DOCTORAL THESIS IN BIOTECHNOLOGY

Chemo-enzymatic cascades for the synthesis of chiral high-value chemicals

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Stockholm, 2019

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TRITA-CBH-FOU-2019:42
ISBN: 978-91-7873-275-3
Printed by Universitetsservice US-AB 2019

Akademisk avhandling som med tillstånd av KTH i Stockholm framlägges till offentlig granskning för avläggande av teknisk doktorsexamen måndagen den 30 september kl. 10:00 i Kollegiesalen, KTH, Brinellvägen 8, Stockholm.

“Der kürzeste Weg zu sich selbst führt um die Welt herum.“

Hermann Graf Keyserling- Das Reisetagebuch eines Philosophen

ABSTRACT

Chiral amines are frequent in today´s top selling pharmaceuticals. Classical organic synthesis of pharmaceuticals is often work intensive involving many synthesis steps, the use of protection group chemistry, heavy metal catalysts and chiral crystallization techniques. In recent years biocatalysts have proven their outstanding ability to synthesize chiral compounds. In this work the possibility of employing biocatalysts as alternative catalysts for API (active pharmaceutical ingredient) synthesis was explored. Three compounds currently on the market were selected as viable case studies: Cinacalcet (a hyperparathyroidism drug), Vyvanse (an ADHD-drug) and Sertraline (an antidepressant). Two enzyme classes were investigated to directly provide the chiral amines - transaminases and imine reductases. Ketoreductases were also investigated to provide the chiral amine via the chiral alcohol. Laccases and hydrolases were employed to complete the synthesis pathways to the final API. In the case of Vyvanse a true one-pot, two-step enzymatic cascade was achieved by a transaminase and hydrolase. For Cinacalcet a chemo-enzymatic cascade could be demonstrated. Both transaminase and ketoreductase gave excellent enantioselectivities and high yield for the key intermediates, which could then be chemically converted into the final API with good yield. For Sertraline the best yield of one diastereomer precursor could be achieved by a ketoreductase, followed by further enzymatic and chemical steps to the final API. Transaminases and imine reductases both have potential in synthesizing the key amine precursors or the APIs themselves. But to date selectivity and yield are insufficient for industrial application in a lot of cases. This work demonstrates the potential of enzymes to serve as viable alternatives to organo-metallic synthesis. Furthermore enzymes have the potential to simplify work-up because of their excellent enantioselectivity. Finally, a scale-up of a one-step transamination to the key chiral precursor of Cinacalcet demonstrated the enzyme´s applicability in larger volume and at higher substrate concentration.

SAMMANFATTNING

Kirala aminer är ofta förekommande i de mest sålda läkemedlen. Deras framställning med klassisk organisk syntes är ofta komplicerad på grund av många syntessteg, användning av skyddsgrupper, tungmetall-katalysatorer och kirala kristallisationstekniker. Under de senaste åren har biokatalysatorer visat sin framstående förmåga att syntetisera kirala aminer. I det här arbetet visas möjligheten att använda biokatalysatorer som alternativa katalysatorer för läkemedelssyntes. Tre substanser som finns idag på marknaden har valts som exempel: Cinacalcet- ett hyperparatyroidism-botemedel, Vyvanse- ett ADHD-botemedel och Sertraline- ett antidpressivt läkemedel. Två enyzmklasser har undersökts som kan syntetisera kirala aminer direkt-transaminaser och iminreduktaser. Ketoreduktaser har också undersökts för att de kan syntetisera kirala alkoholer, vilka kan omvandlas till kirala aminer. Laccaser och hydrolaser har använts för att komplettera hela syntesen till den aktiva läkemedelssubstansen. För Vyvanse kunde en två-stegssyntes i samma reaktionskärl utvecklas med ett transaminas och ett hydrolas. För Cinacalcet kunde en kombinerad kemisk-enyzmatisk katalyserad syntes utvecklas. Både transaminaser och ketoreduktaser uppvisade en enastående enantioselektivitet och hög omsättning för framställningen av nyckelintermediärer som kunde omvandlas till målsubstanserna med organisk kemiska metoder med bra utbyte. För Sertraline kunde den bästa omsättningen med bra selektivitet för en diastereomer erhållas med ett ketoreduktas. Den aktiva substansen Sertraline kunde erhållas med vidare enzymatiska och kemiska steg. Både transaminaser och iminreduktaser har potential för att användas till själva syntesen men tills nu inte med tillräckligt selektivitet och omsättning. Detta arbete visar att enyzmer har ett framtid som möjliga alternativ till organisk-metalliska katalysatorer. Framöver finns möjlighet att de kan minska behovet av upparbetnings-steg på grund av deras enastående selektivitet. Slutligen gjordes en uppskalning av en transaminasreaktion för framställning av en kiral nyckel-byggsten till Cinacalcet. Denna uppskalning visade enzymets tilllämpbarhet att hantera större volymer och högre substratkoncentrationer.

ZUSAMMENFASSUNG

Chirale Amine sind eine häufig vorkommende Stoffklasse in den meistverkauften Arzneimitteln. Ihre klassische, organische Synthese beinhaltet oft viele Reaktionsschritte, Anwendung von Schutzgruppenchemie, Schwermetallkatalysatoren und chiralen Kristallisationstechniken. Untersuchungen in den letzten Jahrzehnten zeigten wiederholt die Fähigkeit von Biokatalysatoren, chirale Moleküle zu synthetisieren. In dieser Arbeit werden Möglichkeiten dargestellt, wie Biokatalysatoren als alternative Katalysatoren in Synthesewegen für Wirkstoffe angewendet werden können. Als Wirkstoffe wurden exemplarisch die Moleküle Cinacalcet, Vyvanse und Sertraline ausgewählt. Es wurden zwei Enzymgruppen- Transaminasen und Iminreduktasen - untersucht, die direkt chirale Amingruppen synthetisieren können. Zusätzlich sind Ketoreduktasen getestet worden, die chirale Alkohole synthetisieren können, die später wiederum in chirale Amine umgesetzt werden. Außerdem sind Laccasen und Hydrolasen für ergänzende Schritte im vollständigen Syntheseweg zum finalen Wirkstoff betrachtet worden. Für Vyvanse konnte eine echte zweischrittige, enzymatisch katalysierte Eintopfreaktion mit einer Transaminase sowie einer Hydrolase entwickelt werden. Verschiedene chemo-enzymatische Kaskaden wurden für Cinacalcet entwickelt. Transaminasen und Ketoreduktasen zeigten beide eine exzellente Selektivität, sowie hohe Umsätze für wichtige Zwischenprodukte, die wiederum chemisch effizient in den Wirkstoff Cinacalcet umgesetzt wurden. Die höchste Ausbeute für ein Alkoholdiastereomer, ein Zwischenprodukt des Wirkstoffs Sertraline, erzielte eine Ketoreduktase. Dieses Alkoholdiastereomer konnte über weitere enzymatische und chemische Schritte zum Wirkstoffmolekül umgewandelt werden. Transaminasen und Iminreduktasen ließen Potenzial erkennen, für den Umsatz zu Zwischenprodukten oder dem Wirkstoff, jedoch waren Umsatz und Selektivität zu gering. Diese Arbeit zeigt, dass Enzyme geeignete Alternativen zu konventionellen, organo-metallischen Katalysatoren sind. Sie haben das Potenzial, durch ihre herausragende Enantioselektivität den Umfang von Aufreinigungsschritten zu reduzieren. Die abschließende Durchführung des Scale-up einer Transaminationsreaktion zu einem wichtigen Zwischen-produkt (Cinacalcet Synthese) zeigte die Anwendungsfähigkeit des Enzyms in höheren Volumina und höheren Substratkonzentrationen.

Public defence of dissertation

This thesis will be defended on September 30th, 2019 at 10:00 am in Kollegiesalen, Brinellvägen 8, Stockholm, for the degree of "Teknologie Doktor" (Doctor of Philosophy, PhD) in Biotechnology.

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LIST OF APPENDED PAPERS

Paper I

Chemoenzymatic Approaches to the Synthesis of the Calcimimetic Agent Cinacalcet Employing Transaminases and Ketoreductases

Lisa Marx, Nicolás Ríos-Lombardía, Judith F. Farnberger, Wolfgang Kroutil, Ana I. Benítez Mateos, Fernando López-Gallego, Francisco Morís, Javier González-Sabín, Per Berglund,
Adv. Synth. Catal. **2018**, *360*, 11, 2157-2165

Paper II

Fractional Factorial Experimental Design and Scale-Up of a Transaminase Process

Lisa Marx, Philipp Süss, Per Berglund. *Manuscript*

Paper III

Chemoenzymatic Synthesis of Sertraline

Lisa Marx, Nicolás Ríos-Lombardía, Philipp Süss, Matthias Höhne, Francisco Morís, Javier González-Sabín, Per Berglund.
Submitted Manuscript

Paper IV

Enzymatic one-pot two-step cascade for the synthesis of Vyvanse

Lisa Marx, Philipp Süss, Francisco Morís, Javier González-Sabín, Per Berglund. *Manuscript*

CONTRIBUTION TO APPENDED PAPERS

I. Major contribution to design, planning and execution of experiments. Minor contribution to the writing. Designed the graphical abstract.

II. Major contribution to planning and executing the experiments. Performed majority of the writing.

III. Major contributions to design, planning and execution of the experiments. Performed majority of the writing.

IV. Major contributions to design, planning and execution of the experiments. Performed the majority of the writing.

LIST OF ABBREVIATIONS

API	Active Pharmaceutical Ingredient
ATA	Amine transaminase
PLP	Pyridoxal- 5´-phosphate
PMP	Pyridoxamine- 5´phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate
NADH	Nicotinamide adenine dinucleotide
KRED	Ketoreductase
IRED	Imine reductase
AZADO	2-azaadamantane-N-oxyl
TEMPO	2,2,6,6-Tetramethylpiperidin-1-yl)oxyl
DoE	Design of Experiments
CAL-B	*Candida antarctica* lipase

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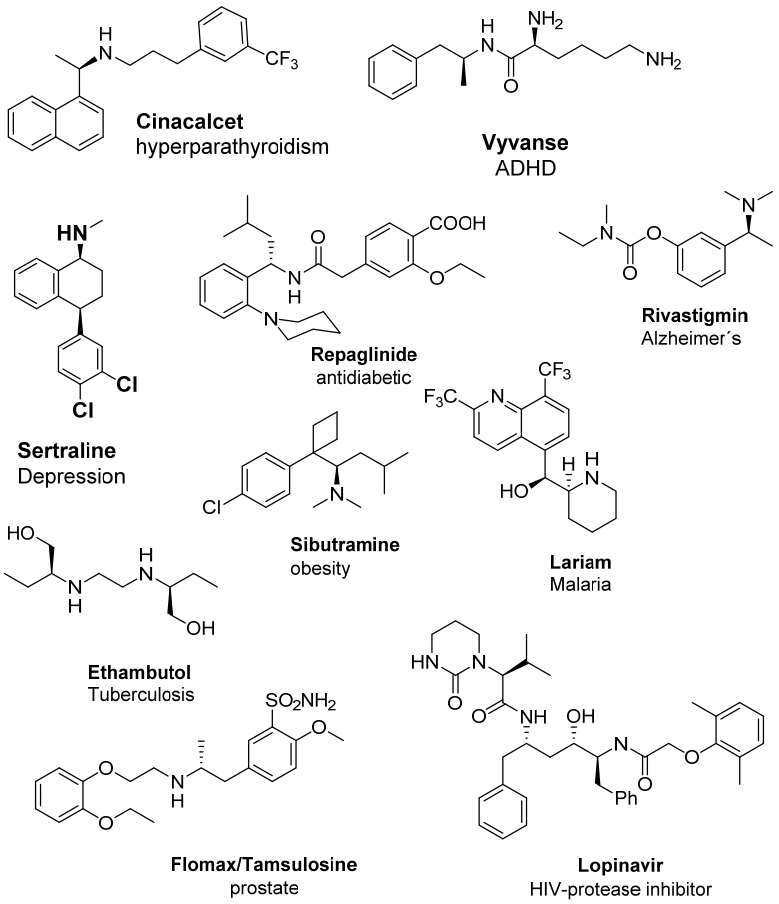
1 INTRODUCTION

Humans have been harnessing the power of nature's own catalysts for thousands of years. First they discovered fermentation for preservation of food and beverages[1]. Here the metabolic pathways of the employed organism- in general yeast and bacteria are exploited to refine or preserve raw edible materials. These metabolic pathways are made up of proteins- more specifically enzymes. Today enzymes are used in various household products, most prominently still in food processing but also in detergent and toiletries[2]. They are also applied as catalysts in chemical production[3]. A special area of employment of proteins is in medicine in the form of vaccines and antibodies[4,5]. A big breakthrough of proteins employed in medicine was the use of pig and later recombinantly produced human insulin for the treatment of diabetes[6,7]. The most important enabling technology for the use of proteins has been the discovery of recombinant cloning and overexpression techniques to be able to produce the desired protein in a controlled manner[8,9]. This enables both academic research and industry to explore the possibilities and advantages of employing biocatalysts in a controlled and specific fashion. In this thesis biocatalysts refers to enzymes used for chemical synthesis in their various formulations. Enzyme formulations are for example wet cells (host organism that contains the overexpressed target enzyme), crude cell extract, varying degrees of purified enzyme and lyophilized formulations thereof[10].

