interesting example for engineering the substrate specificity of MAOs by introducing additional mutations at the entrance of the substrate tunnel that leads to the active site. Using this methodology, previously non-accepted substrates were converted by the newly obtained variants. Furthermore, inversion of the stereo-selectivity was revealed with some of the variants, thereby indicating that the substrate channel from the outer part of the enzyme to the active site can influence the stereoselectivity of the oxidation. Another possibility for improving the atom-economy in the deracemization of amines using MAOs concerns the combination with a photo-redox process as reported by the groups of Wenger and Ward. Kinetic resolution catalyzed by *E. coli* cells expressing...
MAO-N was combined with a visible light-driven reduction of the imine intermediate catalyzed by Na$_3$Ir(ppy)$_3$ or Ru(bpy)$_3$Cl$_2$ as photocatalysts. Addition of ascorbate or thiol donors was required to quench the formed α-amino alkyl radical intermediate. The performance of the system enabled the production of cyclic secondary amines with yields up to >95% and e.e.’s up to >99%. Nonetheless, the reaction was run at a 1 ml scale and at 10 mM substrate concentration only.

Finally, an important application of MAOs relates to the synthesis of Boceprevir, a drug targeting hepatitis C. Boceprevir possesses a bicyclic [3.1.0]proline structural motif. Li’s group reported the development of a MAO-N variant capable of catalyzing the oxidation of 6,6-dimethyl-3-azabicyclo[3.1.0]hexane to 6,6-dimethyl-3-azabicyclo[3.1.0]hex-2-ene. Since the imine product is partly lost because of low solubility and volatility, and since it causes inhibition of the biocatalyst, the authors increased its solubility by in situ formation of amino sulfonate through the addition of sodium bisulfate (Scheme 1.28). The Boceprevir intermediate was obtained with 95% isolated yield and optically pure form.

Scheme 1.28. MAO-N 401-catalyzed synthesis of the sulfonyl intermediate of the P2 intermediate of Boceprevir.
1.5.2 Aerobic Kinetic Resolution of Amino Acids and Synthesis of α-Keto Acids

The oxidation of α-amino acids catalyzed by α-amino acid oxidases (AAOs) can be used for deracemization processes or for obtaining α-keto acids (Scheme 1.29a). Most of the available enzymes act on D-α-amino acids (D-α-amino acid oxidases, DAAOs), thus enabling the use of D-AAOs for the production of enantiopure L-α-amino acids.130

One of the most industrially relevant applications of DAAOs is the production of 7-aminocephalosporanic acid (7-ACA), which is an intermediate for the synthesis of cephalosporin antibiotics. In particular, DAAO was applied in a one-pot process affording 7-ACA from cephalosporin C.131 Another application is the preparation of optically pure fluorinated α-amino acids, which was demonstrated in the kinetic resolution of 3-fluoroalanine (500 mM concentration); >99% e.e. of the desired enantiomer was obtained at 52% conversion after 14 hours, using a DAAO-hemoglobin fusion (Scheme 1.29b).132 As mentioned above, DAAOs are highly specific towards D-α-amino acids.133 Therefore, Asano’s group engineered a variant of DAAO from porcine kidney (pkDAAO) that was applicable in a dynamic kinetic resolution of racemic para-chlorobenzhydrylamine (5 mM) to yield the (R)-enantiomer.134 An interesting approach combines a DAAO from Trignopsis variabilis, an α-amino acid racemase from Vibrio

![Scheme 1.29. a) General reaction catalyzed by α-amino acid oxidases; b) kinetic resolution of 3-fluoroalanine on preparative scale; c) synthesis of α-ketoglutarate from L-glutamate.](image-url)
*cholera* and a catalase; all of the enzymes were immobilized on activated agarose beads and applied for the synthesis of α-keto acids from valine, leucine and nor-leucine.\textsuperscript{135}

Although L-α-amino acid oxidases (LAAO) exist in Nature, they are difficult to produce in a heterologous host and this fact has limited their applicability.\textsuperscript{133} A successful heterologous expression of L-aspartate oxidase from *Sulfolobus tokodaii* in *E. coli* enabled the kinetic resolution of (rac)-aspartate at 50 mM scale. The enzyme exhibited extraordinary thermal stability, since it showed a 2-fold increased activity at 60 and 80 °C compared to 37 °C.\textsuperscript{136} L-glutamate oxidase from *Streptomyces mobaraensis* was produced in *E. coli* and the cells containing the overexpressed enzyme were used for the production of α-ketoglutarate, thus leading to an average space-time yield (STY) of 6.5 g L\(^{-1}\) h\(^{-1}\) at 100 g L\(^{-1}\) loading of glutamate (Scheme 1.29c).\textsuperscript{137}

