



Amine Transaminases in Biocatalytic Amine Synthesis

Henrik Land

KTH Royal Institute of Technology

School of Biotechnology

Stockholm 2016

© Henrik Land

Stockholm 2016
KTH Royal Institute of Technology
School of Biotechnology
Division of Industrial Biotechnology
AlbaNova University Center
SE-106 91 Stockholm
Sweden

Printed by Universitetsservice US-AB
Drottning Kristinas väg 53B
SE-114 28 Stockholm
Sweden

ISBN 978-91-7729-164-0
TRITA-BIO Report 2016:18
ISSN 1654-2312

Front Cover: Crystal structure of *Chromobacterium violaceum* amine transaminase (PDB ID: 4A6T) surrounded by a selection of substrates and products from the projects presented in this thesis.

Abstract

The use of enzymes, nature's own catalysts, both isolated or as whole cells to perform chemical transformations is called biocatalysis. As a complement to classical chemical catalysis, biocatalysis can be an environmentally friendly and more economical option in the production and synthesis of chemicals. Research on the application of amine transaminases in synthesis of chiral amines have exploded over the last two decades and interest from the industry is increasing. Amine transaminases are promising catalysts due to their ability to perform reductive amination of ketones with excellent enantioselectivity.

For a process to be efficient, high substrate specificity of the applied enzyme is an important factor. A variant of *Chromobacterium violaceum* amine transaminase that was obtained through rational design has an increased specific activity toward (*S*)-1-phenylethylamine and a set of 4'-substituted acetophenones. This result makes this variant a promising catalyst for the asymmetric synthesis of similar amines.

Amine transaminase catalyzed asymmetric synthesis of amines generally suffers from unfavorable equilibrium. Two methods that include spontaneous tautomerization and biocatalytic amidation for equilibrium displacement have therefore been developed.

Efficient assays and screening methods are demanded for the discovery and development of novel amine transaminases. For this purpose, a sensitive fluorescence-based assay that holds promise as a high-throughput screening method was developed.

One of the major obstacles for application of enzymes in industrial processes is the instability of the enzyme toward harsh conditions. The stability of *Chromobacterium violaceum* amine transaminase was investigated and improved using co-solvents and other additives. Co-lyophilization with surfactants was also applied to improve the performance of the same enzyme in organic solvents.

Keywords: Amine Transaminase, Biocatalysis, Transamination, Reductive Amination, Enzyme, Enzyme Engineering, Equilibrium Displacement, Screening, Enzyme Stability

Sammanfattning

Användandet av enzymer, naturens egna katalysatorer, både isolerade och som hela celler för att utföra kemiska transformationer kallas för biokatalys. Som ett komplement till klassisk kemisk katalys kan biokatalys vara ett mer miljövänligt och ekonomiskt alternativ vid produktion och syntes av kemikalier. Forskning på tillämpning av amintransaminaser för syntes av kirala aminer har exploderat under de två senaste decennierna och intresset från industrin ökar hela tiden. Amintransaminaser är lovande katalysatorer p.g.a. deras förmåga att utföra reduktiv aminering av ketoner med utmärkt enantioselektivitet.

För att en process ska vara effektiv så måste det applicerade enzymet ha hög substratspecificitet. En variant av amintransaminaset från *Chromobacterium violaceum* som erhöles genom rationell design har en ökad specifik aktivitet mot (*S*)-1-fenyletylamin och en uppsättning 4'-substituerade acetofenoner. Detta resultat gör den här varianten till en lovande katalysator för asymmetrisk syntes av liknande aminer.

Asymmetrisk syntes av aminer katalyserad av amintransaminaser lider generellt av ofördelaktig jämvikt. Två metoder som inkluderar spontan tautomerisering och biokatalytisk amidering för jämviktsförskjutning har därför utvecklats.

Effektiva analys- och screeningmetoder krävs för att kunna upptäcka och utveckla nya amintransaminaser. För detta syfte har en känslig fluorescensbaserad analysmetod som ser lovande ut som screeningmetod för hög genomströmning utvecklats.

Ett av de största hindrena för tillämpning av enzymer i industriella processer är enzymets instabilitet i ogästvänliga förhållanden. Amintransaminaset från *Chromobacterium violaceum*s stabilitet undersöktes och förbättrades genom addition av vattenlösliga lösningsmedel och andra tillsatser. Frystorkning tillsammans med surfaktanter applicerades också för att öka enzymets prestanda i organiska lösningsmedel.

Nyckelord: Amintransaminas, Biokatalys, Transaminering, Reduktiv Aminering, Enzym, Enzymmodifiering, Jämviktsförskjutning, Screening, Enzymstabilitet

List of appended papers

Paper I

Cassimjee K E, Humble M S, Land H, Abedi V and Berglund P. *Chromobacterium violaceum* ω -transaminase variant Trp60Cys shows increased specificity for (S)-1-phenylethylamine and 4'-substituted acetophenones, and follows Swain-Lupton parameterization. *Org. Biomol. Chem.* **2012**, *10*, 5466-5470.

Paper II

Wang B, Land H and Berglund P. An efficient single-enzymatic cascade for asymmetric synthesis of chiral amines catalyzed by ω -transaminase. *Chem. Commun.* **2013**, *49*, 161-163.

Paper III

Land H, Hendil-Forsell P, Martinelle M and Berglund P. One-pot biocatalytic amine transaminase/acyl transferase cascade for aqueous formation of amides from aldehydes or ketones. *Catal. Sci. Technol.* **2016**, *6*, 2897-2900.

Paper IV

Scheidt T, Land H, Anderson M, Chen Y, Berglund P, Yi D and Fessner W-D. Fluorescence-Based Kinetic Assay for High-Throughput Discovery and Engineering of Stereoselective ω -Transaminases. *Adv. Synth. Catal.* **2015**, *357*, 1721-1731.

Paper V

Chen S,^[1] Land H,^[1] Berglund P and Humble M S. Stabilization of an amine transaminase for biocatalysis. *J. Mol. Catal. B: Enzym.* **2016**, *124*, 20-28.

^[1] Shared first authorship

Contributions to appended papers

Paper I

Contribution to design, execution and analysis of kinetic experiments.

Paper II

Design of the cascade and planning of experiments together with Bo Wang. Performed a large amount of the writing.

Paper III

Design of the cascade. Planning, execution and analysis of all experiments. Performed a majority of the writing.

Paper IV

Major contribution to “Determination of Enantioselectivity and Kinetic Constants” and “Determination of Substrate Promiscuity” of the designed assay. Minor contribution to the writing.

Paper V

Major contribution to all planning as well as experiment design and execution together with Shan Chen. Performed a majority of the writing together with Shan Chen.

Papers not included in this thesis

Steffen-Munsberg F, Vickers C, Thontowi A, Schätzle S, Tumlirsch T, Humble M S, Land H, Berglund P, Bornscheuer U T and Höhne M. Connecting Unexplored Protein Crystal Structures to Enzymatic Function. *ChemCatChem* **2013**, 5, 150-153.

Steffen-Munsberg F, Vickers C, Thontowi A, Schätzle S, Meinhardt T, Humble M S, Land H, Berglund P, Bornscheuer U T and Höhne M. Revealing the Structural Basis of Promiscuous Amine Transaminase Activity. *ChemCatChem* **2013**, 5, 154-157.

Steffen-Munsberg F, Vickers C, Kohls H, Land H, Mallin H, Nobili A, Skalden L, van den Bergh T, Joosten H-J, Berglund P, Höhne M and Bornscheuer U T. Bioinformatic analysis of a PLP-dependent enzyme superfamily suitable for biocatalytic applications. *Biotechnol. Adv.* **2015**, 33, 566-604.

List of abbreviations

1-PEA	1-Phenylethylamine
3-HBA	3-Hydroxybenzoic acid
Ac-ATA	<i>Arthrobacter citreus</i> amine transaminase
ACHDA	3-Aminocyclohexa-1,5-dienecarboxylic acid
ATA	Amine transaminase
BN-PAGE	Blue native polyacrylamide gel electrophoresis
Cv-ATA	<i>Chromobacterium violaceum</i> amine transaminase
DNA	Deoxyribonucleic acid
<i>E. coli</i>	<i>Escherichia coli</i>
ee	Enantiomeric excess
IPA	Isopropylamine
L pocket	Large pocket
MNBA	1-(6-Methoxynaphth-2-yl)butylamine
MNEA	1-(6-Methoxynaphth-2-yl)ethylamine
MNPA	1-(6-Methoxynaphth-2-yl)propylamine
MsAcT	<i>Mycobacterium smegmatis</i> acyl transferase
NMR	Nuclear magnetic resonance
OMP	Orotidine 5'-monophosphate
PCR	Polymerase chain reaction
PLP	Pyridoxal 5'-phosphate
PMP	Pyridoxamine 5'-phosphate
S pocket	Small pocket
Sp-ATA	<i>Silicibacter pomeroyi</i> amine transaminase
T_m	Melting point
UV	Ultraviolet
Vf-ATA	<i>Vibrio fluvialis</i> amine transaminase
ΔT_m	Melting point difference

Table of Contents

1. Introduction	1
2. Enzyme kinetics	5
3. Chirality and enantioselectivity	11
4. Enzyme engineering	17
4.1. Rational design.....	18
4.2. Directed evolution.....	18
4.3. Semi-rational design	19
4.4. Screening & selection	20
5. Enzyme stability	21
6. Chiral amines.....	23
7. Amine transaminase	29
8. Present investigation	35
8.1. Rational design for increased substrate specificity (Paper I)	35
8.2. Equilibrium displacement of amine transaminase catalyzed asymmetric synthesis (Paper II & Paper III).....	43
8.2.1. Chemoenzymatic cascade for equilibrium displacement (Paper II).....	43
8.2.2. Enzymatic cascade for equilibrium displacement (Paper III)	47
8.3. Fluorescence-based assay for screening of amine transaminases (Paper IV)	55
8.4. Investigation of amine transaminase stability (Paper V)	61
9. Concluding remarks and future outlook	73
Acknowledgements.....	77
Bibliography	79

1. Introduction

The earliest evidenced signs of life date the first life on Earth to around three billion years ago.¹ Since then, life has been dependent on chemical reactions working simultaneously to convert nutrients into molecules necessary for an organism's survival and function. This process, called metabolism, is crucial for life on Earth and the chemical reactions involved needs to be carefully controlled for the machinery of life to function properly. Many reactions involved in metabolism are however too slow to be able to support life. As an example, the spontaneous decarboxylation of orotidine 5'-monophosphate (OMP) to form uridine monophosphate at non-elevated temperatures has a half-life of 78 million years.² This is not the only example of such a slow reaction and these time spans are obviously too long for life to be supported. However, nature has elegantly solved this issue by evolving its own catalysts. These macromolecular biological catalysts of nature are called enzymes. Enzymes are proteins that catalyze chemical reactions and nature has evolved countless enzymes that catalyze reactions with high specificity in all areas of life.³ One such enzyme is OMP decarboxylase that catalyzes the previously mentioned decarboxylation of OMP. OMP decarboxylase demonstrates a remarkable catalytic power as it enhances the rate of the decarboxylation by 1.4×10^{17} times.² This means that instead of a half-life of 78 million years, this reaction has a half-life of approximately 18 milliseconds in the presence of OMP decarboxylase. The catalysis performed by naturally evolved enzymes makes these virtually non-spontaneous reactions occur in a much shorter time and life on Earth can therefore be supported.