2 ASYMMETRIC SYNTHESIS OF AMINES

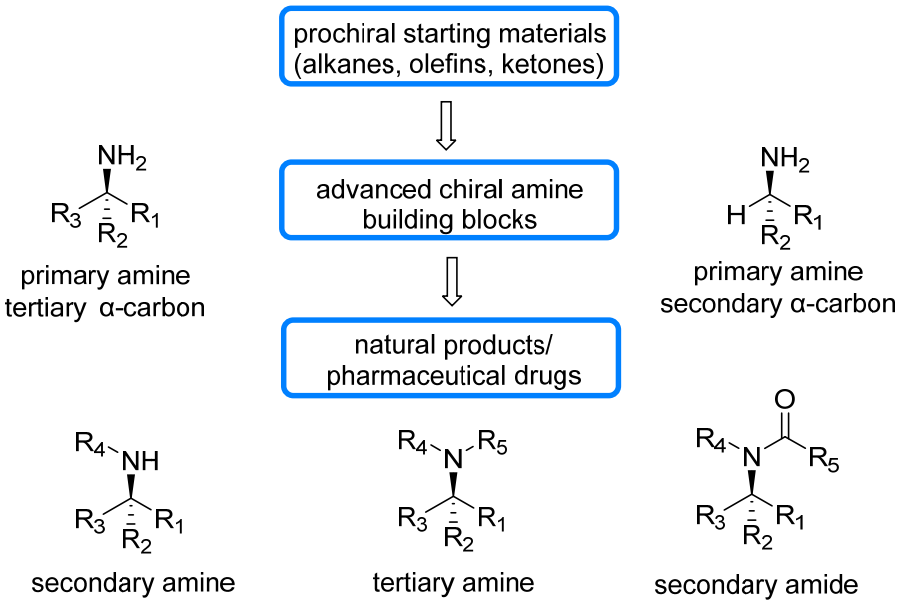
Asymmetric synthesis has been an intensively researched area of organic chemistry over the past decades. By definition an asymmetric synthesis is the conversion of an achiral compound into a chiral compound without the resolution of a racemic mixture[11,12]. From the 1990s chiral amines have emerged as a dominating class of pharmaceuticals (Scheme 1) providing a large variety of treatment for amongst others depression, diabetes, obesity, malaria and tuberculosis[13]. Resulting from this high demand emphasis has been put on finding efficient methods for chiral amine synthesis[14]. A multitude of methods, strategies and catalysts have been developed for synthesizing chiral amines. These methods achieve chiral amines by hydrogenation, transition metal catalysis, organocatalysis as well as by use of protection groups or any combination of the above. In recent years these processes were also developed to be greener and therefore more sustainable[15–17]. Biocatalysts have emerged as new promising, green and sustainable amine synthesizing catalysts and are the focus of this work[18].

All amine scaffolds can be characterized by firstly the nitrogen that can be primary, secondary, tertiary, or even quarternary (ammonium salt), and secondly by stereogenic carbon that bears the amino group, that by necessity can only be secondary or tertiary (Scheme 2)[13]. Widely occurring chiral amine compounds in all living beings are α-amino acids. These in turn serve as the essential building blocks of proteins[19]. They are almost exclusively *S*-amines respective to their α-amino group. Exceptions are the nonchiral glycine and *R*-cysteine. Some pharmaceuticals are derived from amino acids or are small peptides.



Scheme 1. Examples of drugs that are chiral amines[13].

An advantage of using biocatalysts is that prochiral starting materials like ketones and imines can be used for yielding enantiopure compounds. A lot of classical synthesis routes towards complex chiral compounds involve enantiopure reagents. With biocatalysts the stereocenter can be synthesized *de novo*.



Scheme 2. Synthesis strategy for amine-containing pharmaceuticals[13].

Another strategy for synthesizing enantiopure amines is kinetic resolution. Here the amine racemate can, for example, be selectively acylated by a lipase. The opposite enantiomer remains as a free amine[20-22]. The concept can be expanded to dynamic kinetic resolution were the remaining free amine is racemized by an additional metal catalyst[23,24]. In this way up to 100% theoretical yield is possible in comparison to 50% for a simple kinetic resolution. Other enantioselective enzymes can be used for (dynamic) kinetic resolution as well, for example ATAs. Here the respective amine enantiomer is converted to the corresponding ketone, leaving the other enantiomer in the reaction solution[25].

3 ENZYMES

Enzymes are the highly versatile catalysts of nature. They can enhance a reaction rate 10^{17} times (Oritidine monophosphate decarboxylase)[19], but even higher rate accelerations have been reported as well. Almost all enzymes are made up of the 20 natural amino acids, except catalytic RNA-oligos with enzymatic activity. Their great variety in reactivity and substrate scope is achieved through their secondary and tertiary structures providing multiple possibilities for orientations of backbone and side-chains of the primary structure of amino acid residues. Further variety is achieved by combining monomers of enzymes into homo- or hetero multimers.

A further frequent feature of an enzyme structure and function is a co-factor. Essential co-factors for humans are colloquially referred to as vitamins which cannot be synthesized by the human metabolism in adequate amounts[26]. They have to be taken up via food and drink. The co-factor is usually involved in the catalytic mechanism and may or may not be depleted in some way. Co-factors are either metals (example: calcium), small organic molecules (example: Pyridoxal-5'-phosphate or PLP)[27] or a hybrid of the two (ex: Heme-group)[28] that are usually a critical part of the enzymes' reaction mechanism. Sometimes they also have a stabilizing function, for example zinc finger motif[29]. Co-factors can be very specific to one reaction, but it is more common that they can catalyse a wide range of reactions depending on the enzyme group[30]. Co-factors are separate molecules or ions and are usually coordinated to the protein by salt bridges, van-der-Waals forces or hydrogen bonds. In some cases they are bound covalently to the enzyme. In other cases as in the catalytic cycle of transaminases with the co-factor PLP (Pyridoxal 5'-phosphate) the bond is broken in the catalytic cycle. If a co-factor, for example NADPH, is depleted during an enzyme catalysed reaction, they are usually regenerated *in vivo* (fermentation) or *in situ* (regeneration system) since it is too expensive to supply them in equimolar amount[31].

Enzymes are frequently activated or regulated by other enzymes by allosteric regulation or cleavage of amino acid chains of pro-enzymes to activate the enzyme assisting it in assuming its active conformation. A special class of proteins are chaperones which are assisting proteins

helping the main enzyme assume the right three-dimensional fold by for example temporarily shielding two regions of the enzyme from interacting with each other. Enzymes can also be activated by their surrounding conditions be it an acidic or hydrophobic environment. A prominent example is the lipase lid that opens upon contact with a hydrophobic surface[19].

3.1 Enzyme promiscuity

Enzymes are usually classified by their natural activity. Enzyme groups are derived from the reaction that they catalyse in the metabolism of the origin organism. But since nature is versatile and adaptable, many enzymes also have the capability to catalyse other reactions than their primary natural activity. These activities other than the main activity in the metabolism are commonly termed promiscuous. Mostly this promiscuous catalytic ability includes reactions on a wider substrate scope and related reaction types or reverse reactions of the primary natural reaction. Promiscuous activities can also be related to the co-factor essentially performing the same catalytic steps, but on a different compound class. In the case of transaminases the absence of significant groups in the substrate molecule like the carboxyl group, that however is not involved in the immediate reaction, is categorized as a promiscuous activity[32,33]. For instance lipases catalyse the cleavage of ester bonds, but can also synthesize esters when substrates are employed in excess or when the reaction is conducted in a water-free reaction media like dry organic solvents. Promiscuity can be divided up in three categories, condition promiscuity, substrate promiscuity and catalytic promiscuity[32].If looking at the category substrate promiscuity, it is not clearly defined how much a molecule have to differ to qualify its conversion into a product as promiscuous- so even a different configuration or substituent is a promiscuous activity. It is not defined when a condition is different enough from the natural environment to be classified as promiscuous: for example should the reaction be conducted non-aqueous conditions or are additional differences required like non-natural pH and temperature? The same argumentation can be applied for condition promiscuity. Several difficulties present itself for drawing the line were the promiscuity begins: for example if additional side-chains of

the molecule are sufficient or is the requirement the absence or presence of an additional reactive group to qualify as promiscuous?

In the age of genome mining the natural activity is not always known and in turn no natural activity can be assigned. For an increasing number of newly discovered enzymes, its original function is not known and indication for activities is discovered via homology searches of sequence and structure. This is further limited by one of the best known biocatalysis specific quotes: “you get what you screen for”[34]. This means it is difficult to discover a natural activity of an enzyme that it is not investigated for. So a promiscuous activity can simply be defined as an activity that the “enzyme was not expected to do”[32].

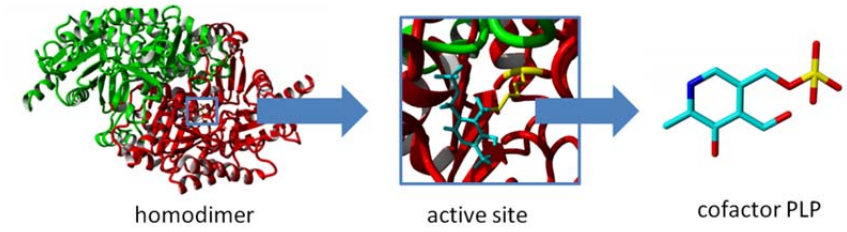
3.1.1 Enzyme classes

Enzymes can be classified by different characteristics like the catalysed reaction, similar fold-types or which co-factor is utilized for catalysis. Here the employed enzymes in this work classified by their respective E-class are introduced briefly.

3.1.2 Amine Transaminases (ATAs)

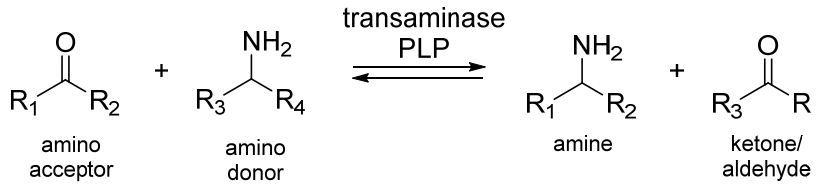
Amine transaminases (EC 2.6.1.X) have the outstanding ability to aminate ketones or aldehydes without an α-carboxyl group with high stereo- and regioselectivity. They represent a special sub-group of amino transferases. Amino transferases or transaminases are commonly categorized by their ability to aminate the α-carbonyl, β-carbonyl or any other carbonyl carbon (Ω-transaminases) of their substrate keto-acid. All of these groups may exhibit a promiscuous activity towards other carbonyl groups than the most common, the α-carbonyl carbon. If no carboxyl-group is required in the molecule for amination, the enzyme is termed an amine transaminase[30]. Amino transferases or transaminases are usually homo-dimeric[35] or homo-tetrameric[36]. The amino group is supplied by a co-substrate from which it is transferred onto the intended

product. For this transfer transaminases require the co-factor PLP (Scheme 3).