Since LAAOs are not easily accessible, L-α-amino acid deaminases (LAADs) constitute a viable alternative. Unlike DAAOs and LAAOs, LAADs utilize cytochrome together with FAD to promote the deamination, hence producing H\(_2\)O instead of H\(_2\)O\(_2\) (Scheme 1.30). Although this feature avoids the need for disproportion of H\(_2\)O\(_2\), the application of LAADs was only marginally investigated so far.\textsuperscript{138} Pollegioni’s group reported the application of purified LAAD from *Proteus myxofaciens* for the kinetic resolution of racemic α-amino acids to yield the D-enantiomer as well as for the chemo-enzymatic stereo-inversion of L-para-nitrophenylalanine.\textsuperscript{139}

![Scheme 1.30. General reaction catalyzed by LAADs.](image-url)
1.5.3 Aerobic Oxidation of Imines

The existence of imines in an aqueous environment is governed by the thermodynamic equilibrium, in which the nitrogen can be displaced by a water molecule. Subsequently, a carbonyl compound is formed and free amine is liberated. Therefore, the oxidation of cyclic imines to amides is a quite elusive biocatalytic reaction. In nature, this reaction is performed by the xanthine dehydrogenase/oxidase (XDH/XOD) enzyme system that is able to use both nicotine amide cofactor and molecular oxygen as electron acceptor. The enzyme system is widely known for its ability to oxidize purines (Scheme 1.31). Turner’s group demonstrated the applicability of XDH in a biocatalytic cascade with a variant of GOx yielding various lactams, albeit the system still needs further research to be applicable on preparative scale.

In this context, we recently reported the oxidation of aromatic and cinnamic aldmines catalyzed by a promiscuous galactose oxidase, whose discovery is further discussed in Chapter 4 of this thesis.67

![Scheme 1.31. Oxidation of hypoxanthine to uric acid by XDH/XOD.](image)

1.6 Biocatalytic Aerobic Oxidation of Alkenes

Biocatalytic oxidation of alkenes at the expense of dioxygen comprises asymmetric epoxidation, mono- and di-hydroxylation as well as C-C double bond cleavage. Biocatalytic epoxidation is a mature methodology, due to the very high productivities as well as chemo- and stereoselectivities. Conversely, biocatalytic di-hydroxylation is currently limited by non-perfect stereoselectivity; therefore, optically pure vicinal diols are most commonly accessed by epoxidation followed by enantioselective hydrolysis. This section highlights the major achievements in this area, including mono-hydroxylation and alkene cleavage.
### 1.6.1 Aerobic Epoxidation of Alkenes

A number of monooxygenase enzymes can catalyze asymmetric epoxidation of alkenes such as styrene monooxygenase, xylene monooxygenase, alkane monooxygenase, alkene monooxygenase and cytochrome P450. The bi-enzymatic system of the styrene monooxygenase (SMO) is currently the most applied epoxidation biocatalyst. SMO comprises a reductase enzymatic unit (StyB)—which performs the reduction of FAD at the expense of NADH—and an epoxidation unit that aerobically generates flavin hydroperoxide (FAD-OOH) and accomplishes the subsequent stereoselective epoxidation of the alkene substrate. Schmid’s group engineered an *E. coli* strain to express the bi-enzymatic system of the SMO from *Pseudomonas* sp. The biocatalyst was applied in a 30 L-scale, bi-phasic aqueous-organic epoxidation of styrene to yield 338 grams of (S)-styrene oxide in >99% e.e. (Scheme 1.32a). In follow-up studies, the productivity of the system was increased first to 8.4 g\text{product L}^{-1}\text{aq. phase h}^{-1} and later the total productivity reached 36.3 g L\text{-1}\text{tot.} Thus,

![Diagram](image.png)