Enzymes are biological macromolecules built up by linear chains of amino acid residues forming a three-dimensional structure that is dependent on the chemical characteristic of each amino acid. Amino acids are compounds that have a common zwitterionic core consisting of an amine- and a carboxylic acid functional group. These cores of the amino

acids are connected via amide bonds to form the backbone of the enzyme. There are 20 naturally occurring amino acids that are usually incorporated into proteins and each amino acid has a unique sidechain attached to the core that can be either hydrophobic, hydrophilic, aromatic, positively charged or negatively charged. The size and molecular weight of the sidechains vary greatly and range from 1 g mol⁻¹ (glycine) to 130 g mol⁻¹ (tryptophan). These 20 amino acids can basically be combined in any combination which means that an enzyme consisting of 400 amino acid residues has 20⁴⁰⁰ possible amino acid sequences. This fact combined with the structural diversity of the incorporated amino acids gives rise to an almost infinite amount of possible enzyme structures.

The macromolecular structure of an enzyme basically acts as a scaffold for catalysis. The place where the actual catalysis takes place in the enzyme is called the active site. This is where the substrate binds in a very specific conformation that is optimal for catalysis. The enzyme catalyzes the transformation by lowering the energy barrier that is necessary to reach for the transformation to take place. This means that less energy needs to be added to form the desired product and this catalytic power makes enzymes interesting as catalysts for industrial transformations. Catalysis has also been classified as one of the twelve principles of green chemistry⁴ and is therefore of great interest for the industry. The remarkable breakthroughs in biotechnology like restriction enzyme analysis⁵⁻⁶, DNA sequencing⁷⁻⁹ and the polymerase chain reaction (PCR)¹⁰ over the last 40 years has made it possible for researchers to exploit and modify the naturally evolved enzymes for synthetic purposes. Since the enzymes found in nature are usually evolved for a very specific reaction they can rarely be applied directly in a synthetic reaction where the product is an unnatural compound. Engineering of the enzymes are therefore often needed.

Biocatalysis is a highly interdisciplinary research field where biochemistry, biotechnology and organic chemistry are all combined. The enzymes that are evolved by nature are studied, modified and applied in chemical synthesis. Biocatalysis has over the last century gone through three waves of development¹¹ where in the first wave extracts of living cells were used to perform simple transformations like in the synthesis of (*R*)-mandelonitrile¹² all the way to mimicking evolution in the current

third wave to create engineered enzymes that can catalyze the transformation of widely different substrates than they were naturally evolved to in very harsh conditions¹³.

This doctoral thesis will deal with the biocatalytic application of amine transaminases in the synthesis of chiral amines. Both engineering and application in chemical synthesis will be presented.

2. Enzyme kinetics

Enzyme kinetics describe the rate at which a certain enzyme performs catalysis. It is a corner stone in the study of enzymes and to understand and correctly apply an enzyme, studying the kinetics is of vital importance. The first steps to describing the kinetics of enzymes were taken by Henri in 1903¹⁴ and Michaelis and Menten in 1913¹⁵. Their combined work together with the added steady-state assumption by Briggs and Haldane in 1925¹⁶ resulted in the now famous and widely used Michaelis-Menten equation (Equation 1).

$$v = \frac{V_{\max}[S]}{K_M + [S]} \quad (\text{Equation 1})$$

The Michaelis-Menten equation (Equation 1) describes how the initial rate (v) is dependent on substrate concentration ($[S]$). By experimentally determining v for a set of $[S]$ one can plot v against $[S]$ to obtain the typical Michaelis-Menten curve for an enzyme catalyzed reaction (Figure 1).

V_{\max} is the maximum velocity that an enzyme can reach for a specific substrate S at a given enzyme concentration $[E]$ (Figure 1). The dependency of V_{\max} toward $[E]$ is shown in equation 2 where k_{cat} is a first-order rate constant usually referred to as a turnover number. It describes the amount of substrate molecules that are transformed into product per time unit.

$$V_{\max} = k_{\text{cat}}[E] \quad (\text{Equation 2})$$

K_M is a kinetic constant that describes the $[S]$ that yields half of V_{\max} (Figure 1). It is a combined constant that consists of the kinetic constants that describe binding of S to E to form the ES complex (k_1), release of S from the ES complex (k_{-1}) and the transformation of the ES complex to product (P) (k_2) (Scheme 1).

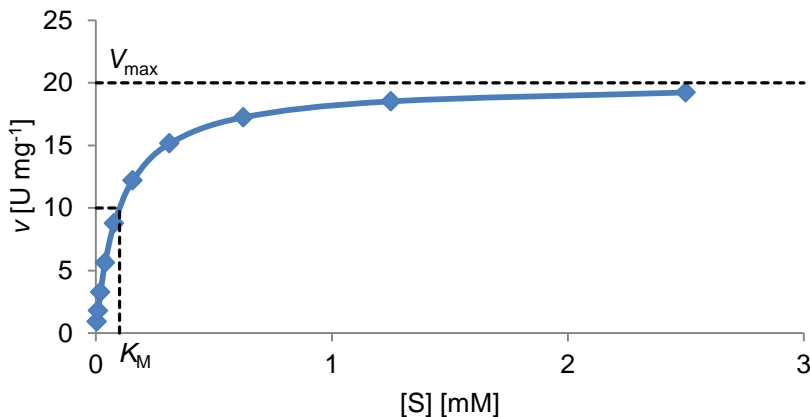
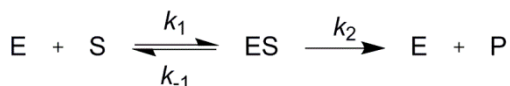


Figure 1. General example of a Michaelis-Menten curve. The arbitrary enzyme in this example has a K_M of 0.1 mM and a V_{max} of 20 U mg⁻¹ enzyme where U (Unit) stands for μmol formed product per minute.

Depending on the complexity of the step described by k_2 , there will be one or several chemical transformations leading to product P. All these steps will have individual kinetic constants which are usually combined to form the first-order rate constant k_{cat} .¹⁷ The combination of these kinetic constants into K_M is described in equation 3.



Scheme 1. Visualization of the kinetic constants that are combined to form K_M . Binding of substrate (S) to the enzyme (E) to form ES is described by k_1 , dissociation of the ES complex to form S and E is described by k_{-1} and transformation of ES to form E and product (P) is described by k_2 .

$$K_M = \frac{k_{-1} + k_2}{k_1} = \frac{k_{-1} + k_{cat}}{k_1} \quad (\text{Equation 3})$$

The kinetic constants k_{cat} and K_M are usually used to compare the activities of different enzymes toward the same substrate or to compare a single enzyme's activity toward different substrates. Comparisons can also be made between different variants. Since they both show different things and since different changes can have effects on only one of the kinetic constants or both at the same time, the best way to compare is by

using the ratio of the two, k_{cat}/K_M . The ratio k_{cat}/K_M is usually referred to as the specificity constant.

The basic Michaelis-Menten equation (Equation 1) works well for describing single-substrate enzymatic reactions with no deviations from the behavior described in that particular equation. However, many modifications of the original equation have been developed that describes for example multi-substrate enzymatic reactions where the equations are different depending on the order of the substrates entering and the products leaving the enzyme.¹⁷ Amine transaminase, the major enzyme described in this thesis, employs a ping-pong bi-bi reaction mechanism. The term bi-bi means that it catalyzes a reaction between two substrates forming two products while the term ping-pong means that the first substrate entering the enzyme is followed by the first product leaving. The second substrate then enters and finally, the second product leaves. The modified Michaelis-Menten equation for the ping-pong bi-bi reaction mechanism is described in equation 4 where the two substrates of the reaction are denoted A and B.

$$v = \frac{k_{\text{cat}}[E][A][B]}{K_M^A[B] + K_M^B[A] + [A][B]} \quad (\text{Equation 4})$$

Another deviation that will be shortly described here is the case of substrate inhibition. In the Michaelis-Menten equation (Equation 1) the amount of S can be indefinitely increased and the only effect is that the rate will go toward V_{max} . There are however many cases where a too high [S] will inhibit the enzymes action. The Michaelis-Menten equation can in these cases be modified (Equation 5) to account for the substrate inhibition.

$$v = \frac{V_{\text{max}}[S]}{K_M + [S] \left(1 + \frac{[S]}{K_I} \right)} \quad (\text{Equation 5})$$

K_I is an inhibition constant that describes the dissociation of the inhibitor from the ES complex, in this case the dissociation of the abundant substrate. When higher concentrations of substrate inhibits an enzyme, the kinetics usually follow the normal behavior of the Michaelis-Menten equation up to a certain concentration threshold where the inhibitory effects become relevant. An example of a Michaelis-Menten curve with

substrate inhibition compared to the non-inhibited case (Figure 1) is shown in figure 2.

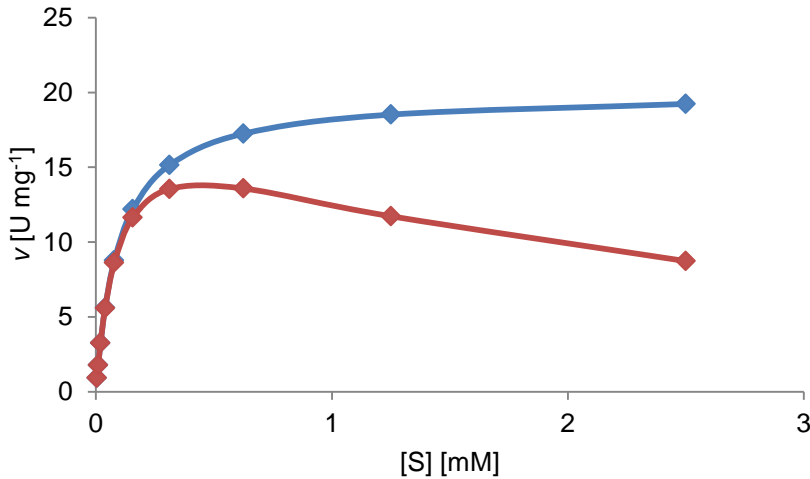
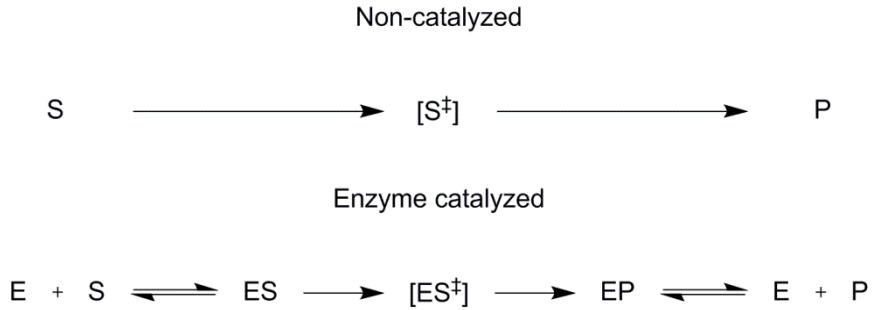


Figure 2. Comparison of the Michaelis-Menten curve from figure 1 (blue) with a Michaelis-Menten curve with substrate inhibition (red). K_i in the substrate inhibition case is set to 2 mM.

In the introduction it was stated that enzymes catalyze reactions by lowering the energy barrier that is necessary to reach for the reaction to take place. This barrier is a high energy state known as the transition state ($[S^\ddagger]$) which is an intermediate that exists for a short time during the transformation of the substrate S into the product P (Scheme 2). In the case of enzyme catalysis the transition state is usually denoted $[ES^\ddagger]$ and exists between ES and EP (Scheme 2).