Scheme 3. Homodimer of the *Chromobacterium violaceum* transaminase (pdb: 4A6T), zoom into the active site where the catalytic lysine (yellow) is bound to the co-factor PLP (blue) and the co-factor PLP in blue (dark blue: nitrogen, red: oxygen, yellow: phosphorus)

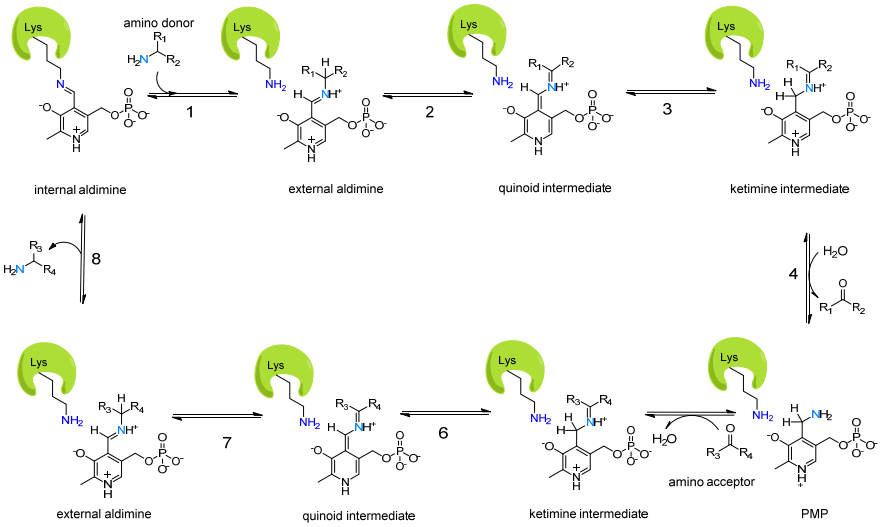
PLP is the active form of the vitamin B_6. PLP -sometimes also named P5P- is a very versatile co-factor. PLP-dependent enzymes are classed in five different fold types and contain a large number of aminotransferases and amine transaminases as well as phosphorylases, lyases, decarboxylases, synthases, racemases and mutases[37]. ATAs achieve the amino group transfer via a ping-pong bi-bi mechanism involving an intermediate state of the co-factor pyridoxal-5´phosphate (PLP). In the transamination reaction (Scheme 4) the amino donor enters the active site and its amino group is transferred to PLP yielding PMP (pyridoxamine phosphate) and leaves the active site as aldehyde or ketone. The amino acceptor (aldehyde or ketone) follows and the amino group is transferred stereo- and regioselectively onto the final product. The reaction is reversible and preferred direction can be achieved by excess of the co-substrate[38]. Transaminases are classified as α-, ω- and amine transaminases depending on whether the amine acceptor has an additional carboxylic group and the position thereof.



Scheme 4. Reaction of a transaminase. The substrates are the amino donor, which can be a ketone or aldehyde, and an amino acceptor, which can be virtually any organic molecule containing a primary amine group. The transaminase or aminotransferase transfers the amino group of the amino donor to the amino acceptor so that the products of this reversible reaction are an amine and a ketone/aldehyde.

The catalytic mechanism is shown in detail in Scheme 5. Central to the catalytic mechanism is the catalytic lysine and the co-factor PLP. In the holoenzyme without substrates present the lysine is covalently bound to PLP in a Schiff-base linkage forming an internal aldimine. Upon entrance of the amino donor this covalent bond between lysine and PLP is broken by the amino donor which forms a Schiff base with PLP instead (Scheme 5. 1). The α-proton of the amino donor is extracted in the next step by the catalytic lysine assisted by a water molecule (Scheme 5. 2). The abstraction of the proton triggers a rearrangement of the PLP-bound amino donor to a planar quinonoid (Scheme 5. 2). Further redistribution of electrons leads to the re-abstraction of the former α-proton on the planar quinonoid structure forming a ketamine (Scheme 5. 3). Hydrolysis completes the first half of the reaction resulting in the leaving aldehyde or ketone, formerly the amino donor and the aminated form of the co-factor-pyridoxamine-5´-phosphate (Scheme 5. 4). Now the amino acceptor enters the active site and all reaction steps up to this point take place in reverse. The ketone or aldehyde is attacked by the amino group of PMP to form a ketimine (Scheme 5. 5). Now the proton is abstracted by the catalytic lysine forming the planar quinonoid (Scheme 5. 6). In the next step chirality is introduced into the molecule if the amino acceptor is a ketone. Due to the specific spatial orientation of catalytic lysine, planar quinonoid and amino acceptor towards each other the proton can only be re-abstracted by the carbon formerly next to the carbonyl-group of the ketone or aldehyde of the amino acceptor in a specific face orientation (Scheme 5. 7). The resulting external aldimine in turn is attacked by the

catalytic lysine resulting in leaving of the final amine product (Scheme 5. 8).



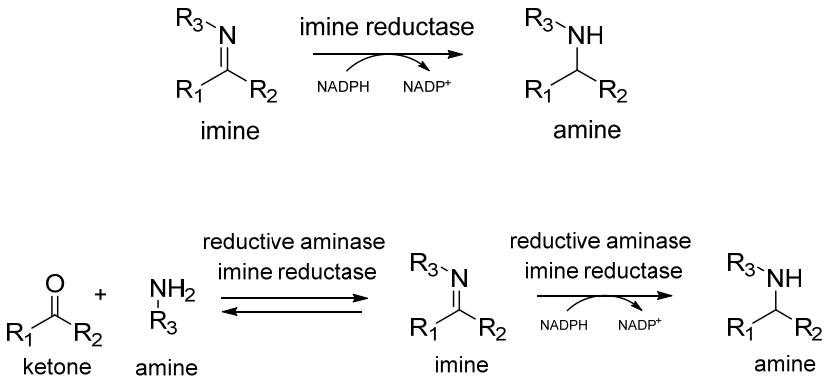
Scheme 4. Reaction mechanism of a transaminase. Displayed is the half reaction, for a full catalytic circle the reaction first has to occur in the forward direction and then in reverse to yield a holoenzyme with the catalytic lysine bound to the co-factor PLP[39].

The outstanding enantioselectivity of transaminases is achieved by different fold types in regard to whether the *R*- or *S*- enantiomer is the desired product. *S*-transaminases exclusively belong to the fold type I of PLP-dependent enzymes and *R*-transaminases exclusively belong to the fold type IV[30].

3.1.3 Imine Reductases (IREDs)

Imine Reductases (EC 1.5.1.48) catalyse the reduction of an imine- cyclic or non-cyclic- to an achiral or chiral amine with moderate to excellent enantioselectivities[40]. It utilizes the co-factor NADPH for hydride transfer (Scheme 5). NADPH (Nicotinamide adenine dinucleotide phosphate), only differs from NADH in one added phosphate group. In the metabolism NADH can be converted to NADPH. NADPH or $NADPH+H^+$ is the reduced form and $NADP^+$ the oxidized form. The related co-factor NADH, short for nicotinamide adenine dinucleotide, is involved in many redox reactions in living beings. It can be referred to as NADH, the reduced form or as NAD or NAD^+, the oxidized form depending on which state the specific enzyme utilizes for their reaction. Enzymes that use NADH or NADPH in their redox-reactions as a co-factor always show a clear preference for one of these co-factors.

Imine reductases are homodimers with a Rossmann motif for NAD(P)H binding. The catalytic mechanism is proposed to be acid mediated by an aspartic acid residue[41]. The formed iminium ion is stabilized by the same residue. This is necessary for the hydride transfer from Nicotinamide adenine dinucleotide phosphate in its reduced form (NADPH) to the substrate and subsequent imine reduction. To this day the reaction mechanism is not fully elucidated and remains subject of further study.



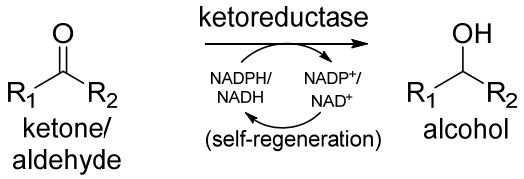
Scheme 5. Imine reductase activity (top) converting an imine to a chiral amine. Many imine reductases also have a promiscuous activity of reductive aminase which can catalyse both the imine formation of a ketone and amine as well as the imine reduction to a chiral amine (bottom).

The resulting oxidized nicotinamide co-factor NADP+ can be reduced for multiple catalysis cycles by co-factor regeneration system involving for example an alcohol dehydrogenase, glucose dehydrogenase or glucose-6-phosphate dehydrogenase. They need a sacrificial co-substrate like isopropanol or glucose[42,43]. In recent years success has been achieved in engineering imine reductases in also utilizing NADH for performing the reduction[44]. A considerable portion of the investigated imine reductases also show reductive aminase activity since they are capable to use the corresponding scaffold ketone and the corresponding amine to form non-cyclic chiral amines (Scheme 5 bottom). The imine is formed in the active site and then reduced to the corresponding amine[45]. The applicability of imine reductases has been successfully shown by employing them in cascades in cells. [10]

3.1.4 Ketoreductases (KREDs)

Ketoreductases (EC 1.1.1.x) reduce ketones to their corresponding alcohols. They employ the co-factor NADPH or NADH for hydride

transfer. Specificity for NADH or NADPH as co-factor depends on the enzyme (Scheme 6).



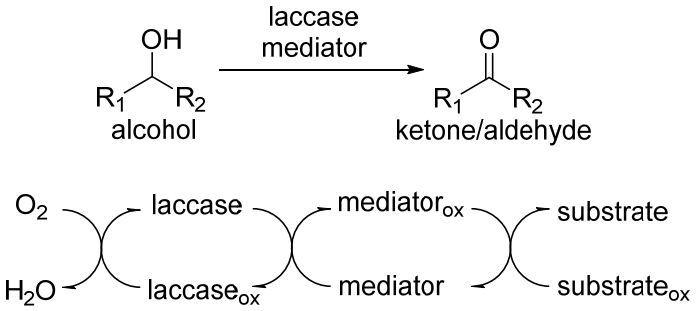
Scheme 6. Reaction of a ketoreductase. A ketone is reduced to a chiral alcohol. When an alcohol is employed as "sacrificial" co-substrate, ketoreductases can self-regenerate the co-factor (coupled-substrate recycling)[46].

Usually ketoreductases (KREDs) are monomers with an α/β-barrel motif. The catalytic mechanism follows an ordered bi-bi mechanism[47]. The co-factor $NADP^+$ or NAD^+ is reduced to NADPH or NADH and has to be regenerated. It is possible to re-reduce the co-factor for multiple uses in catalysis cycles either by employing the KRED itself in a cycle of self-regeneration or employing an additional alcohol dehydrogenase employing a sacrificial co-substrate like isopropanol in both cases. The catalytic mechanism involves a conserved tetrad of tyrosine (Tyr), lysine (Lys), aspartic acid (Asp) and histidine (His)[48]. KREDs generally have a wide substrate promiscuity- meaning they accept a wide variety of substrates and exhibit excellent enantioselectivity for the synthesis of a broad range of chiral alcohols. KREDs have been employed successfully in cascades together with transaminases[49].

3.1.5 Laccases

Laccases (EC 1.10.3.2), particularly the commercially available *Trametes versicolor* laccase has been thoroughly investigated in the past and several useful oxidation applications could be demonstrated[50]. This enzyme employs copper as a co-factor[51]. Laccases have a broad substrate spectrum that can be further enhanced by employing a Laccase-Mediator-System (LMS)[50]. Laccases need a mediator molecule as electron shuttle to molecular oxygen. Mediator molecules in general serve as electron

transporters for redox reactions that can increase laccase activity (Scheme 7). Characteristics for a good laccase mediator would be stable oxidized and reduced forms and being a good substrate for the enzyme without inhibiting it[50]. Mediators commonly used in laccase oxidations are HBT (1-hydroxybenzotriazole), ABS (2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)), AZADO (2-Azaadamantane-N-oxyl) or TEMPO (2,2,6,6-Tetramethylpiperidine 1-oxyl).