**Scheme 1.32.** Biocatalytic aerobic epoxidation catalyzed by styrene monooxygenase (SMO) from *Pseudomonas* sp. a) *E. coli* cell/SMO applied in a feed-batch 30 L bioreactor; b) the elucidated substrate scope.
the bio-epoxidation of styrene derivatives became a superior method compared with chemical epoxidation both from an environmental and an economic perspective. The same SMO was also applied as isolated crude enzyme in a 200 ml scale reaction, albeit the productivity was shown to be lower (1 g l⁻¹ h⁻¹). In particular in more recent studies by Li’s group, the substrate scope of the SMO from *Pseudomonas* sp. was elucidated, in which the enzyme was implemented in cascade reactions to produce optically active diols, α-hydroxy amines, α-hydroxy carboxylic acids and α-amino carboxylic acids (Scheme 1.32b). The substrate scope comprises a variety of halo-, methyl-, methoxy-substitute styrenes, α- and β-methylstyrenes, indene and 1,2-dihyronaphthalene. Mutti’s group recently engineered a chimera of the SMO from *Pseudomonas* sp. (Fus-SMO) by genetically fusing StyA and StyB using a designed linker. Remarkably, Fus-SMO exhibited a 3-fold increase of the catalytic activity and improved coupling efficiency (NADH consumption vs. product formation) compared with the natural system, in which discrete reductase and epoxidase units are present. Fus-SMO was also 110-fold more active than other native fusion enzymes. Fus-SMO and formate dehydrogenase were co-expressed in *E. coli* and applied as a self-sufficient biocatalytic system for epoxidation on greater than 500 mg scale. Other SMOs such as the enzyme isolated from *Pseudomonas* sp. LQ26 epoxidizes non-conjugated secondary allylic alcohols with excellent stereoselectivities. Non-heme iron-dependent monoxygenases can also epoxidize styrenes as well as more general types of alkenes. However, the applicability of these enzymes is limited because of the moderate chemoselectivity (i.e., alcohols are also formed) and stereoselectivity (i.e., e.e. hardly ever reaches 95%). In some cases, the narrow substrate scope or the impossibility to express the enzyme in a heterologous host such as *E. coli* constitute additional drawbacks. The heme-dependent cytochrome P450 can catalyze alkene epoxidation on a variety of structurally diverse substrates comprising styrenes, terminal alkenes, cyclic alkenes and unsaturated fatty acids. The inherent limitation of these natural enzymes for epoxidation is the non-perfect stereoselectivity and the low product titers. However, directed evolution studies on P450 demonstrated that variants with high stereoselectivity can be created, thus opening the way for possible larger scale synthetic applications in the future.
1.6.2 Aerobic Mono- and Di-hydroxylation of Alkenes

Mono- or di-hydroxylation at the aromatic C=C bond constitutes an attractive form of functionalization, through de-aromatization of the target compound.\textsuperscript{154}

1.6.3 Aerobic Mono-Hydroxylation of Phenols and Alkenes

Only very few examples of biocatalytic hydroxylation of phenols are reported in literature. Enzymes catalyzing this transformation are only biochemically characterized and their use in chemical synthesis remains largely unexplored. Narayan’s group used three flavin-dependent monooxygenases (TropB from \textit{Talaromyces stipitatus}, SorC from \textit{Penicillium rubens} and AzaH from \textit{Aspergillus niger}) for the biocatalytic site-selective dearomatization of substituted phenols, which generally proceeded within 1 h at 2.5 mM substrate concentration. The applicability of the biocatalytic reaction was demonstrated in 1 g scale synthesis utilizing lyophilized cells expressing the enzymes. At 5 mM substrate concentration, 99\% conversion could be reached for three of the tested substrates (Scheme 1.33).\textsuperscript{155}
The anti-Markovnikov redox hydration of alkenes has not been observed in Nature. However, Arnold’s group engineered a P450 variant from *Labrenzia aggregate* (further called anti-Markovnikov oxygenase, aMOx) that was capable of catalyzing the anti-Markovnikov oxidation of nine substrates including ortho-, meta- and para- substituted compounds as well as both 1,1-disubstituted and internal alkenes. The highest TTN of 4500 was observed with para-chlorostyrene, leading to 92% product isolated yield.

Scheme 1.33. Hydroxylation of phenols catalyzed by flavin-dependent monooxygenases.
Although this methodology is still limited by the substrate scope and relatively small substrate loading (5 mM), it appears quite promising considering the possibilities for further protein engineering.