All the states depicted in scheme 2 exist at different levels of free energy (G) and the enzyme enhances the rate of the reaction by stabilizing the transition state, thereby lowering the difference in free energy ($\Delta G^{[ES^\ddagger]}$) between ES and $[ES^\ddagger]$ compared to the difference between S and $[S^\ddagger]$ in the non-catalyzed reaction ($\Delta G^{[S^\ddagger]}$) (Figure 3).



Scheme 2. Comparison of the steps involved in a non-catalyzed and enzyme catalyzed conversion of S to P.

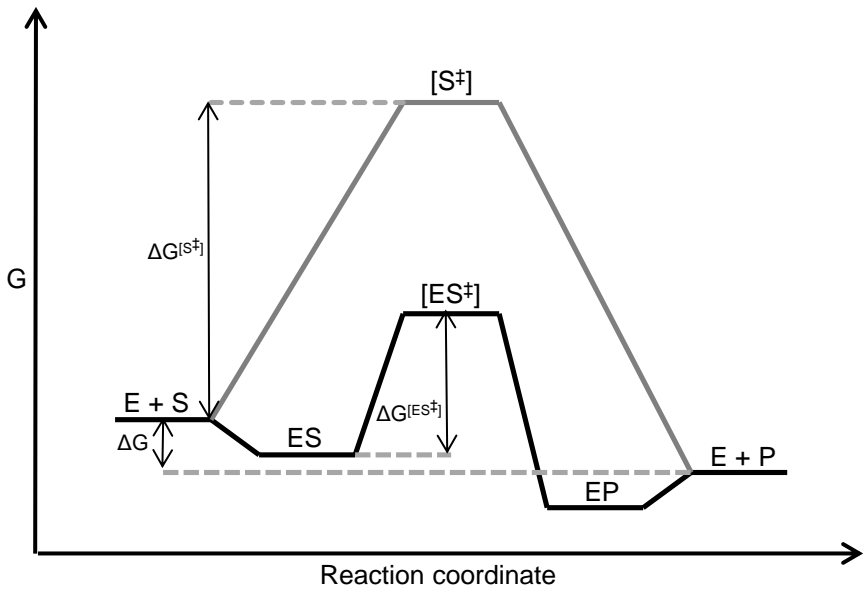


Figure 3. Energy diagram displaying the differences in free energy (ΔG) between a non-catalyzed (grey) and an enzyme catalyzed (black) conversion of S to P.

Chemical reactions are often reversible and depending on the free energies of the substrate S and product P there will be a point where the concentrations of S and P yields the same reaction rate in both directions and the concentrations of S and P will therefore be constant. S and P are here said to be in equilibrium and this is described by an equilibrium constant (K_{eq}) (Equation 6).

$$K_{\text{eq}} = \frac{[\text{P}]_{\text{eq}}}{[\text{S}]_{\text{eq}}} \quad (\text{Equation 6})$$

The difference in free energy for the total reaction (ΔG) is the same for the non-catalyzed reaction and the enzyme catalyzed reaction (Figure 3) which means that ΔG is path independent and does not depend on the intermediate states of the reaction.¹⁷ This leads to the conclusion that the addition of an enzyme to a chemical reaction increases the rate but does not influence the equilibrium.

Kinetic constants can be used in many applications and one of those is the Swain-Lupton plot¹⁸. The Swain-Lupton equation (Equation 7) is a refined version of the Hammett equation¹⁹ that describes the effects of aromatic substitutions on chemical reactions. It is based on the assumption that field and resonance effects from the aromatic substitutions are the only significant factors that have an effect on the reaction.¹⁸

$$\log \frac{k}{k_0} = fF + rR \quad (\text{Equation 7})$$

k is the first order rate constant for the substituted species and k_0 is the first order rate constant for the unsubstituted species. F and R are field and resonance constants that can be calculated and has been previously determined for a large set of substituents.^{18,20} By determining rate constants for a set of substituted species in a certain reaction one can numerically determine the coefficients f and r and thereby assess the contributions of field and resonance on the specific reaction.

3. Chirality and enantioselectivity

Chirality is a common phenomenon in nature and chemical synthesis. It means that compounds that at a first glance might look identical are actually non-identical mirror images of each other. Chiral compounds can be chiral due to the presence of one or more stereocenters. The most common stereocenter is a saturated carbon atom that has four different substituents. This means that if two of the substituents are substituted with each other, the resulting compound will not be identical to the first compound. They will be non-identical mirror images of each other. A common example to visualize chirality is the human hands. They both look the same but are actually non-identical mirror images of each other. This results in the fact that a left hand only fits in a left glove and vice versa.^[2] A chemical example of chirality is the compound 1-phenylethylamine (1-PEA). It contains a carbon stereocenter with a phenyl-, a methyl-, an amine- and a hydrogen substituent. By substituting the amine and the hydrogen substituent of (*S*)-1-PEA with each other, the mirror image compound (*R*)-1-PEA is created (Figure 4). By rotating (*R*)-1-PEA one can clearly see that the compounds are not identical. Mirror image compounds like these are called enantiomers.

Chiral compounds are very abundant in nature. The naturally occurring amino acids that make up the structure of proteins are all chiral (except for glycine) and it is only the L-enantiomer ((*S*)-enantiomer in the case of canonical α -amino acids, except for L-cysteine which is an (*R*)-enantiomer) of each amino acid that is incorporated into proteins. The binding sites of proteins are usually very specific for a certain compound and when this compound is chiral the binding sites often only bind one of the enantiomers (just like a right hand only fits in a right glove). Since many pharmaceuticals are designed to target a specific enzyme or receptor, chirality is very important in the synthesis of pharmaceuticals.

[2] The word chiral actually comes from the Greek word for "hand"

Different enantiomers of compounds can have very different biological activities and the synthesis of pure single enantiomers is therefore of great importance.

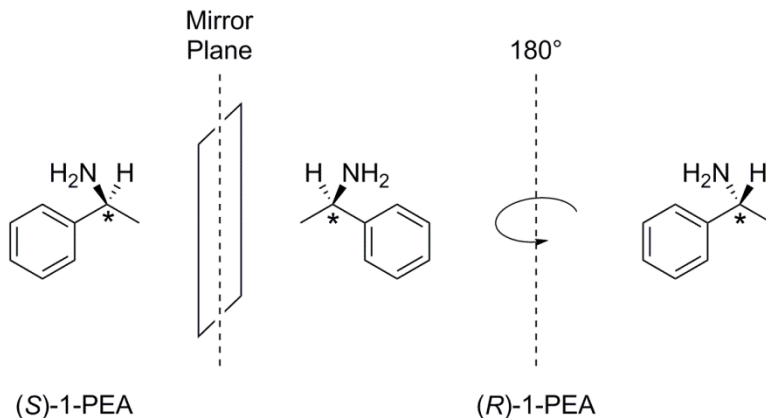
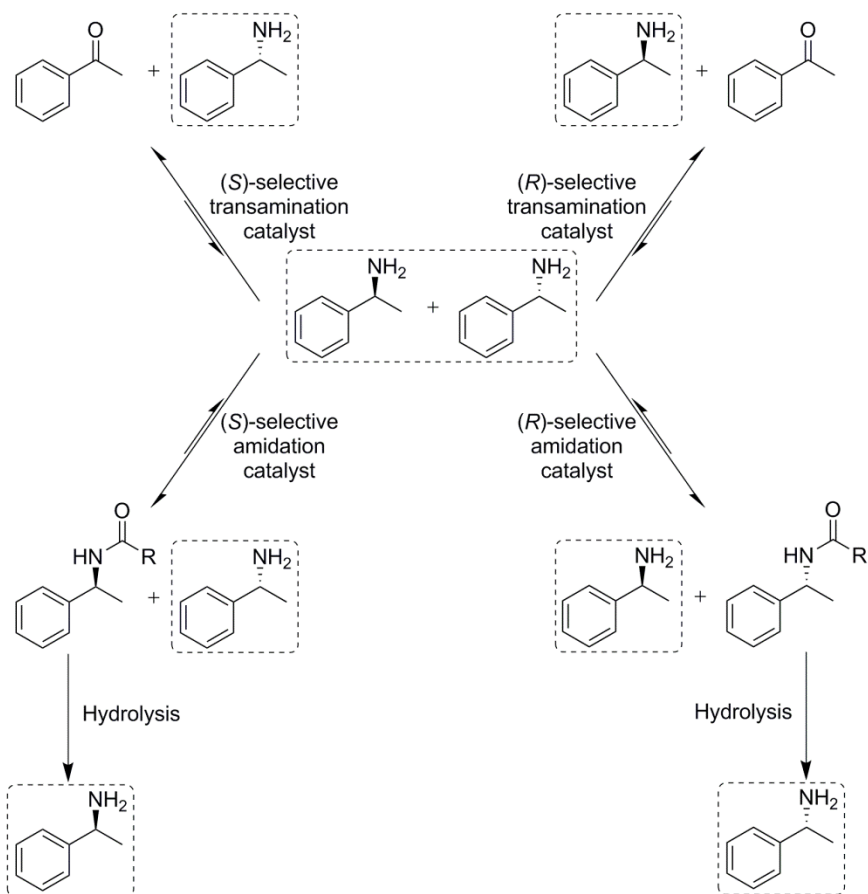


Figure 4. The structural difference between the two enantiomers of 1-PEA is here illustrated by creating the mirror image of (S)-1-PEA and rotating it 180°. The difference between (S)-1-PEA and (R)-1-PEA is then clear as the amine function is pointing in different directions.

The synthesis of chiral compounds is often accomplished via asymmetric synthesis starting from a prochiral compound. However, if a prochiral compound would react with a non-chiral nucleophile catalyzed by a simple metal catalyst to form a chiral compound with nothing else present; a 50/50 mixture of both enantiomers (racemate) would be formed. The reaction would not be enantioselective (selective against a single enantiomer). Chemists have solved this by adding chiral ligands²¹ that coordinate to the metal catalyst and force the prochiral compound to bind in a certain conformation, thereby enhancing the synthesis of one of the enantiomers. Chiral organic catalysts can also be used to perform enantioselective asymmetric synthesis in a similar fashion.²²

Enzymes perform catalysis in their active site and the amino acid residues lining the active site form a scaffold where the catalysis takes place. This usually means that enzymes catalyzing reactions involving chiral substrates or products are very enantioselective and one of the benefits of biocatalysis is often said to be the excellent enantioselectivity of enzymes.²³



Scheme 3. Comparison between amine transaminase (top) and lipase (bottom) catalyzed kinetic resolution of 1-PEA. Lipase catalyzed amidation does not destroy the chirality of the reacting enantiomer and hydrolysis of the amide to obtain both pure enantiomers is therefore possible.

Asymmetric synthesis has been mentioned as a method to synthesize chiral compounds starting from a prochiral compound. It is a method that introduces chirality and it has a 100% theoretical yield. However, there are other methods to produce enantiomerically pure compounds using enantioselective catalysts. Kinetic resolution is a form of enantiomeric enrichment where the starting substrate is a mixture of enantiomers, typically a racemate. By introducing an enantioselective catalyst that preferably reacts with one enantiomer to form any given

product, the remaining enantiomer can be isolated after the reaction is completed (Scheme 3).

Kinetic resolution has one major drawback and that is that the maximal theoretical yield is 50% starting from a racemate. This can however be somewhat circumvented if the resolving reaction does not destroy the chirality of the reacting enantiomer. This is the case in lipase catalyzed kinetic resolution of amines where the product is a chiral amide that can be isolated and hydrolyzed to form the enantiomerically pure amine (Scheme 3).²⁴ A stereo inverting step can also be introduced to continuously racemize the substrate as it is kinetically resolved. This method for deracemization is called a dynamic kinetic resolution²⁵ and is an improvement of kinetic resolution allowing a 100% theoretical yield.