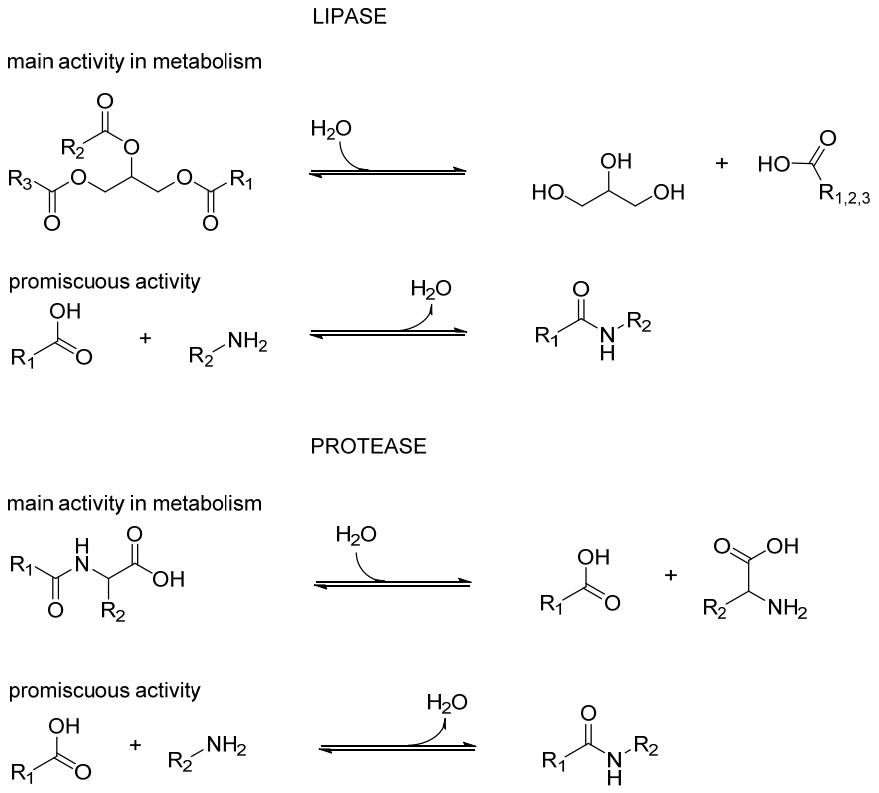
Scheme 7: laccase mediator system. The system comprises of several concurrent redox reactions oxidizing the substrate in a "domino" fashion[52].

As water is the only by-product of this reaction, laccases can be regarded as particularly environmentally sustainable oxidants.

3.1.6 Lipases and Proteases

Lipases, EC 3.1.1.3, and proteases, EC 3.1.4.x both belong to the hydrolase main enzyme class[53,54]. They are widely employed in industry and are among the most investigated classes of enzymes to date. They act on a wide variety of substrates and show reaction promiscuity as they also can catalyse transesterification and amidation (Scheme 8)[55–57]. One prominent example is *Candida antarctica* lipase B (CAL-B). This enzyme´s extraordinary stability and versatility has been demonstrated in a plethora of industrial and research applications[58,59]. Frequent

applications include (dynamic) kinetic resolution either as transesterification, hydrolysis or esterification.



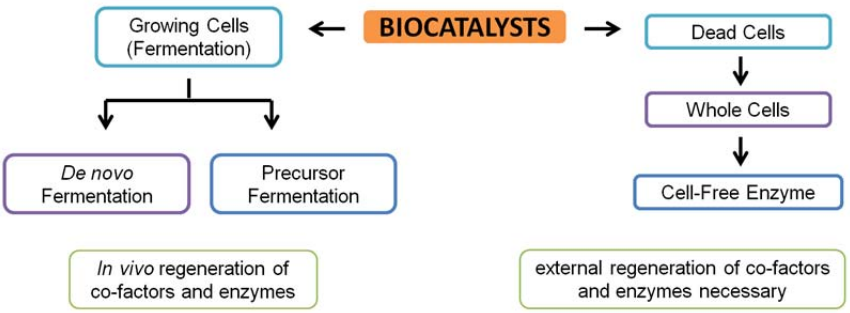
Scheme 8. Exemplary reaction schemes for the main activity of the enzyme and (intended) promiscuous reactions of lipases and proteases screened for in the 2nd cascade step.

3.1.7 Enzyme formulation

When developing a new process, one must also consider catalyst formulation. While for extensive enzyme characterization studies the purified enzyme will always be the first choice, however, it is not certain

that this is the best formulation for industrial application. Here other considerations come into play like cost efficiency of the catalyst production process or stabilizing influence of the crude cell extract or stabilizing effects of the immobilization material.

When considering a bioprocess a fermentation has many advantages over a purely enzymatic process. The catalyst self-multiplies and multiple step pathways can be conducted in a single cell. No co-factor regeneration is needed since regeneration mechanisms are built into the metabolism. It is however still a major challenge to control and tune fermentation processes. To avoid some of the pitfalls like dominating unwanted side-product formation, one can employ an enzyme process instead. In an enzyme process there are several degrees of purity of enzyme one can employ in an industrial process. One of the simplest approaches is to employ whole cells wherein the desired biocatalyst is overexpressed (Scheme 9). Here one can distinguish between wet cells or lyophilized cells, wet cells being the most simple purification process of centrifuging the cells to separate them from the fermentation medium. Lyophilized whole cells in turn can be easier to handle in organic solvent reaction conditions and can be stored and transported easier. The next step of purification would be cell lysis where the cells' membranes and other precipitating cell's contents are separated from the supernatant containing the target catalyst. Prerequisite for this formulation is the solubility of the biocatalyst. Here in turn the crude cell extract can be just employed as is in liquid formulation or again lyophilized. It is also necessary to check whether the enzyme in question tolerates lyophilisation. If the target biocatalyst needs to be further purified one can also precipitate the enzyme from the crude cell extract supernatant. In case of excretion of the target enzyme into the fermentation medium, it can be directly precipitated from the medium. This holds the advantage as precipitation condition will be tailored to the enzyme, that even soluble host cell protein can be easily separated from the target enzyme and unwanted side reactions can be avoided.



Scheme 9. Different ways biocatalysts can be employed[60].

For better recyclability and sometimes improved stability, enzymes can be immobilized. There is a plethora of methods reported in literature[61] which include but are not limited to entrapment, cross-linking adhesion or covalent bonding to various support materials. The best immobilization method always depends on the enzyme in question as immobilization can have a profound effect on stability and activity of the enzyme. Furthermore the immobilization strategy also has to be compatible with the intended process parameters. Great success has been achieved in prolonging the catalysts lifetime and recyclability employing immobilization methods [62]. Immobilized biocatalysts are an established technology in chemical and especially pharmaceutical production today [63]. To establish the use of biocatalysts further, it is paramount that they are very stable, versatile and easy to handle. This can be best described by this quote by Francis Arnold (Nobel lecture 2018) that we need:

“Biocatalysts so simple, even chemists can use them.”

3.1.8 Finding the right catalyst

There are three basic approaches to find a biocatalyst suitable for the task at hand. Prerequisite for this classification is that one already knows the target compound. The first approach is the metagenomics or even environmental mining approach where it is also possible to discover

completely new activities formerly not achievable by other catalysts, as enzymes can create unique micro-environments in their active sites [64]. If certain additional characteristics are sought after like stability or temperature tolerance one can choose the environmental circumstances to fit those requirements. An example would be the interest in enzymes isolated from microorganisms living in extreme environments like “black smokers”-underwater volcanic hydrothermal vents. Here biocatalysts have to perform in extreme conditions. Also metagenomes can be screened for certain pre-identified motifs to test later for the desired activity.

Another approach is to dive into nature´s plethora of already available characterized biocatalysts and screen for the catalyst best suited for the substrate(s) and reaction conditions for the application. This approach was until a few decades ago the only approach for finding an isolated biocatalyst until enzyme engineering techniques and structure determination techniques reached a level where they could be routinely employed for tailoring a biocatalyst to specific needs. Engineering a biocatalyst towards a specific application is the third approach one can take to have the best catalyst for its specific application. All approaches have their advantages and disadvantages. The advantage of the second approach is that, if lucky, a suitable catalyst can be found quickly. If one takes the example of the extensively researched class of lipases it is quite likely that in a sufficiently large panel of even commercially available enzymes a suitable candidate can be found. The disadvantage is however that this might not be the case and either the substrate or the reaction conditions can be a problem and no candidate is found. A further dilemma is the fact that enzymes in their natural cell environment usually do not come in contact with high concentrations of the substrates and are not optimized by evolution to have the highest possible reaction rate or even display substrate inhibition. Mild reaction conditions are often cited as an advantage of enzymatic reaction and contribute to their low environmental impact but process parameters might require the enzyme to work at harsher conditions as it might be its optimum. Therefore special focus has been put on discovering new enzymes of extremophiles-for example in very hot or saline environments[65] as these enzymes are thought to be more robust at high temperatures than enzymes in mesophile´s metabolisms.

The advantage of the third approach is one can have a truly tailor-made biocatalyst and does not have to be content with the templates provided by nature. The advantage here is that a poorly suited biocatalyst can be engineered towards the specific substrate and ideal process conditions- which might greatly differ from the natural environment of the enzyme. Basic prerequisite for choosing a suitable enzyme scaffold here is that the enzyme can catalyse the general reaction- for example amination, oxidation or alkylation- one is looking to achieve. As the building blocks for enzymes are always the same 20 amino acids – leaving incorporation of unnatural amino acids out of the picture – one has sufficient possibilities to tailor the enzyme to the process. This leads to a major disadvantage of this approach: the screening demands for large libraries can be enormous. Therefore a lot of effort is put into reducing the number of variants in a screening without risking on missing out on a possible positive hit. To date it is unfortunately not possible to fully rationally design an enzyme with regard to all properties –for example stability and specificity- therefore a mixed approach of random mutagenesis and rational design is chosen today[66]. Approaches to make random mutagenesis more efficient are to target specific regions of the enzyme or improve mutagenesis efficiency by avoiding silent mutations and amino acid bias. Design of smart libraries or so called semi-rational approach usually involve iterative circles of *in silico* enzyme modelling, rational design and random mutagenesis methods. If for example optimizing for stability modelling can help to identify unstable regions like large unordered loops or strengthen interactions in weakly interacting parts of the enzyme. Then these regions can be altered using rational design like deleting sequences for large loops or random mutagenesis to improve for example the interaction between weakly interacting regions.

Full rational design is further limited by today´s structure determination methods which in general rely on X-ray crystallography and require high-quality crystals of the enzyme. Even with a high resolution structure the intricate interactions between amino acid residues may not be represented entirely correct. Since an enzyme is such a complex molecule it cannot be entirely predicted by today´s computing power what influence one or several amino acid changes have on the properties of the enzyme. Therefore current smart library approaches cannot forgo random mutagenesis methods altogether. In conclusion, for each project where a

biocatalyst application is investigated has to be judged by its unique requirements to pick the right approach.

3.1.9 Commercial enzymes

There are a few advantages and disadvantages which have to be considered when employing commercial enzymes. One big advantage is of course the availability which skips complication and cost of ordering a gene, cloning, finding a good expression system as well as a suitable overexpression and purification strategy. Furthermore commercial enzymes are already characterized and therefore these standard conditions can be used as a reference starting point for a screen for conversion to the target compound. Usually they are also optimized or chosen for further beneficial characteristics like stability. Often the information on amino acid sequence or origin organism is not available, therefore linking possible homologues and related publicly available research on it cannot be linked to the enzyme in use.

Furthermore the formulation is mostly not pure, but often a form of cell free extract formulation of the producing organism is sold. One of the disadvantages is often not exactly specified what is in the enzyme formulation- whether it is for example spray-dried crude cell extract or purified enzyme. This in turn means that unwelcome side reactions can occur catalysed by residual house-keeping enzymes in the powder or liquid formulation. Furthermore residual production molecules can cause unwanted cross-reaction and can interfere with optimization strategies.