**1.6.4 Aerobic Dihydroxylation of Alkenes and Arenes**

Cis-dihydroxylation of aliphatic alkenes and arenes is catalyzed by dioxygenases (DOs) that commonly contain a Rieske-type Fe-S cluster in their active site (Scheme 1.35a). These enzymes play important roles in many biosynthetic pathways, thus bearing the future promise of catalyzing very selective hydroxylation reactions. Rieske-type DOs are difficult to purify, therefore mainly bacterial strains and various cell preparations are used. For instance, a fermenter-based procedure for the large scale synthesis of cyclic diols was implemented using a *Pseudomonas putida* UV4 strain
producing a toluene dioxygenase.\textsuperscript{159} In 1996, Gibson’s group reported the stereoselective dihydroxylation of styrene vinyl group catalyzed by a naphthalene dioxygenase from \textit{Pseudomonas} sp., albeit with non-perfect e.e. (78\%).\textsuperscript{160} Hauer’s group recently revived this research line by studying wild-type Rieske DOs and creating engineered variants, which were applied for the asymmetric dihydroxylation of aliphatic and aromatic substrates. Although chemo- and stereoselectivities were generally not perfect, the applicability was demonstrated in a 70 mg scale synthesis.\textsuperscript{161} However, a number of toluene dioxygenases were engineered to accomplish the dihydroxylation of arenes and styrenes in a high stereoselective manner.\textsuperscript{162} A site-directed mutagenesis study focused on naphthalene dioxygenase from \textit{Pseudomonas} sp. NCIB 9816-4.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Scheme_1.png}
\caption{Dihydroxylation using dioxygenases and examples of their application in the total synthesis of natural products.}
\end{figure}
revealed the amino acid residues in the active site which influence chemo-, stereo- and regioselectivity for a panel of representative substrates. At this moment, the main application of aerobic biocatalytic dihydroxylation reactions lies in the preparation of building blocks for the chemo enzymatic synthesis of various natural products as demonstrated by the chemo-enzymatic total synthesis of ent-Hydromorphone. In the first step of the synthesis, the 2',3'-dihydroxylation of 1-phenyl-2-bromoethane was performed by fermenting E. coli cells overexpressing the toluene dioxygenase from Pseudomonas sp. (Scheme 1.35b). A further example is the chemo-enzymatic synthesis of Pleiogenone A, in which the starting diol was obtained through the 1',2'-dihyroxylation of benzoic acid catalyzed by R. neuthropus B9 (Scheme 1.35c). The dihydroxylation products produced by Rieske-type dioxygenases constitute important intermediates for the synthesis of natural products and other bioactive molecules; these intermediates are commonly not accessible by chemical methods as they lack of the required selectivity. Therefore, Rieske-type dioxygenases are useful and applicable biocatalysts, for which the use of densely populated bacterial cultures or large scale fermentation processes can compensate for the low substrate loadings and conversion rates.
1.6.5 Aerobic Biocatalytic Alkene Cleavage

The oxidative cleavage of alkenes is in general performed with ozone (i.e., ozonolysis) or with stoichiometric amounts of inorganic oxidants. Alkene cleavage reactions that use dioxygen are highly desirable because of either improved safety (compared with ozonolysis) or atom-efficiency (compared with other chemical methods). The biocatalytic aerobic alkene cleavage was observed with a number of iron-dependent enzymes (heme and non-heme). In general, it appears that iron-dependent alkene cleaving enzymes are either active on specific families of substrates or produce a mixture of products. However, two other enzymes were thoroughly studied for this reaction because of their high chemoselectivity, namely AlkCE from *Trametes hirsuta* and TM1459 from *Thermotoga maritima*. AlkCE from *Trametes hirsuta* was initially applied in the form of lyophilized cells of the fungal strain, which could cleave a variety of vinyl aromatic compounds with high conversion and chemoselectivity (Scheme 1.38a). For instance, AlkCE cells were able to oxidative cleave 1-allyloxy-2-vinylbenzene into benzaldehyde in a preparative scale reaction (Scheme 1.38b). The catalytic cycle of AlkCE was lately elucidated, and finally the enzyme was isolated and characterized. Both AlkCE and TM1459 contain a Mn^{III} prosthetic group.
in their active site.\textsuperscript{172,173} In contrast to AlkCE, TM1459 is a peroxidase-like enzyme that needs tert-butyl peroxide to maintain the active Mn\textsuperscript{III} species and dioxygen to perform the catalysis.\textsuperscript{173} Therefore, the need to add an excess of the peroxide donor as well as its low productivity still hinders its wide applicability.\textsuperscript{174}