Two of the most common ways to report enantioselectivity are enantiomeric excess (*ee*) and the E-value²⁶⁻²⁷. The value *ee* describes how much more of one of the enantiomers that is present in a mixture of enantiomers²⁸, i.e. the excess of one enantiomer over the other and can be calculated using equation 8. For example, a racemic mixture has an *ee* of 0% while a mixture with 90% (*R*)-enantiomer and 10% (*S*)-enantiomer has an *ee* of 80%. The value *ee* is a property of the product and is most commonly used to report enantioselectivity in asymmetric synthesis.

$$ee = \frac{([R]-[S])}{([R]+[S])} * 100 \quad (\text{Equation 8})$$

$$E = \frac{\left(\frac{k_{cat}}{K_M}\right)_{fast}}{\left(\frac{k_{cat}}{K_M}\right)_{slow}} \quad (\text{Equation 9})$$

Enantioselective catalysts are rarely completely selective toward a single enantiomer and the enantioselectivity is dependent on how much faster the catalyst reacts with the preferred enantiomer. The E-value, or the enantiomeric ratio, is a ratio showing how much faster the faster enantiomer is reacting in an enantioselective reaction, either in asymmetric synthesis where the enantiomers are produced or in any kind of enantiomeric enrichment, like kinetic resolution, where the enantiomers are the substrates. The E-value can therefore be determined using rate constants and in enzymatic catalysis the specificity constant

$(k_{\text{cat}}/K_{\text{M}})$ for each enantiomer is commonly used (Equation 9). Since *ee* of the product is constantly changing during the course of kinetic resolution, the E-value (which is a property of the enzyme and not the product) is the preferred way to report enantioselectivity in that case.

4. Enzyme engineering

Many enzymes are naturally evolved to perform very specific tasks in the chemistry of life. This means that they are specific toward certain substrates and cannot perform catalysis on any other substrates. They are also specific with regards to reaction conditions, such as; temperature, pH and substrate concentration. All enzymes are however not as specific. There are promiscuous enzymes that can have a broader tolerance against reaction conditions and different substrates and in some cases they can even catalyze different types of reactions.²⁹⁻³⁰

Even though there are many natural products produced in the chemical industry, a large part of the produced chemicals are non-natural in the sense that they are either completely artificial or derived from natural compounds. In fact, out of all approved pharmaceuticals globally between 1981 and 2014, only 4% are completely unaltered natural products while 73% are either artificial, derived from natural products or mimics thereof.³¹ It might therefore be challenging to find an enzyme in nature that can be used in the synthesis of a certain non-natural compound and even if a promiscuous enzyme is found that can catalyze the desired reaction; the activity is probably too low for industrial implementation. Substrate specificity is however not the only issue when an enzyme needs to be applied in industrial transformations. Reaction conditions usually also differ greatly from the natural conditions where the enzyme is evolved to function.³² These issues combined means that enzymes directly derived from nature rarely can be applied in industry in their wild-type form.

As previously mentioned, modern biotechnological breakthroughs have made it possible to overcome the challenges of industrial application of enzymes. Enzymes can be engineered by substituting nucleotides in the enzyme-encoding gene leading to an altered amino acid sequence of the enzyme. This can be done to change the architecture of the active site to

allow binding of different substrates or to introduce other modifications that improves the applicability of the enzyme. Three commonly applied strategies; rational design, directed evolution and semi-rational design, for enzyme engineering will hereafter be described. Screening methods to evaluate the produced variant libraries of directed evolution and semi-rational design will also be mentioned.

4.1. Rational design

As the name implies, rational design is a method where rational choices are made on how to engineer an enzyme for a desired property. One or several amino acid substitutions can be made to induce a hypothesized change in the enzyme's performance. This method is not very work intensive as only a few variants are created and the evaluation of said variants is therefore in most cases straightforward. However, to be able to make rational choices in the engineering of an enzyme, a lot of knowledge about the enzyme under study needs to be acquired. Information about the 3D structure, which has become available through breakthroughs in X-ray crystallography³³⁻³⁴, NMR spectroscopy³⁵⁻³⁶ and homology modelling³⁷, is necessary to predict which amino acid substitutions will yield the desired outcome. One also needs to know where and how substrates bind and how catalysis is performed. All this information is possible to obtain with today's technology and in many cases it is already available for the enzyme of interest. Rational design is most commonly applied in active site engineering to change the substrate specificity of an enzyme³⁸⁻³⁹, invert the enantioselectivity⁴⁰⁻⁴² or enhance/introduce catalytic promiscuity⁴³⁻⁴⁴.

The typical workflow of a rational design project starts with study of the enzyme structure and mechanism of action followed by prediction of mutations. Mutations are incorporated into the enzyme encoding gene via site-directed mutagenesis⁴⁵⁻⁴⁶ and the mutated genes are transformed into an expression host. Variants are verified by DNA sequencing and they are then individually expressed, purified and analyzed for the desired activity.

4.2. Directed evolution

Directed evolution can in many ways be seen as the opposite of rational design. Mutations are randomly incorporated into the enzyme encoding gene followed by screening of a large population of variants for the

desired activity. This makes the method very work intensive but a minimal amount of information about the enzyme is required. The method revolutionized enzyme engineering in the 1990's⁴⁷ as it makes it easier to induce enzyme improvements that are challenging to predict using rational design. Enzymes are built up by hundreds of amino acid residues and they are all connected via networks of chemical and physical interactions. This means that amino acid substitutions far away from the active site may have effects on enzyme activity that are impossible to predict with the present knowledge of how enzymes function. Directed evolution can be used in all applications of enzyme engineering but is especially useful when major improvements in stability⁴⁸ is desired or when the enzyme's activity in harsh process conditions needs to be improved¹³.

The typical workflow of a directed evolution project starts with randomization of the gene of interest using methods like error-prone PCR⁴⁹⁻⁵¹ where conditions are applied that induce random errors by the DNA polymerase or DNA shuffling⁵² where several homologous enzyme encoding genes are randomly digested and reassembled to form chimeric genes. The generated libraries of random variants are thereafter transformed into an expression host and screened for the desired activity by expressing a set number of variants individually and analyzing them. Screening will be more thoroughly discussed later in this chapter.

4.3. Semi-rational design

Rational design and directed evolution are both at individual ends of the spectrum of enzyme engineering methods and there are many methods that apply a combination of both. These methods are usually referred to as semi-rational and one common method of semi-rational design is saturation mutagenesis of one or several amino acid residues in an enzyme. This approach to enzyme engineering combines the rational predictions of rational design with the randomization of directed evolution and this means that a library of variants is still created but the size will be significantly reduced. Site-directed saturation mutagenesis of one or a few adjacent amino acid residues can be performed using complementary degenerate primers⁵³ and if the residues are distant from each other they can still be simultaneously saturated using the megaprimer PCR method⁵⁴.

A typical workflow of semi-rational design will usually be a combination of rational design and directed evolution where mutation sites are first rationally decided. The resulting condensed library will then be transformed into an expression host and subsequently screened for the desired activity.

4.4. Screening & selection

Whenever a library of variants is created by random mutagenesis or similar methods it is of great importance to have a robust and high-throughput screening method that can locate improvements in said library. As the degree of randomization gets higher, the library quickly becomes enormous. As an example, an enzyme that consists of 400 amino acid residues was earlier stated to have 20^{400} (2.6×10^{520}) possible amino acid sequences. The magnitude of that number can be realized by comparing it to the total number of atoms in the known universe which is estimated to be between 10^{70} and 10^{80} .⁵⁵ This leads to the realization that a screening method should be able to screen a large enough number of variants to get a sufficient statistical coverage of the library in the shortest possible time. The classical way of performing screening is to use multiwell plates⁵⁶ that have either 96- or 384 wells. Each well is individually inoculated with a transformed variant and after cultivation each variant is evaluated depending on the characteristics of the involved substrates and products by e.g. change in visible color, UV-absorbance or fluorescence using plate readers. This method can be performed manually but the throughput is much higher using robotic systems.⁵⁷ Solid-phase screening methods⁵⁸⁻⁵⁹ have also been developed where the screening is directly performed on agar-plates making it easier to perform high-throughput screening. These methods normally use reporter enzymes to induce color formation on colonies with the desired activity and the coloration can be visualized either with your eyes or by using digital imaging⁵⁷.

5. Enzyme stability

As previously mentioned, enzymes are evolved to function in the conditions of the environment they are evolved in. These conditions differ widely as there are examples of extremophilic organisms⁶⁰ that live in extreme environments and have subsequently evolved to survive there. However, most enzymes are evolved to function in the physiological conditions where their host organism lives which usually consist of neutral pH and moderate temperatures. These conditions usually differ from the conditions applied in industry where temperatures are often elevated and pH can be either acidic or alkaline. Organic solvents are also commonly used either as the only solvent or in high concentrations to help dissolve water-insoluble substrates.³²

These factors combined results in the fact that wild-type enzymes rarely can be applied directly in the harsh conditions of an industrial process as they are not stable enough. Enzyme stability can be divided into thermodynamic (conformational) stability and kinetic (long-term) stability.^{32,61} The thermodynamic stability of an enzyme is a measurement of its reversible unfolding in different conditions while the kinetic stability shows how long the enzyme is active before it is irreversibly denatured. The stability of an enzyme in an industrial process is usually referred to as operational stability and involves stability toward temperature, organic solvents and time. Operational stability is a function of both thermodynamic and kinetic stability.⁶²⁻⁶⁴

There are many ways of improving enzyme stability and the most commonly used methods are enzyme engineering, immobilization and medium engineering (addition of co-solvents or additives).⁶⁵ Enzyme engineering can be used by applying directed evolution^{13,48} or by using rational design to deliberately introduce stabilizing interactions like disulfide bonds⁶⁶. Enzyme immobilization is a method where the enzyme is either immobilized on a carrier by physical, ionic or covalent

interactions, encapsulated in a polymer network or cross-linked forming enzyme aggregates.⁶⁷⁻⁶⁸ The immobilization also transforms the enzyme from a homogeneous catalyst in aqueous solution into a heterogeneous catalyst which facilitates repeated usage of the enzyme.²³ The use of additives to increase enzyme stability is well-known and the addition of glycerol to enzyme solutions for long-time storage has been applied at least since the 1950's.⁶⁹

An enzyme stability investigation of the amine transaminase from *Chromobacterium violaceum* (Cv-ATA) and its stability improvement by addition of additives and co-solvents⁷⁰ are demonstrated in **Paper V**.

6. Chiral amines

The chiral amine is an important motif in many fine chemicals such as pharmaceuticals⁷¹⁻⁷² and agrochemicals⁷³. The enantioselective synthesis of chiral amines have for a long time been a synthetic challenge but many successful methods have been developed over the last 20 years.⁷⁴ Enantiomerically pure active ingredients are important both in the pharmaceutical and agrochemical industry as two enantiomers of the same compound can have very different biological effects⁷⁵⁻⁷⁶. The application of the single enantiomer that has the desired activity instead of the racemate can not only avoid harmful side effects but also significantly reduce the amount of active ingredients ending up in the environment.

Examples of pharmaceuticals that consist of chiral amines are Cinacalcet⁷⁷ against hyperparathyroidism, Tamsulosin⁷⁸ against benign prostatic hyperplasia, Rivastigmine⁷⁹ against Alzheimer's disease, Sertraline⁸⁰ against depression, Sitagliptin⁸¹ against diabetes type 2, Lisdexamfetamine⁸² for treatment of ADHD and the antiplatelet agent Clopidogrel⁸³ (Figure 5).