4 CASCADES

Two differing concepts of cascades that both involve enzymes exist: regulatory cascades of enzymes in the metabolism and cascades for synthesis of desired chemical compounds wherein the enzyme is the catalyst[19]. In a narrower sense cascades can also be defined as a set of chemical reactions performed in one pot without the need of isolating the intermediates[67]. Cascades can also be categorized by whether they are run sequentially, where reagents are added step-wise or in relay fashion were all reagents are present from the beginning. A special case is the coordinated cascade where two or more catalyst and reagents catalyse a reaction together (Scheme 10).[68] Cascades are also characterized by their type of catalysts, for example being purely enzymatic or a mix of metals, organocatalysts and enzymes. For a mixed-catalyst cascade it is often a challenge to find a good compromise of reaction conditions suitable for all of them. This is usually solved by a sequential approach or compartmentalization.

4.1.1 Regulatory cascades

In the organism regulatory cascades mostly amplify a molecular signal to start a process like for example blood coagulation. In a series of steps started by activation of a protein by a signal molecule further proteins are modified and activated. In most cases the signal is amplified by the starting protein activating several further proteins which in turn activate multiple proteins down the signal pathway line. The chemical equivalent for this kind of biological cascade would be a domino reaction initiated by a single catalyst[69].

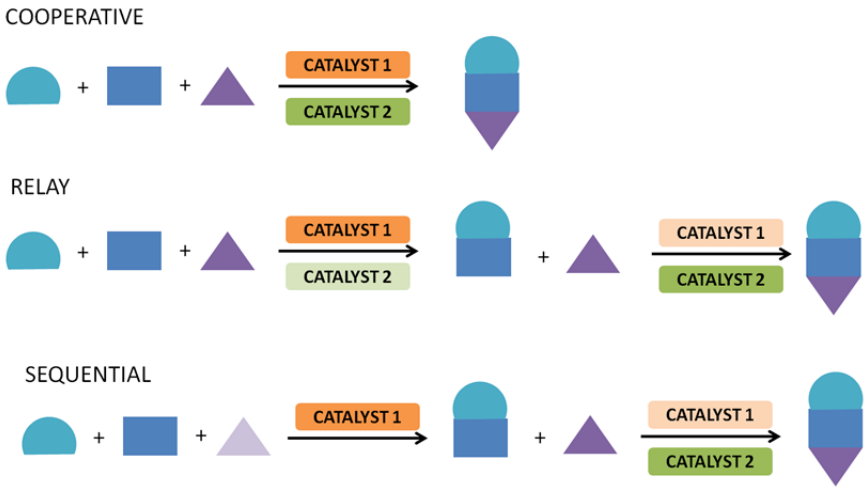
4.1.2 Enzymatic cascades for synthesis

Catalytic enzymatic cascades for synthesis employ enzymes as the catalyst. A catalyst by definition is a molecule propagating the reaction

without being depleted in the process. A metabolic pathway in the cell can be understood in that way. A regulatory cascade of the specific enzymes in contrast determine or adjust the concentrations and availability of the metabolites. Enzymes are also employed in synthetic chemistry synthesis pathways in the same fashion as metals or organocatalysts. In this case enzymes convert the substrate to the desired product or intermediate and then a second enzyme or other catalyst converts the molecule further or vice versa[61,64, 67–78]. Developing a cascade usually involves additional work in research and development but cascades also have advantages over step-by-step processes. One-pot cascades avoid laborious and work- and material intensive work-up steps after each synthesis stage of the target compound. Furthermore they provide a solution for work with unstable intermediates as those can immediately be interconverted to stable follow-up products.

4.1.3 Modes of cascades

There are three different modes of cascades that can be run in a one-pot process- cooperative, relay and sequential[82]. In cooperative mode two catalysts work on two or more substrates simultaneously (see Scheme 10). That means both catalysts are present at the start of the reaction and both catalyst are involved in the same catalytic cycle activating different functional groups to achieve the final product. In relay mode both catalysts and all substrates are present in the reaction from the start. The final product is however achieved via an intermediate which reaction is catalysed by one catalyst. The intermediate in turn is then converted by the other catalyst to the final product. In contrast to the cooperative reaction the synthesis proceeds in a step-wise fashion instead of simultaneously. Important for a relay mode is the compatibility of all catalysts and substrates in the solution to avoid cross-reactivities. If there are issues like catalyst incompatibility or substrate toxicity or cross-reactivity a one-pot process can still be achieved in sequential mode. In that case the reaction also proceeds in a step-wise fashion, but only the substrates and catalyst needed to achieve the intermediate are present at first. If the concentration of intermediate is sufficient or the reaction is completed the second catalyst and optionally further substrates are added to the reaction.



Scheme 10. Cooperative, relay and sequential cascades.

Furthermore, catalyst and substrate incompatibility can be avoided by compartmentalization. This can be achieved through membranes and insets in the reactor system.

5 AIM AND MOTIVATION

In this thesis the applicability of enzymes for novel synthesis routes for APIs is investigated. Employing a biocatalyst in a synthesis route contributes to making it more sustainable and thereby contribute to the UN sustainable development goals. For this three enzyme types of special interest have been selected- transaminases, imine reductases and keto reductases. All of these enzymes have demonstrated their outstanding ability of selectively synthesizing chiral amines in the case of transaminases and imine reductases and chiral alcohols in the case of ketoreductases in numerous proof-of-concept studies. Now, three chiral amine APIs currently on the market- Cinacalcet (for hyperparathyroidism), Vyvanse (for ADHD) and Sertraline (antidepressant), were selected to investigate the capability of enzymes to synthesizing these APIs or key intermediate precursors.

The ultimate goal was to develop a one-pot process for the final API from an achiral precursor in excellent enantioselectivity and full conversion. This would give the enormous advantage of minimizing the need for work-up steps during the process and would potentially also decrease and facilitate the need for hazardous downstream processing to yield a GMP-compliant high purity API. For achieving a one-pot process with for example two enzymes it was also of interest to look for cross-reactivities and general compatibility of the enzymes' optimal operating conditions.

If no suitable one-pot enzymatic cascade could be found, a combination of methods for chemo-enzymatic pathway posed a viable alternative to achieve the goal of synthesizing the API. Here the additional challenge is the compatibility of conditions for enzymatic synthesis which often takes place in aqueous and ambient reaction conditions compared to classical organic chemistry methods which often involve organic solvent, metal-catalysts and harsh reaction conditions like high temperatures.

Finally it was also a goal to see how an enzyme would react to scale-up and process intensification for the actual reaction required to achieve a valuable API or key intermediate. In previous studies substrates have been employed in relatively low concentration of around 20 mM in aqueous medium to provide proof of concept for the enzymes ability to convert the respective compound. This concentration is not economically

viable and therefore further investigation was necessary on how the selected enzyme in this study behaves towards higher substrate concentrations. Increasing substrate concentration also poses secondary issues like the substrates solubility limit and pH change and control during the process. In conclusion, synthesis and scale-up of the respective process for economically interesting compounds by biocatalysts were the main aims of this study.

6 PRESENT INVESTIGATION

Three enzyme types have been in particular focus in this work: transaminases, imine reductases and ketoreductases. Three APIs have been chosen as case studies: Cinacalcet, Sertraline and Vyvanse. They are all chiral amines and represent either pharmaceuticals that generated most revenue or have been most prescribed in recent years[83].

First, the employment of transaminases in Paper I-IV for achieving key chiral precursors of the target APIs is discussed. In Paper I a chemo-enzymatic synthesis route towards Cinacalcet was developed. Paper II concentrates on optimizing and scaling the transaminase synthesis step using Design of Experiments of the transaminase mediated synthesis route towards Cinacalcet. After this, the employment of imine reductases is discussed to explore the synthesis of Cinacalcet (Paper I) and Sertraline (Paper III). Thirdly, completing the enzyme groups, ketoreductase application is discussed for Paper I (Cinacalcet) and Paper III (Sertraline). In the chapter cascades it is discussed whether Paper I (Cinacalcet), Paper III (Sertraline) and Paper IV (Vyvanse) are true (chemo-)enzymatic cascades. Benefits and problems of the developed cascades and synthesis routes are discussed. Finally all synthesis routes are discussed with regards to contributing to a greener chemistry and thereby to the UN sustainable development goals.

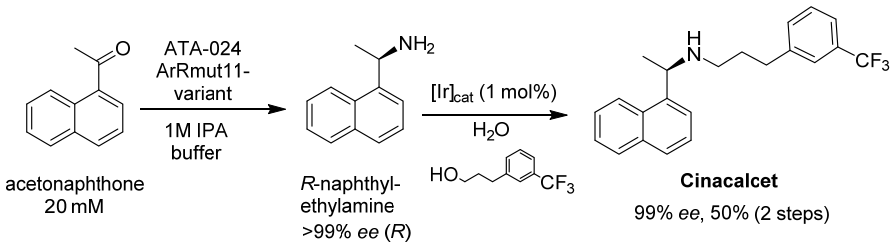
6.1 Present investigation: transaminases

Transaminases have the capability of catalysing the stereospecific amination of a ketone by help of an amino donor (chapter 3.2.1). Since all studied compounds have an amine or amide group, transaminases have been employed in the synthesis strategies for all papers presented in this study.

6.1.1 Paper I

The goal of the project is to develop an efficient chemo-enzymatic cascade (Scheme 11) for Cinacalcet- a hyperparathyroidism drug. These routes

provide alternative routes employing biocatalysts to chemical routes using for example metal catalysts[84]. The first step for the transaminase employing approach starts from 1-acetonaphthone to 1-(*R*)-naphthylethylamine catalysed by a *R*-selective transaminase. The second step is a chemical step directly coupling the amine and the complementary alcohol. The thermodynamic equilibrium of the first step of the cascade is strongly shifted towards the substrate. Therefore an equilibrium shift method is needed. Several possibilities were explored: excess of co-substrate (amino donor) and employment of a suitable co-product removal.[85] The enzyme was employed as whole cell biocatalyst, meaning the enzyme was overexpressed in *E. coli* BL21. This enzyme formulation was chosen with regard to later scalability (Paper II: scale-up). Whole cells are thought to have higher operational stability than purified enzyme, as has been shown in previous studies for similar processes[10]. It also a lower work-load to produce the amounts of biocatalyst required for bigger batches.



Scheme 11. Explored chemo-enzymatic cascade to Cinacalcet. First step is catalysed by ArR mut11 transaminase variant and the second step is catalysed by an Iridium catalyst.

The highest yield for the complete proof of concept cascade was 50% providing the product API with 99% *ee* (*R*) in two separate consecutive steps. The highest HPLC yield for the transaminase step was 98% for ATA-024 (Codexis).

6.1.2 Paper II

Paper II focuses on the transaminase step presented in Scheme 11. A Design of Experiment software (DesignExpert 11) was used to elucidate the best process conditions for producing the key intermediate *R*-naphthylethylamine in 30 mL reaction volume. Optimization factors included percentage of DMSO, pH, temperature and pressure. Pressure was studied as it was thought to help with the equilibrium shift of the transamination reaction. Since isopropylamine is converted to acetone, low pressure should promote evaporation of the co-product acetone and therefore pushing the reaction equilibrium further to the product side. The influence of DMSO is evaluated because the substrate acetonaphthone is poorly soluble in water. In fact it was difficult to determine whether the reaction solution was actually homogenous because of the employment of whole cells. Therefore a heterogenous reaction system was assumed and total yield was only determined by NMR at the end point of 24 h. It is questionable whether a homogenous reaction system is actually necessary since a certain amount of substrate will be dissolved in the water phase especially with the co-solvent DMSO.