1.7 Aims of this thesis

Amine dehydrogenases constitute a notable group of enzymes due to their ability to efficiently catalyze the synthesis of primary amines from carbonyl compounds using ammonia (Scheme 1.37). They have initially been engineered from amino acid dehydrogenases by the mutation of two residues in the active site in 2012.\textsuperscript{160} Since then, the research efforts focused on engineering and discovery of novel AmDHs and their application. In general, the progress on AmDHs resulted into several paradigm-shifting applications such as the “hydrogen-borrowing” (i.e., however, more properly defined as “hydride-borrowing”) amination of alcohols using ADH and AmDH in tandem.\textsuperscript{175} The simultaneous use of two dehydrogenases that perform oxidation of alcohol and consecutive reductive amination of the carbonyl compound intermediate enabled the internal recycling of the cofactor. However, the overall alcohol amination process is reversible, meaning that in certain cases the unfavorable thermodynamic equilibrium can impede substrate conversion. Thus, additional studies demonstrated that the reaction equilibrium can be shifted towards the amination reaction by employing the NADP-dependent ADH and NAD-dependent AmDH together with orthogonal cofactor-specific recycling systems (Scheme 1.38).\textsuperscript{85} The cofactor regeneration NADP\textsuperscript{+} was enabled by the oxidase from \textit{E. coli} (YcnD), which utilizes

\begin{center}
\includegraphics[width=0.5\textwidth]{scheme.png}
\end{center}

Scheme 1.37. General scheme of reaction catalyzed by the amine dehydrogenases.
molecular oxygen and produces hydrogen peroxide. Section 1.4 discussed a class of enzymes that performs the oxidation of alcohols using molecular oxygen: the alcohol oxidases. The replacement of AOx for ADH-YcnD system would diminish the number of required enzymes. Since there have not been previous reports on the combination of AOx with AmDH, it seemed an interesting alternative to be explored. Chapter 2 discusses our attempts to combine AOx and AmDH in a cascade using either flavin- or copper-dependent AOxs. Chapter 3 follows up on observations and experimental challenges discussed in Chapter 2, and reports on a reductive amination process in flow utilizing agarose-entrapped amine dehydrogenase and formate dehydrogenase. In Chapter 4, we studied the oxidation of benzyl alcohol using a galactose oxidase variant, which resulted in an unexpected over-oxidation of the obtained benzaldehyde into benzonitrile and benzoic acid.

Considering the available arsenal of oxidoreductases described in this introduction, we conceived a multi-enzymatic process for the synthesis of a nylon-6 monomer (i.e., linear structure, 6-aminohexanoic acid) from cyclohexanol (Scheme 1.39). However, we had to seek first a suitable catalyst to perform the amination. We selected the ε-(deaminating)-L-Lysine dehydrogenase from *Geobacillus stearothermophilus*
Interestingly, the enzyme proved to be a highly evolvable scaffold as it was suitable for a few diverse enzyme engineering projects in our group. All of these studies were based on an enzyme’s homology model. Therefore, we decided to gain a better insight into the biochemical structure of this enzyme and validate the computational homology model by determining experimentally the 3D structure. Hence, **Chapter 5** describes the crystallographic study on the best performing variant – LysEDH_F173A (i.e., LE-AmDH-v1). Finally, **Chapter 6** describes the work for nylon-6 synthesis by implementing the previously discussed oxidoreductases. The outlook from the work presented in **Chapters 2-6** is discussed in **Chapter 7**.

Scheme 1.39. Modular biocatalytic cascade affording 6-aminohexanoic acid from cyclohexanol. (LysEDH). Interestingly, the enzyme proved to be a highly evolvable scaffold as it was suitable for a few diverse enzyme engineering projects in our group. All of these studies were based on an enzyme’s homology model. Therefore, we decided to gain a better insight into the biochemical structure of this enzyme and validate the computational homology model by determining experimentally the 3D structure. Hence, **Chapter 5** describes the crystallographic study on the best performing variant – LysEDH_F173A (i.e., LE-AmDH-v1). Finally, **Chapter 6** describes the work for nylon-6 synthesis by implementing the previously discussed oxidoreductases. The outlook from the work presented in **Chapters 2-6** is discussed in **Chapter 7**.

Scheme 1.39. Modular biocatalytic cascade affording 6-aminohexanoic acid from cyclohexanol.

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1.8 References

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