Examples of agrochemicals containing chiral amines are not as prevalent but they include the antifeedant (+)-Dihydropinidine⁸⁴, the herbicides Bialaphos⁸⁵ and Bromobutide⁸⁶ as well as Fluvalinate⁸⁷ which is used to control *Varroa destructor* (varroa mites) in honey bee colonies (Figure 6).

As previously mentioned, methods for the synthesis of these high-value chiral amines are of great importance and the developed methods most commonly include addition of nitrogen from a nitrogen source to alkanes, alkenes or ketones, reduction of imines and enamides as well as resolution of racemic amines.⁷⁴ Chemical methods will not be thoroughly described here but they include resolution by diastereomeric

crystallization, addition of carbanions or radicals to imines, organocatalytic reactions applying chiral Brønsted acids or Lewis bases, organocatalytic Mannich reactions, transition-metal catalyzed hydrogenation, asymmetric reductive amination, hydroamination and C-H amination.^{71,74}

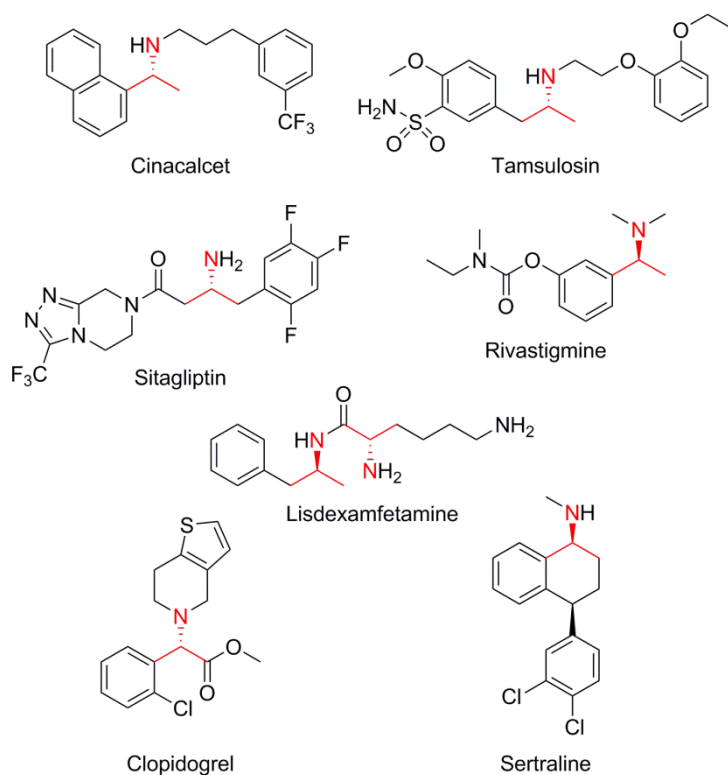


Figure 5. Examples of pharmaceutical compounds that consist of chiral amine moieties. The stereocenters and their substituents are marked in red.

Biocatalysis is growing in the field of chiral amine synthesis and several different synthesis routes and catalysts have been discovered and developed over the last two decades.⁷¹⁻⁷² This has already resulted in biocatalytic processes that can compete with the more established chemical methodologies.⁷¹⁻⁷² The discussion about biocatalytic examples of chiral amine synthesis will be divided with regards to starting material (amine, ketone, imine, alkane and alkene) and all examples are illustrated in Figure 7.

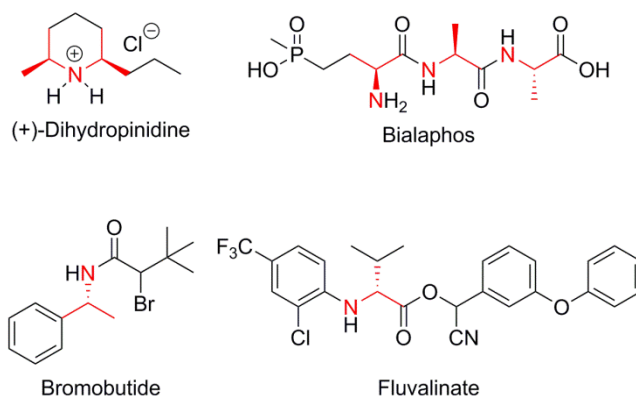


Figure 6. Examples of agrochemical compounds that consist of chiral amine moieties. The stereocenters and their substituents are marked in red.

An early example of biocatalytic synthesis of chiral amines is the kinetic resolution of racemic amines employing lipases⁸⁸⁻⁹⁰ where an (*R*)-selective lipase acylates the (*R*)-amine and leaves the enantiomerically pure (*S*)-amine unreacted. This process has been further developed into a dynamic kinetic resolution by the addition of amine racemizing metal catalysts⁹¹⁻⁹² enabling 100% theoretical yields. Another early example of kinetic resolution of amines is the use of amine transaminase (ATA) to turn the reactive enantiomer into the corresponding ketone and leave the enantiomerically pure non-reactive enantiomer.⁹³ Monoamine oxidase has been extensively investigated over the last decade for the synthesis of chiral amines.⁷¹ The engineered monoamine oxidases enantioselectively oxidize amines into their corresponding imines and in combination with a non-selective reduction agent racemic amines can be deracemized with a 100% theoretical yield.⁹⁴ Also using an amine as starting material, a recently investigated norcoclaurine synthase enantioselectively forms a C-C bond between a non-chiral amine and an aldehyde to make a chiral secondary amine.⁹⁵

The reaction catalyzed by ATAs is readily reversible and the catalyst can therefore also be applied in the asymmetric reductive amination of ketones using different amine donors.⁹⁶ This method using ketones as starting material has been very successful and several examples of active pharmaceutical ingredients being synthesized by ATAs have been reported.⁹⁷ A recent example utilizing another biocatalyst for the

asymmetric reductive amination of ketones is a set of amine dehydrogenases that were engineered from amino acid dehydrogenases.⁹⁸⁻⁹⁹ The benefit of this method is that these enzymes use ammonia as nitrogen source.

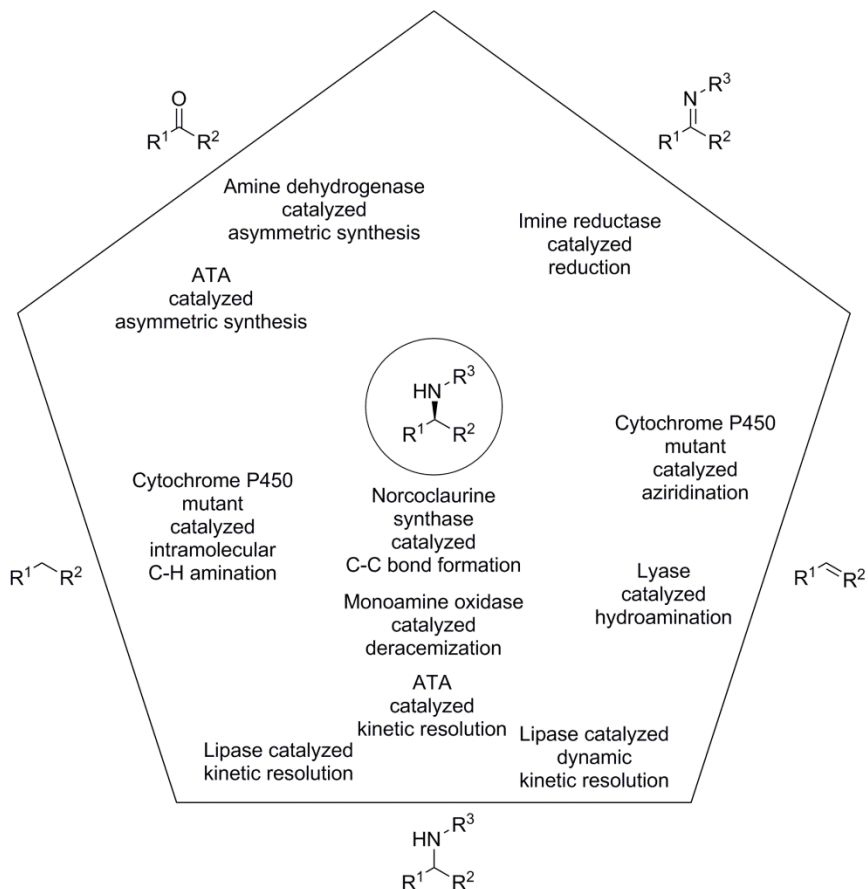


Figure 7. Illustration of different biocatalytic synthesis routes to chiral amines. The different enzymes are grouped regarding to their starting substrate.

A more recently discovered class of enzymes that will be important for the biocatalytic synthesis of secondary amines is the NADPH-dependent imine reductases that enantioselectively reduces imines to form the corresponding secondary amines.¹⁰⁰⁻¹⁰¹

Amines, ketones and imines have now been discussed as starting materials for the synthesis of chiral amines and they are all possibly cheap and readily available but there are other substrates that would be interesting as well. C-H amination of alkanes and hydroamination of alkenes are both highly desirable reactions. Cytochrome P450 was recently engineered to perform intramolecular C-H amination between an alkane and an azide functional group.¹⁰² Biocatalytic hydroamination of alkenes is available but so far only for the synthesis of amino acids and enzyme engineering has only extended the scope for amino acid and nucleophile specificity.⁷² Another recent example with alkenes as starting material is the engineering of cytochrome P450 to enable the aziridination of aromatic alkenes.¹⁰³

Due to their often excellent selectivities, enzymes will become increasingly important as catalysts in the synthesis of enantiomerically pure compounds. This thesis is about the use of ATAs for the synthesis of amines and the following chapter will therefore give a more detailed description of this important enzyme class.

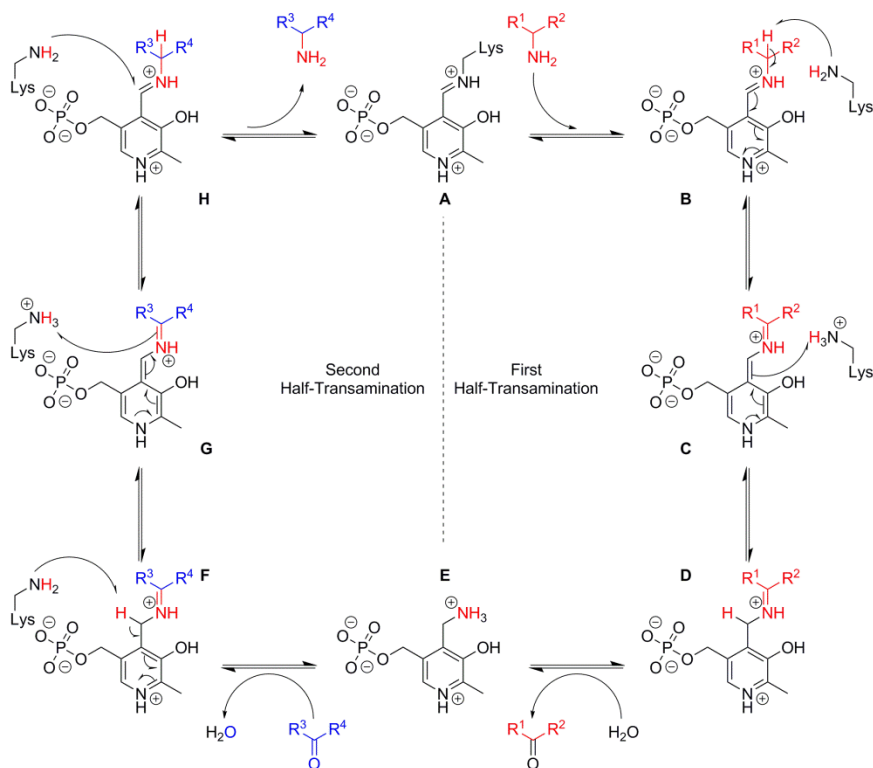
7. Amine transaminase

Amine transaminases (ATAs) have been extensively investigated during the last decade for their application in chiral amine synthesis.^{97,104-105} They are transferases (EC 2.6.1.18) that catalyze the transamination between an amino donor and an amino acceptor (Scheme 4) but they differ from α -transaminases and ω -transaminases in the sense that they are not restricted to substrates bearing a carboxylate group. ω -Transaminases catalyze transamination of amino acids where the carboxylate is located further away from the amino function than the α -position (which is the preferred position of α -transaminases). ATAs belong to a subgroup of ω -transaminases and are therefore commonly referred to as such. This can however be misleading as there are many examples of ω -transaminases that are inactive toward amine substrates not bearing the aforementioned carboxylic function.¹⁰⁶ Both naming conventions are used in the papers presented in this thesis but the enzymes will only be referred to as ATAs in the thesis for clarity. Theoretically any ketone or aldehyde can be subjected to transamination by ATAs and this makes them very interesting for application in synthetic chemistry.⁹⁷



Scheme 4. A general ATA catalyzed reaction where the amine function of the amino donor is transferred to the amino acceptor.