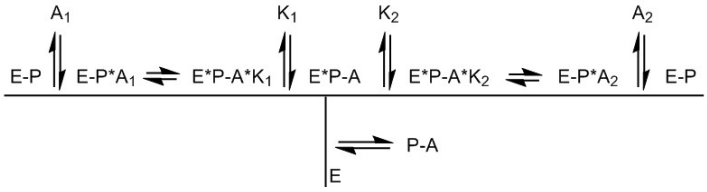
Two different pH were chosen for this study, as this enzyme variant had previously not been extensively studied for its pH optimum. The substrate concentration was increased to 500 mM acetonaphthone. Here 74% yield of *R*-naphthylethylamine from acetonaphthone could be achieved employing only 1.4 equivalents of the amino donor isopropylamine (IPA) in the DoE experiments. Equivalents of IPA were calculated in relation to total amount of IPA in relation to 500 mM starting concentration of the substrate acetonaphthone.

Table 1. Conversion (%) of acetonaphthone to *R*-naphthylethylamine at 24 h for all DoE-experiments

Experiment	pH	T (°C)	Pressure[a]	DMSO (v/v %)	Conversion (%)[b]	Equivalents IPA[c]	STY[d] ($mM*L^{-1}*h^{-1}$)	STY ($g*L^{-1}*h^{-1}$)	TTN[e] ($g*g^{-1}$)
1	9	30	low	30	48	4.9	10	2	0.8
2	9	45	low	10	29	4.8	6	1	0.5
3	7	45	atmospheric	10	4	1.5	1	0	0.2
4	9	45	atmospheric	30	74	1.4	15	3	1.3
5	9	30	atmospheric	10	29	1.6	6	1	0.5
6	7	30	atmospheric	30	67	1.1	14	2	1.1
7	7	30	low	10	12	2.3	3	0	0.2
8	7	45	low	30	43	1.3	9	2	0.7

[a]connected vacuum pump, [b]end-point determination of conversion of substrate into product after 24h, [c]number of equivalents of IPA added in relation to starting substrate concentration of 500 mM, [d]STY -space-time-yield, [e] TTN- total turnover number given in $g_{product}$ per $g_{wet\ cell\ biocatalyst}$.

The highest yield of 74% was achieved employing 1.4 equivalents of IPA. This stands in contrast to previous studies where 5 to 10 equivalents of amino donor was employed as it was thought to be necessary for achieving the highest possible yield[86–88]. One explanation for the low amount of equivalents required for good yield could be the experimental set-up. Due to the set-up of the instrument all reaction components were stirred for one hour to achieve a homogenous mixture before the substrate in DMSO was added. The amino donor IPA was supplied by a pH-stat to adjust for pH shift during the reaction in 30 mL reaction volume (previously 1 mL in Paper I). Possibly, the transaminase was "primed" with amino donor and therefore the reaction took place more efficiently. In view of the results revealing that no large excess of isopropylamine is needed for up to 74% yield when employing 500 mM of substrate concentration, it is now the question whether problem of driving a transaminase reaction to the finish is purely thermodynamic. For 5 out of 8 experiments the equivalents employed range from 1.1 to 1.6 and the NMR yield from 4% to 74% (Table 1). Since the boiling point of IPA is 32° some of it will be lost through evaporation during the 24 h period. That means not all of the 1.4 equivalents are even available for the transaminase reaction. Therefore the following alternative explanations could account for the transaminase behaviour which will be discussed below based on Scheme 12.



Scheme 12. Reaction scheme for a transaminase reaction. E-P: Enzyme-PLP complex, A_1: amino donor, K1: leaving keto product, K_2: amino acceptor, A_2: amine product, P-A: pyridoxamine, E: apoenzyme.

One of the main factors in this reaction is the inactivation rate of the enzyme, in this case the dissociation of PMP. The enzyme can be further inactivated by unfolding, hydrolysis and ultimately denaturation. If the amino acceptor and amino donor pair bind well in the active site of the enzyme and the PLP is firmly bound in its co-factor "pocket" this

dissociation constant is low[89]. Therefore the “thermodynamic” problem can be seen rather as a fit or binding problem. The ArR-mut11 variant used in this reaction has been specifically engineered for this substrate-amino donor pairing. First it was evolved by directed evolution for taking isopropylamine as amino donor. Later an additional rational design engineering approach was applied specifically for the sterically demanding acetonaphthone moiety[90]. Its lack of appearance or no reported conversions in previous publications[91] of acetonaphthone may indicate that a substituent at position 1 of the naphthyl moiety are particularly hard for a transaminase to accommodate. Conversions of compounds substituted at the 2-position of the naphthyl moiety however have been reported, which can be due to the extra degrees of freedom in movement of the side-chain[92]. This possible higher accessibility can explain conversion observed for the 2’-substituted position and not the 1’-substituted position[38,92]. It remains to be shown whether similar results can be shown with a similar well-fitting donor-acceptor pair, for example a wild type enzyme with its natural substrates after pre-incubation of the enzyme with the amino donor. On the other hand, what also remains to be explained are the large differences in yield between seemingly very similar runs, as for example in the runs 3 and 4 (Table 1). The differences lie in the pH and DMSO percentage, however pH does not seem to have a significant effect overall on the runs. Therefore DMSO can be seen to have a major influence on the yield. The reaction mixture is possibly highly inhomogenous (multiple phases). The co-solvent DMSO can facilitate mass transfer between the hydrophobic phase (substrate) and the whole cell biocatalyst making the substrate more readily available for the enzyme to convert. The difference between run 3 and 4 however is 70% difference in yield. Since the reaction mixture is highly inhomogeneous further synergistic effects can be at work when employing a high percentage of DMSO. No definite conclusion can be drawn for the difference between these two runs.

Looking at the good results for the yield of *R*-naphthylethylamine by the optimized transaminase variant for the Cinacalcet intermediate amine, one can also hypothesize that even in wildtype transaminase an optimization for amino donor and acceptor has taken place. It was recently discovered that in the active site of *Aspergillus fumigatus R*-ATA there is an arginine residue (Arg126) that when mutated to an alanine significantly reduces the ability of this enzyme to employ pyruvate as an

amino acceptor. Alanine, the amino donor for the reverse reaction, is a popular amino donor for many ATA-studies. One could speculate from this example that every transaminase has an optimal amino donor and acceptor pair in which combinations it displays its highest activity. The apparent preference of wild-type transaminases for alanine as amino donor could be a result of the enzyme's natural evolution towards employing this abundant amino acid. Another indicator for this hypothesis is the fact that the optimized ArR-mut11 accepts isopropylamine readily as its amino donor while it shows poor acceptance of alanine as amino donor[86]. In comparison, many wildtype transaminases show poor acceptance of isopropylamine as an amino donor, but good activity when alanine is employed as amino donor. It can be hypothesized that each single residue at the active sites of the transaminase plays a role in determining the specificity of the transaminase for certain amino donors and acceptors by virtue of their coordination and interaction with the respective molecule. The arrays of amino donors or acceptors do not necessarily have to be similar with regards for example to size, as shown for the acetonaphthone-isopropylamine pair.

To date the standard enzymatic procedure to select for enantiomer of a chiral amine is the kinetic resolution. Here a comparison is made to a recently published dynamic kinetic resolution by CAL-B (*Candida antarctica* lipase B), most commonly used as the immobilised formulation Novozyme-435 (Table 2)[23]. The enzyme selects for one enantiomer whereby the undesired enantiomer is racemized making full conversion theoretically possible.

Table 2. Comparison of the TA-process to a CAL-B DKR[23]

Enzyme Process	Conversion (%)	*ee*	STY ($mM*l^{-1}*h^{-1}$)	STY ($g*l^{-1}*h^{-1}$)	TTN ($g*g^{-1}$)
ATA-process	74%	>99%	15	3	1.3
CAL-B DKR	92%	95%	0.8	0.1	2.2

In this practical example, employing a similar molecule to *R*-naphthylethylamine nearly full conversion - 92% - is achieved. The space-time-yields are better for the ATA-process by roughly 15 fold. On the other hand TTN (total turnover number) is higher for the CAL-B

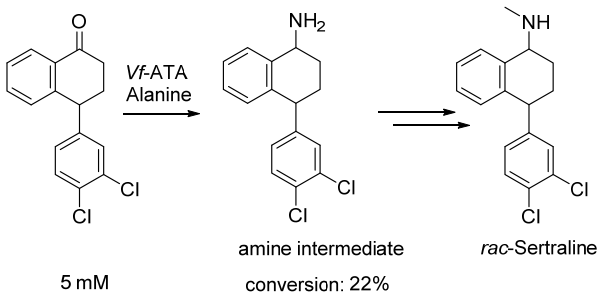
DKR, pointing towards higher catalyst efficiency and stability. For simplifying reasons only CAL-B weight was included in the calculation excluding the racemizing catalyst (for example a metal complex organic molecule). One could also argue that generally the workload for producing a whole cell biocatalyst is lower than creating an immobilised enzyme catalyst. But since Novozyme-435 is a staple immobilized enzyme, the process is streamlined and therefore this argumentation speculative. One of the key advantages here is the excellent enantioselectivity of the transaminase, staying reliable at >99% *ee* at all explored scale levels. In this example the CAL-B DKR only achieves 95% *ee* which is not ideal for a pharmaceutical application. In conclusion one could say that the transaminase process can be seen as a viable future alternative to a used CAL-B DKR.

During the work-up process of the 30 mL reaction volume of all DoE experiments (Table 1), a clear second phase was observed after filtration of the cells and increasing the pH to basic conditions (pH 12). This phase is a mixture of product and substrate solubilized into each other. Therefore, the effect of the pull on the reaction system cannot be estimated since for the enzyme present as wet cell catalyst, only the solubilized substrate and product is thermodynamically accessible. This second phase can also be achieved by centrifugation, however, the ketone/amine mixture has a high density and precipitates even below or in the cell debris pellet of the stopped reaction. Here, part of the substrate and product also remain in the aqueous phase due to the employment of the co-solvent DMSO. Therefore, it would be interesting to know whether the putative mass transfer issue for which DMSO is currently employed cannot be remedied by more vigorous stirring instead of adding 30% DMSO. In the experiment, addition of DMSO seemed to be beneficial for the yield. On the other hand using high percentages of DMSO is not environmentally friendly as the solvent is recommended to be substituted for a greener solvent[93]. Furthermore it could contribute to the low isolated yield of the work-up. For all the DoE experiments product remained in the water phase and in the cell phase. DMSO is often employed as a co-solvent that helps with solubilizing hydrophobic substrates in water. But during the work-up process the hydrophobicity of the substrate is exploited during extraction for the separation of product from reaction medium. Therefore it would be interesting to see whether detergents or simple vigorous agitation can achieve the same effect as

DMSO. Another problem that materialized during the work-up strategy testing was the employment of wet whole cells. It was difficult to separate liquid from the cells and additional washing revealed that substrate remained in the filter cake of denatured wet cell catalyst. To decrease the amount of biological mass in the reaction solution that is not catalyst, one could think about an alternative enzyme formulation like crude cell extract, precipitated lyophilized enzyme or even immobilized enzyme. In this fashion, the work-up process could be simplified and could lead to reduction of polishing steps in the final work up were host cell protein might need to be removed.

6.1.3 Paper III

Sertraline is a pharmaceutical used for treating depression, addiction and anxiety disorders. In literature only one chemo-enzymatic approach is described[94] in 10 steps. The chemical process described by Pfizer[95] from the same starting ketone-racemate as shown below has 3 steps and yields 36% product after a final crystallization. A chemical route via the sertraline-alcohol is also described[96]. The final steps towards Sertraline are also supposed to be employed here (*S*-ketone to Sertraline). The racemic substrate ketone is synthesized from 1-naphthol and 1,2-dichlorobenzene with $AlCl_3$ and is a cheap and readily available starting material. A particular feature of the target API Sertraline is its two stereocenters (Scheme 13). One is located at the amine function and one at the 4´-position at the chlorinated aryl moiety. The best HPLC yield that could be achieved for the amination of the starting ketone was 22% after 3 days employing *Vibrio fluvialis* amine transaminase (*Vf*-ATA).

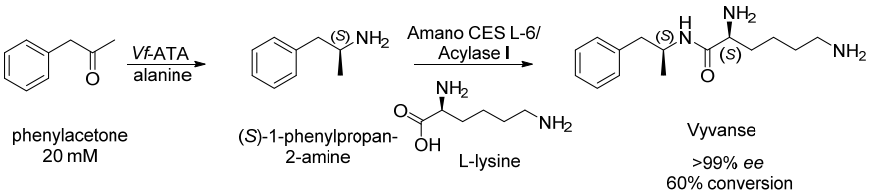


Scheme 13. ATA-catalysed synthesis of amine intermediate of Sertraline.

No selectivity for one specific diastereomer was detected, therefore full diastereomer peak determination on HPLC was not carried out. It could however be concluded that the transaminase could not select for the aryl stereocenter. This stereocenter is possibly not in close proximity to the active site with its excellent ability for stereoselectivity but could be situated in the substrate channel. The substrate channel may not have the three-dimensional structural prerequisites for only allowing the *S*-ketone and not the *R*-ketone to enter the active site and be converted. There are a plethora of enzymes that can select for multiple stereocenters. In our case, the lack of stereoselectivity made us not to continue to explore the transaminase route further.

6.1.4 Paper IV

Vyvanse, also known as lisdexamphetamine, is used for treating ADHD in adults and children over 6 years old. It is also used to treat binge eating in adults. Vyvanse acts as a central nervous system stimulant and contributes to hyperactivity and impulse control. In paper IV a one-pot cascade using *Vibrio fluvialis* amine transaminase (whole cell biocatalyst) and commercially available lipase could be designed reaching up to 60% yield (Scheme 14).



Scheme 14. Vyvanse one-pot cascade. The first step is catalysed by *Vibrio fluvialis* ATA and the second step is catalysed by a hydrolase (Amano CES L-6 lipase or Acylase I).

During the monitoring of the formation of (S)-1-phenylpropan-2-amine for all transaminases tested in the various reaction conditions, a distinctive behaviour could be observed: (S)-1-Phenylpropan-2-amine quickly increased up to 71% of converted substrate (15.8 mM concentration) within the first hour of the reactions. After that the concentration of (*S*)-1-phenylpropan-2-amine decreased to a level as low as 17% after 24 h. There are several possibilities to explain this, though no definite conclusion could be made. The first explanation could be that a kind of "overshooting" takes place. The reaction occurs in the desired direction to relatively high yields of 60-70%. After 1 h the concentration decreases, which could indicate that now the equilibrium concentrations of the reaction are established. This means that after 1 h the rate of the unwanted reverse reaction of amine product to starting ketone is taking place in a noticeable rate. The second explanation might be that the salt of the amine intermediate is formed and precipitation takes place. The sample would only show the solubilized fraction of the (*S*)-1-phenylpropan-2-amine while the precipitate would not show up in the analysis. A support for this explanation is the pKa of (*S*)-1-phenylpropan-2-amine of 9.9 while the reaction was conducted at pH 9, the optimum for most of the transaminases.

6.1.5 Outlook

Several observations made in these transaminase studies would be interesting to investigate further. In paper II the DoE experiments employing the pH-stat simultaneously for regulating the pH and

providing extra supply of amino donor revealed that yields of up to 74% could be achieved by only employing 1.4 equivalents of the amine donor isopropylamine. It would be interesting to investigate how much excess of amino donor is actually necessary to achieve satisfactory yield and what additional factors it is depending on[97]. It would be interesting to study whether the equivalents needed for a specific reaction differ depending on whether enzyme, PLP and amino donor are pre-incubated for a certain amount of time. Here, one could investigate if a certain transaminase is particularly suited for a certain amino acceptor and amino donor pairing. In turn this would mean that for a certain reaction a certain transaminase variant can be designed that is tailored to its intended amino acceptor and amino donor pairing. In the past it has been shown that custom-designing a transaminase for even what is considered a sterically challenging substrate is possible[98]. It could also be an interesting study to develop a transaminase that is tailor-made for the Sertraline amine intermediate (paper III) that can select for both stereocenters. It should be possible to create a variant that selectively can synthesize the *S,S*-diastereomer. Another interesting aspect is that two of the three substrates employed in this work (paper I, II and IV), acetonaphthone and phenylacetone are liquids. For maximum process efficiency one could also envisage the possibility of conducting a transamination in neat substrate. Here the pH control in the reaction mixture and the solubility of the amino donor has to be paid special attention to. It might be easier to employ an amino donor that is liquid like isopropylamine. Enzyme solubility, formulation and stability are also factors of special interest for this in-neat synthesis set-up. Exploratory experiments have been conducted with lyophilized whole cell transaminase and isopropylamine, but no conversion could be observed. Generally, it would also be interesting for further process development how the transaminases employed here would perform in, for example, immobilised form. The immobilised enzyme could then be tested in a flow reactor set-up. Immobilized enzymes might also facilitate the work up process for example in the case of *R*-naphthylethylamine, as it can be filtered off from the reaction mixture. Another consideration is the optimum pH or more specifically whether the reaction should be run at optimum pH for the amine or for the enzyme. In transamination reactions it is an advantage if the amine is unprotonated due to the reaction mechanism of the transaminase. In the case of (*S*)-1-phenylpropan-2-amine its pKa is

around 9.9. Here, it was interesting for us to know whether the *Vibrio fluvialis* transaminase is also active at higher pH than pH 9. For further study and alternative process options, the transaminase was immobilized on two different carriers. We found that the transaminase was active up to pH 11. A new flow reactor set-up was tested using the immobilized transaminase as solid phase. Here, we could observe GC yield up to 85% for the first 3 h. These trial results show promise in exploring more options in how to design the Vyvanse process either in step-wise flow chemistry or one-pot stirred reactor set-up. With regards to the work-up of the reactions for paper II new reactor set-ups might be of interest. The reactions for paper II have been conducted in stirred round bottomed flasks. In contrast to that, it would be interesting to know how a continuous flow reactor would perform in comparison. For that the transaminase has to be immobilised in some way which would give the advantage of having a catalyst-free reaction mixture for the work-up. In this study as in previous experiments, the biocatalyst was used in wet cell form. It might be interesting to know whether the same results can be achieved using immobilized enzyme or no DMSO, just a heterogenous mixture of the reaction.

6.2 Imine Reductases

Imine reductases (IREDs) have the capability of synthesizing chiral amines from either their imine precursors or from the ketones and amine substrates (chapter 3.2.2). For each of the evaluated pharmaceuticals, Cinacalcet, Vyvanse and Sertraline, this catalyst would make a cascade obsolete, as the target compound can be synthesized from prochiral compounds in one step. Given that a suitable IRED with sufficient selectivity would be found, this would simplify the process. As most IREDs can either employ a ketone and amine substrate pair or an imine, both synthesis starting points can achieve success.

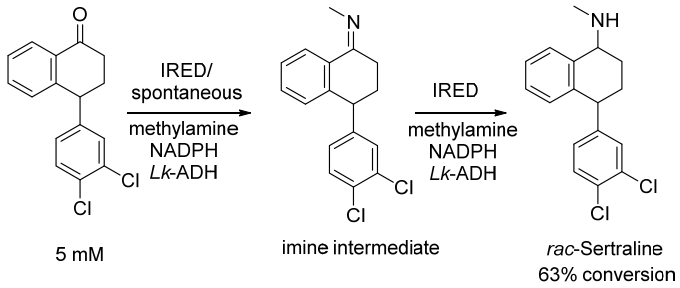
6.2.1 Paper I

For the one-step synthesis of Cinacalcet no suitable IRED was found. This poses the question whether the substrates, either ketone and corresponding amine or imine, are too sterically challenging for the enzyme. Conversions could be achieved with ketone amine pairs of approximately the same size[99,100].

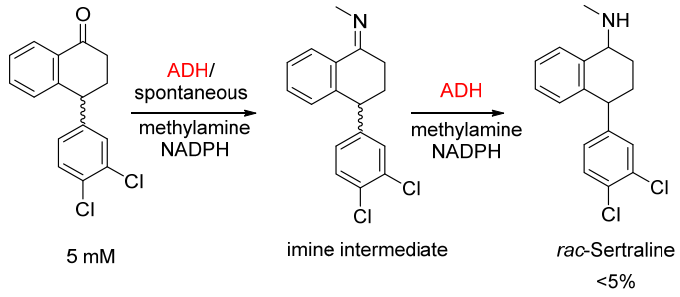
6.2.2 Paper II

Conversion to racemic Sertraline could be found both from the precursor ketone and methylamine as well as the pre-synthesized imine precursor (Scheme 15. A). Conversion took place with either no stereoselectivity for any diastereomer or poor diastereoselectivity for the ketone and amine. Most possibly, the IREDs could not select for the aryl stereocenter of the substrate. Further investigation into this lack of selectivity revealed that *Lactobacillus kefir* alcohol dehydrogenase (*Lk*-ADH), employed in the co-factor recycling system, has imine reductase activity[101]. This can be seen as a side reaction or promiscuous activity of *Lk*-ADH. *E. coli* whole cells without any overexpressed enzyme also showed activity towards the imine substrate. Here, one can speculate that there are ADHs present in *E. coli* host cells that may potentially also catalyse the reaction.

A



B



Scheme 15. A) IRED-catalysed synthesis of *rac*-Sertraline. B) Promiscuous activity of ADH employed in the NADPH-co-factor regenerating system.

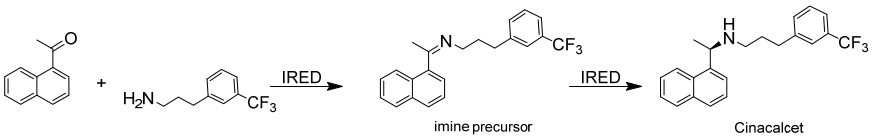
This seems to be an example where one cannot cleanly draw the line between enzyme classes. Firstly, the IRED can also be described as a reductive aminase since both the imine substrate and the ketone and complementary amine substrate can be converted. Secondly, it has also been reported that IREDs have alcohol dehydrogenase activity[41]. Complementarily, it has been shown that alcohol dehydrogenases can also perform iminium reductions probably due to their similarity in reaction mechanism related to their common co-factor NADPH[101].

6.2.3 Outlook

Imine reductases have good potential for one-step synthesis routes from API imine precursors[40,99,102]. Alternatively, the synthesis from the corresponding ketone and amine pair can be another option when exploiting their reductive aminase ability of a wide array of IREDs[43,45,103,104]. But a general solution has to be found for NADPH co-factor recycling, since the employed *Lk*-ADH as well as other dehydrogenases show promiscuous IRED activity[101]. Here, it would be interesting to investigate whether a substrate-coupled regeneration system is possible. It is successfully employed for ketoreductases, which are also NADPH dependent oxidoreductases. In the case of imine reductases, a sacrificial amine instead of a sacrificial alcohol would be necessary. It would conveniently avoid introducing a second enzyme into the reaction with all the problems it may entail as described above (Scheme 15). It would also improve the synthesis from an overall economic and environmental viewpoint, as the cost and the environmental impact of producing and using a second catalyst is avoided.

It is also worth to look into the promiscuous imine reduction capability and selectivity of available SDRs (short chain dehydrogenases/reductases), a part of which are classified as ADHs [48]. This enzyme class has been under investigation for a long time and a large and widely varied panel is commercially available. An even larger number is described in the literature. In the spirit of “teach an old dog new tricks” this source of already available and characterized biocatalysts should not be left untapped. Here one should generally assume greater promiscuity of enzymes than might be obvious from previous studies, as it has been the case for *Lk*-ADH and GDH. In the case of GDH this enzyme was assumed to be specific for its reaction in the metabolism of catalysing the reduction of glucose to gluconolactone. GDH has been a popular choice for NADPH co-factor recycling systems because of its assumed specificity and its cheap, readily available and easy-to-handle substrate glucose [40]. In the light of recent discoveries that the reaction spectrum of biocatalysts is actually broader than previously thought, it is necessary to revisit the concept of co-factor recycling systems and how to make sure to avoid unwanted side-reactions catalysed by said co-factor recycling systems. Turning a set-back into a possibility it is also worth testing employing an

ADH for the synthesis of any of the target compounds in this study since promiscuous imine reductase activity has already been observed. Furthering the concept one can also envision a process were an alcohol and amine stereocenter are set in a one-pot process. Here one could employ a ketoreductase that has the ability of substrate-coupled co-factor regeneration providing NADPH for both reactions[105]. Coming back to this work´s case study molecules: imine reductase routes could also be envisioned for Cinacalcet and Vyvanse imine intermediates or corresponding ketone and amine pairs (Scheme 16).