ATAs are dependent on the cofactor pyridoxal 5'-phosphate (PLP) that is one of the most versatile and prevalent cofactors available¹⁰⁶ and examples of reactions catalyzed by PLP-dependent enzymes include transamination, racemization, decarboxylation, elimination and substitution¹⁰⁷. The reaction mechanism employed by ATAs has been assumed¹⁰⁸ to be the same as in α -transaminases¹⁰⁹ and this assumption has been strengthened by the use of quantum chemical methods¹¹⁰.



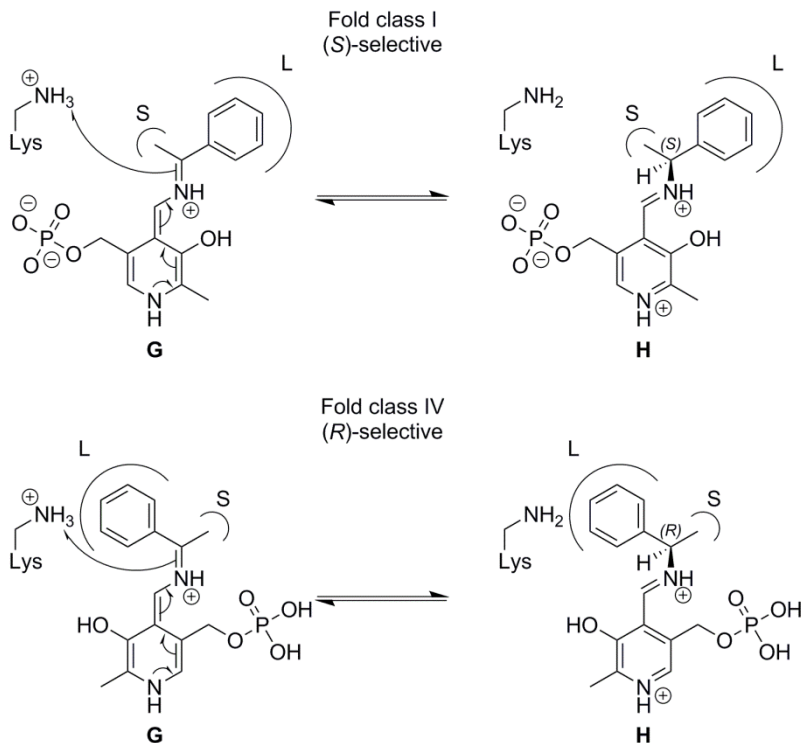
Scheme 5. Proposed ATA reaction mechanism. The amino donor and its corresponding carbonyl co-product are marked in red while the amino acceptor and the amine product are marked in blue. The atoms from the substrates keep their coloring throughout the mechanism for clarity.

The enzyme applies a ping-pong bi bi¹⁷ reaction mechanism which means that the first substrate (amino donor) enters the active site followed by the first product leaving. The second substrate (amino acceptor) then enters the active site and the second product finally leaves. A detailed ATA reaction mechanism consisting of two half-transaminations is described in scheme 5 and it starts with the resting state where a catalytic lysine residue binds to PLP with a Schiff-base linkage to form an internal aldimine (**A**). The amino donor now enters the active site and forms a Schiff-base linkage with PLP (external aldimine) (**B**), replacing the catalytic lysine. The next step is abstraction of the α -proton from the substrate. This is performed by the basic catalytic lysine with the aid of a water molecule.¹¹⁰ The proton abstraction leads to rearrangement of the

electrons in the system and a planar quinonoid (**C**) is formed. The electrons are redistributed again so that the previously abstracted proton now can be abstracted from the catalytic lysine to another carbon in **C** to form a ketimine (**D**). The first half-transamination can now be completed as **D** is hydrolyzed. The first product ketone or aldehyde leaves the active site and the aminated form of the cofactor, pyridoxamine-5'-phosphate (PMP) (**E**), is formed. The second half transamination is basically the reverse of the first half as the donated amino group gets incorporated into the amino acceptor substrate. The amino acceptor ketone or aldehyde enters the active site and gets attacked by the amino group in **E** to form a ketimine (**F**). This is followed by the reverse of the previously mentioned protonation/deprotonation sequence through a planar quinonoid (**G**) where the protonation introduces enantioselectivity as the proton only can be attached from one of the faces depending on the transaminase. The final external aldimine (**H**) gets attacked by the catalytic lysine and **A** is formed again as the final product amine is expelled from the active site.

There are two major fold classes of ATAs with opposite enantioselectivities within the fold classes of PLP-dependent enzymes. The (*S*)-selective ATAs belong to fold class I while the (*R*)-selective ATAs belong to fold class IV.¹¹¹ Even though these two fold classes are structurally very different, both with regards to tertiary- and active site structure, the basic mechanism of substrate binding/coordination and enantioselectivity works in a similar fashion. The Schiff-base bound cofactor PLP is surrounded by the amine transaminase active site which consists of two binding pockets. These two binding pockets usually differ in size and are hereby denoted as the small pocket (S pocket) and the large pocket (L pocket). The S pocket generally only accepts a methyl group substituent well while the L pocket accepts larger substituents like aromatic rings and longer aliphatic chains. This is the basis of how the two different ATA fold classes display opposite enantioselectivities^{38,112-113} and this is explained in scheme 6 by using the transformation of intermediate **G** to intermediate **H** in the ATA mechanism (Scheme 5) with the benchmark product 1-PEA as an example. The substrate is forced to bind in a certain conformation due to the sizes of its substituents and as the proton that is donated from the catalytic lysine only can come from one face of PLP (*si*-face for (*S*)-selective ATAs and *re*-face for (*R*)-selective ATAs¹¹⁴), only one enantiomer is formed. A rationally designed

mutation (W60C) in the L pocket of *Cv*-ATA with increased specificity for (*S*)-1-PEA is demonstrated in **Paper I**.



Scheme 6. Depiction of the binding pockets in both ATA fold classes. The reaction mechanism of both fold classes is the same but the different binding of substrates enables opposite enantioselectivity.

As mentioned in the previous chapter, the ATA reaction is readily reversible and there are therefore two possible modes of action to obtain chiral amines. ATAs can be used in the kinetic resolution of racemic amines as well as in the asymmetric synthesis of chiral amines from their corresponding ketones. As the theoretical yield of asymmetric synthesis is 100%, it is the preferred method. However, the equilibrium of transamination is usually (depending on the substrate) heavily shifted toward the ketone as it is thermodynamically favored. As an example, the equilibrium constant (K_{eq}) for the asymmetric synthesis of (*S*)-1-PEA

using acetophenone (30 mM) as amino acceptor and L-alanine (300 mM) as amino donor was determined to be 8.81×10^{-4} .^{96,115} Many innovative solutions to this issue have been reported and they include enzymatic cascades where the co-products are removed to shift the equilibrium. Examples include reduction or decarboxylation of pyruvate using lactate dehydrogenase^{96,116}, alanine dehydrogenase¹¹⁷ or pyruvate decarboxylase¹¹⁸ as well as alcohol dehydrogenase catalyzed reduction of acetone¹¹⁹. Non-enzymatic methods for equilibrium displacement include spontaneous chemical transformation of co-products¹²⁰⁻¹²⁴ (**Paper II**) and in situ evaporation of acetone^{13,125}. Application of acetolactate synthase for the removal of pyruvate in combination with in situ extraction of product amine has also been reported.¹²⁶ In the cases where the product amine is not the final desired product, equilibrium displacement by removing the product amine can be performed. Examples of this include spontaneous lactamization¹²⁷, imination⁸⁴ and enzyme catalyzed amidation¹²⁸ (**Paper III**).

As previously mentioned, ATAs have been extensively explored during the last decade and this intense research is continuing. Not already mentioned breakthroughs in ATA biocatalysis include structure investigations^{111,129-141}, immobilization¹⁴²⁻¹⁵⁵ as well as the development of kinetic assays and screening methods^{48,59,121,156-164} (**Paper IV**). The extensive research on ATAs has also led to successful examples of ATA catalyzed synthesis of several active pharmaceutical ingredients and building blocks thereof.⁹⁷ The industry clearly has a large interest for ATAs as a vital part of the biocatalytic toolbox for amine synthesis and more breakthroughs and industrial processes involving ATAs will probably be seen in the future.

8. Present investigation

8.1. Rational design for increased substrate specificity (Paper I)

Generally, the successful use of ATAs in a synthetic application requires enzyme engineering, both for increased stability and specificity for the substrate of interest. Previous work related to the present investigation includes the directed evolution of an amine transaminase from *Arthrobacter citreus* (*Ac*-ATA) which yielded a variant with increased stability and activity⁴⁸ as well as a study by Cho *et. al.*³⁸ where the L pocket of *Vibrio fluvialis* amine transaminase (*Vf*-ATA) was rationally engineered for increased activity toward aliphatic and aromatic amines. The best performing variant created by Cho *et. al.* was a substitution of tryptophan 57 to a glycine (W57G) and the corresponding position in *Ac*-ATA (tyrosine 60) was substituted by a cysteine (Y60C). In a study performed by our group⁴² homology models of *Vf*-ATA and *Ac*-ATA were structurally aligned to a homology model of *Cv*-ATA for the purpose of locating the position in *Cv*-ATA that corresponded to the previously mentioned amino acid substitutions. The corresponding position in *Cv*-ATA was found to be W60 and it was hypothesized that substitution of W60 to smaller residues would increase the enantioselectivity of *Cv*-ATA toward (*S*)-amines. Variants W60A and W60C were created and evaluated in a kinetic resolution between pyruvate and the three amines 1-PEA, 1-aminotetralin and 2-aminotetralin. W60A showed low cultivation yields and the activity toward the selected substrates was lower than for the wild-type enzyme. However, the W60C substitution resulted in drastically increased E-values for all three amines.

In the present investigation, *Cv*-ATA W60C was further investigated with regards to kinetics, pH dependence and PLP dependence. The specificity constants ($k_{\text{cat}}/K_{\text{M}}$) of (*S*)-1-PEA, the commonly applied amino donor isopropylamine (IPA) and a set of 4'-substituted acetophenones were determined. Any possible alterations of the reaction mechanism in the

variant were investigated by applying the Swain-Lupton equation on the 4'-substituted acetophenone specificity constants.

The pH dependence of *Cv*-ATA W60C was measured spectrophotometrically by following the asymmetric synthesis of (*S*)-1-PEA from acetophenone and IPA at different pH-values. The pH-optimum of *Cv*-ATA wild-type using the same assay has previously been determined to be 8.3.¹⁰⁸ *Cv*-ATA W60C however shows a pH-optimum of 7.0 (Figure 8).

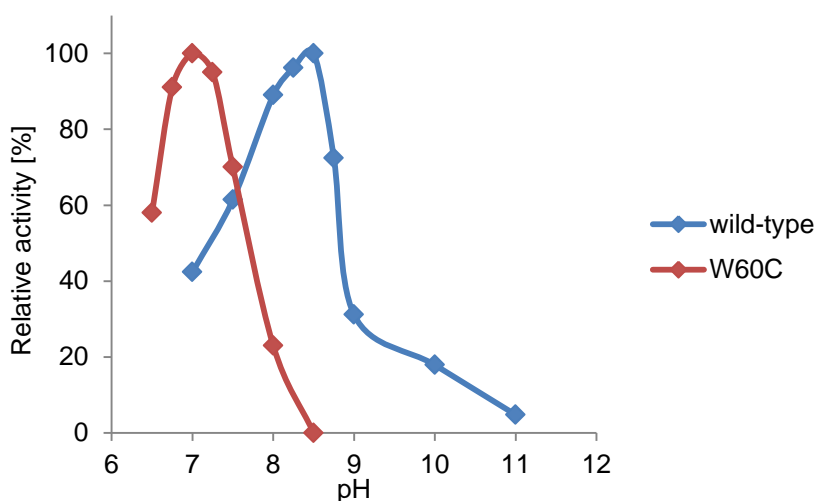


Figure 8. pH-dependence of asymmetric synthesis of (*S*)-1-PEA catalyzed by *Cv*-ATA wild-type (blue) and W60C (red).

Cv-ATA wild-type has previously been reported to be inhibited by an excess of PLP.¹⁰⁸ This means that the enzyme needs to be desalted after addition of PLP to remove any excess in order to reach full activity. W60C, however, behaved completely different as it needed approximately 200% excess of PLP to display full activity and when the enzyme/PLP ratio was 1:1, only 20% activity was achieved (Figure 9).

By using a previously published active site quantification method for ATAs¹⁰⁸ we were able to determine proper rate constants, which is necessary for comparing enzyme variants. The method is based on the absorbance of the enzyme bound PLP which has a maximum at 395 nm.

However, after incubation with PLP followed by desalting to avoid any free PLP, *Cv*-ATA W60C displayed an absorbance maximum at 407 nm. This might indicate altered binding of PLP in the variant W60C. The active site quantification of *Cv*-ATA W60C was therefore modified to be measured at 407 nm.

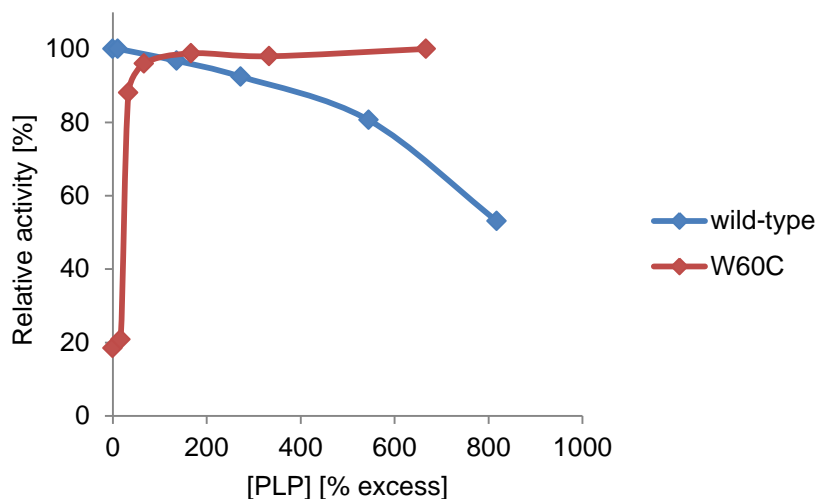


Figure 9. Influence of excess PLP on the asymmetric synthesis of (*S*)-1-PEA catalyzed by *Cv*-ATA wild-type (blue) and W60C (red).

The enzyme kinetics of *Cv*-ATA wild-type and W60C was investigated with regards to the commonly used amine substrates (*S*)-1-PEA and IPA. As previously mentioned, ATAs perform catalysis through a ping-pong bi-bi reaction mechanism. This means that equation 4 has to be applied. In this equation, E stands for enzyme while A and B stand for amino donor and pyruvate, respectively. By keeping one substrate (B) at a constant concentration, pseudo one-substrate kinetics can be applied and equation 4 can be rewritten for simplicity:

$$v = \frac{k_{\text{cat}}[E][A]}{K_M^A + [A] \left(1 + \frac{K_M^B}{[B]} \right)} \quad (\text{Equation 10})$$

Since the concentration of B is being held constant, the bracket in equation 10 can be treated as a constant. By dividing equation 10 with this constant, equation 11 arises:

$$v = \frac{\frac{k_{\text{cat}}[E][A]}{\left(1 + \frac{K_M^B}{[B]}\right)}}{\frac{K_M^A}{\left(1 + \frac{K_M^B}{[B]}\right) + [A]}} \quad (\text{Equation 11})$$

If the measurements are performed at low concentrations of A, [A] in the denominator of equation 11 can be ignored. This finally leads to equation 12:

$$v = \frac{k_{\text{cat}}}{K_M^A} [E][A] \quad (\text{Equation 12})$$

By studying the reaction rates at low substrate concentrations the specificity constant can now easily be deduced by plotting reaction rate against substrate concentration. This was done for (S)-1-PEA and IPA using pyruvate as amino acceptor (Table 1) and the reaction rates were either measured using the acetophenone assay¹⁵⁹ in the case of (S)-1-PEA or by end-point measurement of formed alanine with ninhydrin at different time points in the case of IPA (this was only possible due to the fact that IPA is one of few amines that does not form a colored complex with ninhydrin¹⁶⁵). Proper substrate concentrations of IPA were decided by adding it to the reactions with (S)-1-PEA and treating it as a competitive inhibitor. K_I for IPA was determined and since IPA was competing with (S)-1-PEA, K_I equals K_M for IPA.

As can be seen in table 1, the specificity for (S)-1-PEA is increased 29 times in the variant W60C compared to wild-type while the specificity for IPA is decreased 8 times. Furthermore, relative specificity constants for a set of 4'-substituted acetophenones were investigated (Table 2) by employing several ketones simultaneously and thereby obtaining their relative specificity constants in relation to spectrophotometrically determined specificity constants for the unsubstituted acetophenone. The reactions were run with IPA as amino donor and relative rates were recorded by following the reactions on HPLC.

Table 1. Experimentally determined kinetic constants for Cv-ATA wild-type and W60C toward substrates (S)-1-PEA and IPA using pyruvate as amino acceptor.

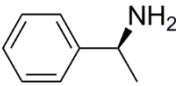
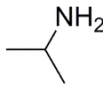
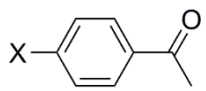
				
	k_{cat}/K_M [s ⁻¹ mM ⁻¹]	k_{cat}/K_M [s ⁻¹ mM ⁻¹]	K_M [mM]	k_{cat} [s ⁻¹]
Cv-ATA wild-type	0.68	0.20±0.004	73	15±0.3
Cv-ATA W60C	20	0.024±0.002	190	4.6±0.4

Table 2. Specificity constants for Cv-ATA wild-type and W60C toward a set of 4'-substituted acetophenones.

	k_{cat}/K_M [s ⁻¹ mM ⁻¹]	
X:	Cv-ATA wild-type	Cv-ATA W60C
NO ₂ -	0.030	0.16
Cl-	0.029	n.d. ^b
Br-	0.028	0.039
CN-	0.026	0.10
F-	0.020	n.d. ^b
H-	0.0082^a	0.034^a
Me-	0.0050	0.024
OH-	0.0024	0.013
MeO-	n.d. ^b	0.011

^a Experimentally determined values that were subsequently used to obtain the relative values of all other substituted acetophenones. ^b Not determined, no observed reaction.

The relative specificity constants in table 2 were obtained from the relative rates of the 4'-substituted acetophenones by applying equation 13 and equation 14 for the two competing substrates ketone C and ketone D assuming pseudo one-substrate conditions for every individual ketone substrate.¹⁶⁶

$$v_C = \frac{k_{\text{cat}}^C [E] K_M^D [C]}{K_M^D [C] + K_M^C [D] + K_M^C K_M^D} \quad (\text{Equation 13})$$

$$v_D = \frac{k_{\text{cat}}^D [E] K_M^C [D]}{K_M^C [D] + K_M^D [C] + K_M^D K_M^C} \quad (\text{Equation 14})$$

By employing both ketones at the same concentration, division of equation 13 with equation 14 yields equation 15 that enables calculation of each individual 4'-substituted acetophenone's specificity constant from the relative rates and the previously determined specificity constant of the non-substituted species.

$$\frac{v_C}{v_D} = \frac{\left(\frac{k_{\text{cat}}}{K_M}\right)^C}{\left(\frac{k_{\text{cat}}}{K_M}\right)^D} \quad (\text{Equation 15})$$

By looking at table 2 and the pattern of which substituents have a positive effect on the specific activity and vice versa, one can see that electron withdrawing substituents have a positive impact while electron donating substituents have a negative impact on the wild-type. The reason for this could be that the electron withdrawing substituents make the carbonyl carbon more electrophilic, thereby improving the nucleophilic attack from PMP (Scheme 5, intermediate **E** to **F**). However, this step is not rate determining¹¹⁰ and the differences in electrophilicity will not make a major contribution to the reaction rate. The highest energy barrier to climb in the second half-transamination is instead in the following step (Scheme 5, intermediate **F** to **G**)¹¹⁰ where an electron withdrawing substituent would make the deprotonation more favorable. This could explain the higher specific activities for acetophenones with electron withdrawing substituents in the wild-type. In the variant W60C the situation is however different. The specific activity toward all substituents were approximately equally increased except for the halides where it was decreased for the chloro and fluoro substituent and only slightly increased for the bromo substituent (Table 2). The reason for this was not further investigated.