Scheme 16. Possible synthesis of Cinacalcet employing IREDs either starting from the corresponding ketone and amine pair or the imine precursor.

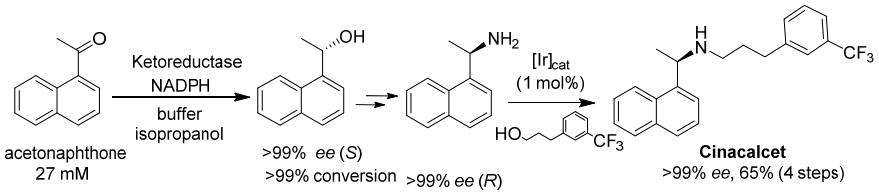
Imine reductases are promising catalysts for chiral amine synthesis, however, their catalytic specificity and capability has to be further elucidated and new solutions have to be found for co-factor regeneration.

6.3 Ketoreductases

The general strategy behind employing a ketoreductase as a biocatalyst was to first synthesize a chiral alcohol precursor of the target molecule. Then the stereocenter of the alcohol is chemically aminated with inversion via a S_N2-mechanism. As it turned out, this approach only gave the racemic amine in the approaches tested in this work. This result is also previously described in the literature for comparable reactions[106]. Therefore, additional steps had to be added to achieve the full cascade to the desired API (paper I and III). The KREDs used in this work were from the commercially available kit from Codexis. The enzymes had the capability of self-regenerating the co-factor NADPH or NADH, depending on the enzyme employed. This requires a sacrificial co-substrate, in this case 2-propanol. The co-substrate can be tolerated up to 50% by the enzymes according to the manual (Codex® KRED Screening kit).

6.3.1 Paper I

An alternative approach to the transaminase-based approach is the ketoreductase-based approach to Cinacalcet (Scheme 17).



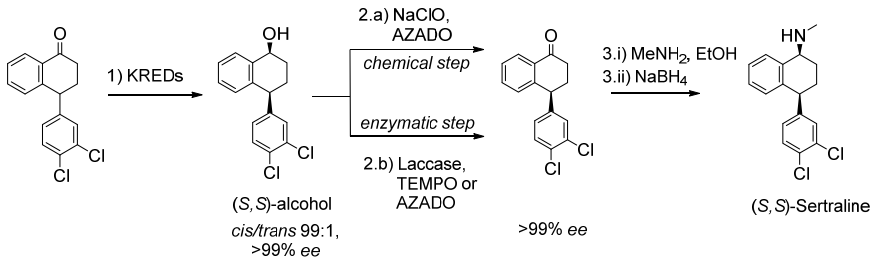
Scheme 17. Chemo-enzymatic cascade to Cinacalcet employing ketoreductases. In total 4 steps via the azide intermediate between the alcohol and amine intermediates.

A number of KREDs showed excellent selectivity for acetonaphthone. As it turned out during the attempt of employing a direct amination by mesylation or tosylation, racemization of the intermediate product (*S*)-1-(1-naphthyl)ethanol took place. Therefore, an alternative synthesis route to Cinacalcet had to be chosen via the alcohol and azide to the precursor

amine. This precursor amine can directly be synthesized by transaminases as described before (chapter 3.2.1). However, this alternative ketoreductase route had a higher overall yield with 65% even though it comprises of 4 isolated steps instead of 2 (transaminase route).

6.3.2 Paper III

Similar to Paper I, a ketoreductase-route was investigated for the synthesis of Sertraline. Initially, the Sertraline (*R*,*S*)-alcohol intermediate seemed to be the ideal candidate for the synthesis as this would be the ideal configuration for a later direct amination with stereoinversion. The racemic ketone sertraline precursor (Scheme 18) was the intended substrate for this synthesis. Early on in the project, it was determined that the aryl-stereocenter does not racemize at reaction conditions suitable for enzymes, so separation of the *R*-ketone from the product is always necessary even with employment of TAs and IREDs. Furthermore, this means that only a theoretical maximum conversion of 50% with regards to the substrate could be achieved. As it turned out, the (*S*,*S*)-alcohol was the preferred isomer obtained by several KREDs in excellent *ee* (%). This however required the additional step of re-oxidation of the purified alcohol to the *S*-ketone, destroying one of the stereocenters in the process to finally achieve the intended API. The two first steps can therefore also be seen as a kinetic resolution of the racemic precursor ketone. A final isolated yield of 16% could be achieved, but chemical chiral amine synthesis methods for the last step were not investigated in detail and could hold great potential for improving this yield.



Scheme 18. KRED and Laccase mediated synthesis pathway to *S,S*-Sertraline. The second step can be alternatively catalysed by NaClO/AZADO instead of a laccase.

6.3.3 Outlook

The KRED kit employed for this part of the work is robust, fast and selective. It is however not clear what the enzyme powder of the KRED kit consists of exactly when the exact composition of the catalyst formulation is not known. It can be difficult to optimize a reaction with regards to further scale-up and applicability of the process. It is important to take into account that one is always depending on the company producing the biocatalyst, which can be very costly and bring its own issues with supply, quality and reproducibility between batches. For the Sertraline project, it would be interesting to see whether *R*-selectivity could be achieved for the aryl-stereocenter which could make step 2 of the current cascade in Paper III obsolete. In this way the *S*-ketone can be directly separated from the reaction mixture and employed for further synthesis. As no success was achieved in enantioselectively synthesizing the amine from the respective alcohol in Paper I, it is questionable whether this approach would yield a viable two-step synthesis route consisting of reduction and amination. This would also require a catalyst achieving the *R,S*-alcohol instead of the *S,S*-alcohol. As the catalysts employed are commercial catalysts of which neither structure nor sequence is available, it would be necessary to identify a different ketoreductase for a potential enzyme engineering study.

6.4 Cascades

One of the goals of this work was to develop cascades to make the chosen target molecules as this holds many advantages for process efficiency and sustainability. Of the synthesis pathways and cascades presented in Paper I, III and IV to their respective target molecules Cinacalcet, Sertraline and Vyvanse, only the cascade to Vyvanse in paper IV is a true one-pot cascade. All other synthesis routes and cascades need intermediate purification steps or at least a removal of catalyst and a solvent change. In the case of Paper I there are arguments for and against whether one can call this a chemo-enzymatic cascade or rather a synthesis route. The argument in favour in the case of the transaminase route is that only the biocatalyst of the first step is removed and then the second step is conducted without any further purification steps. This fits into the concept of a sequential cascade (chapter 4.2). An attempt to run this cascade as a one-pot process was not successful. Previous work on combination of metal and enzyme catalysis show that the metal catalyst can be inactivated by the enzyme and vice versa [107]. In the case of the ketoreductase route in paper I, purification steps are needed for the chemical synthesis steps from the Cinacalcet alcohol intermediate to the *R*-naphthylethylamine. Here, the term of a synthesis route may be more appropriate. The same argument can be brought forward for the most successful synthesis route to Sertraline (Paper III)employing KREDs were the *R*-ketone has to be separated from the reaction mixture after step one (Scheme 18). The argument in favour of this route is that the alcohol and ketone are easy to separate in contrast to a chiral resolution process that would be necessary to separate the *S*-ketone from the *R*-ketone. Therefore, this step can also be seen as a possibility for a kinetic resolution. In any case, purification is necessary in this process and therefore this can be seen as a synthesis route rather than a cascade. In Paper IV a successful one-pot cascade was developed. Here all required reaction components are present from the start and a successful synthesis of the final API Vyvanse takes place. Here only purification at the end is necessary, as it would for all other cascades and synthesis routes in paper I and III. The advantage with a one-pot cascade is that all materials and work hours necessary for intermediate purification steps are not needed. This lowers the general environmental impact and greatly improves the sustainability of such a production process.

7 CONCLUDING REMARKS AND OUTLOOK

The aim of this work was to develop new enzymatic cascades towards pharmaceuticals. Two concepts are combined to potentially lower environmental impact and improve process efficiency. This ties well into the 17 UN sustainable development goals[108] and increases the understanding and applicability of enzymes for achieving these goals. Biocatalytic processes have the potential to be environmentally friendly which goes with goal 6 “clean water and sanitation”, 14 “life below water” and 15 “life on land”. Since the biocatalysts themselves are biologically degradable and environmentally friendly, they tie well into goal 11 “sustainable cities and communities” and goal 12 “responsible consumption and production”. Here, one can say a contribution towards a greener chemistry is made. With regards to the synthesis route, this conclusion is not as clear cut. Whether a process is truly greener than the state-of-the-art can only be determined by a detailed Life Cycle Analysis (LCA). For that, exact process parameters and specifics around chemicals employed need to be known which are not necessarily at hand for a proof-of-concept synthesis route or a small pilot process. But a few general conclusions can be drawn when looking for example at solvents employed and number of steps necessary to achieve a target compound. Here, one can look at a step-by-step synthesis in comparison to a one-pot synthesis. Generally, it can be said that the efficiency and straight-forward synthesis of a true one-pot process as has been developed in paper IV provides synthesis strategy with a more efficient use of resources. A better use of resources contributes to UN sustainable development goal 8 “decent work and economic growth”. In combination with the focus on pharmaceuticals as example compounds it also ties into UN sustainable development goals 3 “good health and well-being” and can contribute in conjunction with goal 8 “decent work and economic growth” (UN sustainable development goal 8) to better availability for everyone and lower cost for generics.

Whether a process is actually more environmentally friendly depends often on its efficiency but also on its employed materials. This can be illustrated best with taking an example from the processes developed in

this work. Often DMSO is employed as a co-solvent which, however, is classified as a solvent that should be substituted if possible[109]. The main reason for employing this co-solvent is the poor solubility of the substrates in water. Here, one can think of looking into either more environmentally compatible substitutes or different process conditions like in-neat catalysis or biocatalysis in more environmentally friendly solvents or ionic liquids[110]. It is also important to think about how to combine the best from all disciplines of chemistry. Even though biocatalysis provides a myriad of options for new synthesis pathways it might not be the best choice for certain necessary synthesis steps. If a one-pot process is aimed for combining an enzyme and another catalyst the compromise between optimal reaction conditions or finding suitable conditions at all can be challenging[111]. Enzymes originally perform well in aqueous medium and at ambient temperature, organocatalysts are often better at higher temperatures and in organic solvents. Bridging these seemingly opposite reaction conditions is a challenge, especially when the processes also have to meet certain requirements. This challenge could also be seen as an opportunity to think across disciplines in chemistry and come up with new innovative solutions for reaction and reactor design. It also requires more communication between disciplines to understand the requirements of the other to find the optimal and most sustainable solution for the process at hand.

8 ACKNOWLEDGMENTS

First I want to thank my main supervisor Per Berglund taking me on as PhD student and giving me the liberty for my research in the laboratory and elsewhere.

Thank you to all the past and present members of the biocatalysis group, it was fantastic working with you in the lab and it made my start and “homecoming” much easier. I also want to thank everyone from floor 1 and the Glycoscience group for help in the lab and out-of-lab fun.

Then I would like thank my two secondment hosts and all of the colleagues there, Entrechem and Enzymicals. I want to especially thank Philipp and Javier for being my supervisors there.

My thanks also goes to all my fellow BIOCASCADES ESRs, you were wonderful and it was so much easier to go through all of it with you. The meet-ups for seminars and conferences were always truly special.

Vielen Dank auch an meine Familie, die alle Umzüge, Höhen und Tiefen jeglicher Art mitgemacht und mich immer unterstützt haben.

I want to thank the Knut and Alice Wallenberg Foundation and the Sillén foundation for financially supporting my participation in conferences. The EU-Cost Action CM1303 Systems Bicatalysis is gratefully acknowledged for supporting attending their summer school.

My thanks also goes to the H2020 Marie Curie European Industrial Doctorate "Biocascades“(H2020-MSCA-ITN-2014 grant number: 634200) for financially supporting my PhD studies.

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