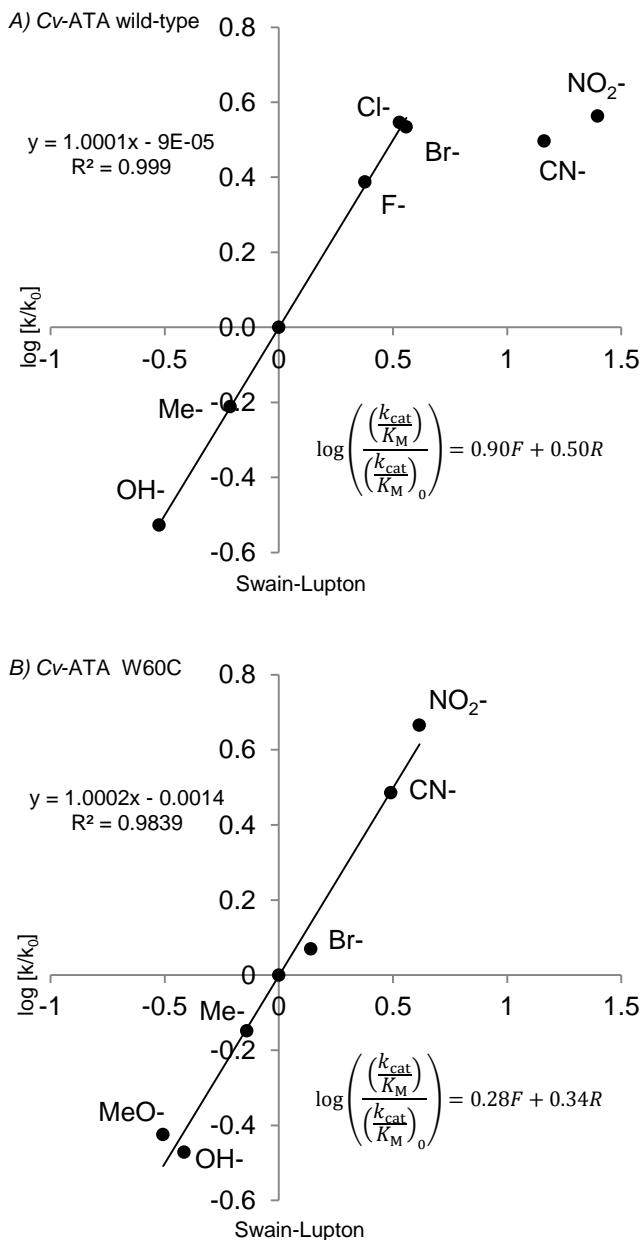


Figure 10. Swain-Lupton plots for Cv-ATA wild-type (A) and W60C (B) toward a set of 4'-substituted acetophenones based on the specificity constants listed in table 2.

As previously mentioned, it was hypothesized that the variant W60C binds PLP differently compared to the wild-type enzyme. This could result in differences in the reaction mechanism and the previously determined specificity constants for a set of 4'-substituted acetophenones were therefore used to construct Swain-Lupton plots (Figure 10) in order to investigate differences in field and resonance contribution between wild-type and W60C. This was done by treating the specificity constants as first-order rate constants and performing least-square fitting of the Swain-Lupton equation (Equation 7)

As can be seen in figure 10, there are two outliers (NO₂ and CN) in the wild-type Swain-Lupton plot. These two were omitted (but remained in the figure for clarity) from the least-square fitting to achieve higher accuracy in the calculation. The variant W60C did however not show the same behavior as all substituents showed linearity and could be included in the Swain-Lupton plot. This is probably due to the increased size of the L pocket which means that these large substituents can be properly accommodated by the active site. The calculated field (*f*) and resonance (*r*) coefficients (Figure 10) show that the resonance contribution in W60C has increased from 36% (wild-type) to 55%.

In conclusion, Cv-ATA shows increased specificity for (*S*)-1-PEA as well as decreased specificity for IPA. It also shows a generally increased specificity for most of the investigated 4'-substituted acetophenones which means that W60C should be a better catalyst for the asymmetric synthesis of these compounds (however not with IPA as amino donor). In addition, the variant is more practical in the sense that it tolerates an excess of PLP which means that the enzyme does not need to be desalted prior to use. The increased resonance contribution of the 4'-substitutions in combination with major changes in pH- and PLP-dependence suggests an altered reaction mechanism. This hypothesized change in the reaction mechanism has not been further investigated but the changed pH-optimum might suggest that the introduced cysteine residue actively participates in catalysis. The change in PLP-dependence and the change in absorbance maximum for the enzyme-PLP complex suggest altered binding of PLP and the cysteine might be involved in this as well.

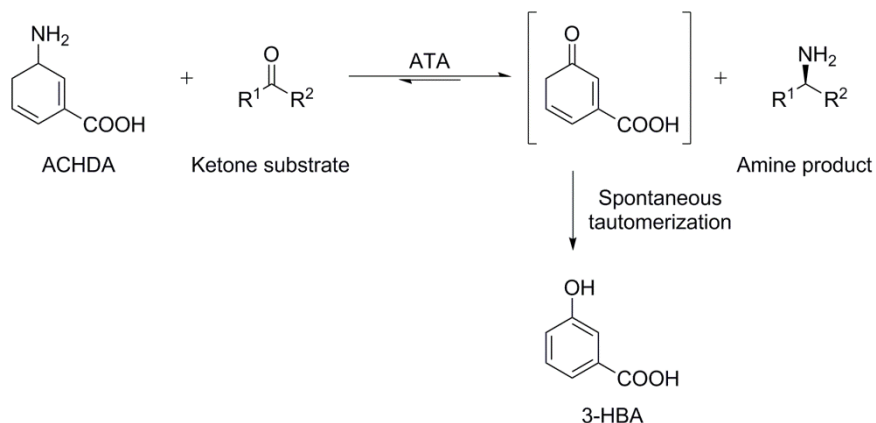
8.2. Equilibrium displacement of amine transaminase catalyzed asymmetric synthesis (Paper II & Paper III)

One common issue with ATA catalyzed asymmetric synthesis is the previously discussed unfavorable equilibrium between the ketone/aldehyde substrates and amine products. One solution is of course to choose an amino donor which has an even more displaced equilibrium toward its corresponding product¹⁶⁷ but this is not always practical. Methods to displace this unfavorable equilibrium to achieve high conversions to the desired product have been thoroughly investigated as discussed in chapter 7. This section will describe two projects on the subject of equilibrium displacement. In **Paper II**, the equilibrium is displaced by using an amino donor whose corresponding ketone product spontaneously tautomerizes into its corresponding alcohol. When the amine is not the final desired product it can be desirable to introduce equilibrium displacement by removing the product amine from the reaction mixture. This is done in **Paper III** where the transamination is followed by amidation. If an amide is the desired product, this solves both the issue of transamination equilibrium displacement as well as the amide synthesis in a one-pot one-step fashion.

8.2.1. Chemoenzymatic cascade for equilibrium displacement (Paper II)

As previously mentioned, there has been a lot of focus on developing methods for ATA equilibrium displacement. Before this study, the main focus was enzymatic conversion of the co-product^{96,116-119,126}. However, there are some drawbacks with these multi-enzymatic systems. Enzymes usually have a narrow range of conditions where they display optimal activity and when several enzymes are used in one-pot the reaction conditions have to be compromised to fit all enzymes. In addition, most of the published methods for enzymatic equilibrium displacement use more than one extra enzyme in order to recycle expensive co-factors. This means that the enzymes are not displaying full activity which might lead to higher enzyme loading. We thereby developed an equilibrium displacement cascade where the second step is purely chemical and works at a larger range of conditions. This means that the ATA can perform catalysis in optimal conditions. The developed chemoenzymatic cascade includes application of the amino donor 3-aminocyclohexa-1,5-

dienecarboxylic acid (ACHDA) together with a ketone and an ATA. ACHDA is converted into its corresponding ketone which spontaneously tautomerizes to form 3-hydroxybenzoic acid (3-HBA) (Scheme 7). This effectively displaces the equilibrium by removing the product ketone and increases the conversion of ATA-catalyzed asymmetric synthesis.



Scheme 7. ATA equilibrium displacement by employment of ACHDA as amino donor. The formed co-product ketone spontaneously tautomerizes into 3-HBA, effectively displacing the equilibrium toward the product amine.

An initial test of the developed system was performed by running a reaction between ACHDA (0-40 mM) and acetophenone (5 mM) catalyzed by *Cu*-ATA W60C. The data showed that even when an equivalent amount of ACHDA (5 mM) was applied, 98% conversion to (*S*)-1-PEA was achieved while 1.5 equivalents of ACHDA and above resulted in >99% conversion (Figure 11a).

Inhibition caused by the final co-product 3-HBA was investigated by measuring the initial reaction rate of the above mentioned reaction in the presence of an increasing concentration of 3-HBA (Figure 11b). No significant inhibition took place in concentrations up to 15 mM and after that the activity declined down to approximately 40% at 25 mM 3-HBA. This means that at higher substrate concentrations than the ones used in this paper, product inhibition might be an issue.

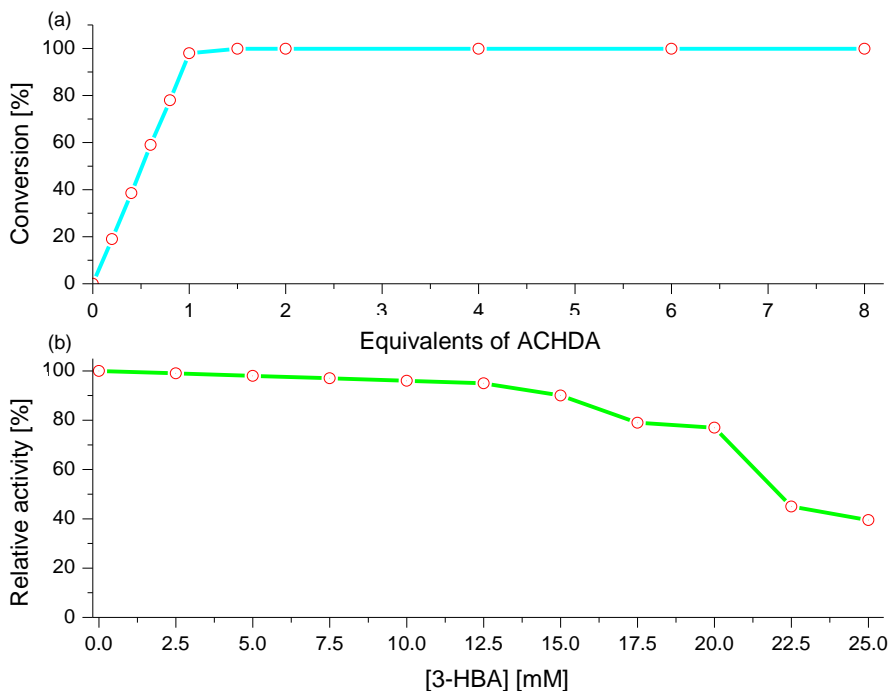



Figure 11. (a) *Cv*-ATA W60C catalyzed asymmetric synthesis of (*S*)-1-PEA from acetophenone and varied amounts of ACHDA. (b) Inhibition of *Cv*-ATA W60C caused by the co-product 3-HBA.

The tautomerization of the co-product ketone into 3-HBA is virtually irreversible due to the fact that 3-HBA is aromatic while the ketone is not. However, in theory the tautomerization is reversible and a test of the reversibility of the reaction was conducted by running a reaction with *Cv*-ATA W60C with (*S*)-1-PEA and 3-HBA. Results showed that even after 36 hours of reaction, no reaction had occurred as no acetophenone was detected.

A final test of the system was performed to evaluate the acceptance of ACHDA by other enzymes than the already tested one. Other variants of *Cv*-ATA (wild-type and F88A/A231F⁴²) as well as commercial ATA-113 ((*S*)-selective), ATA-117 ((*R*)-selective) and ω -TA-001 ((*S*)-selective) were tested with the standard reaction including acetophenone and ACHDA (Table 3, entries 1-8). The amino donor ACHDA was applied at 1 and 1.05

equivalents and the latter was enough to achieve >99% conversion. This amino donor concentration was therefore used in all following reactions.

Table 3. Asymmetric synthesis of a set of 4'-substituted 1-phenylethylamines from their corresponding acetophenones and ACHDA catalyzed by ATA.



Entry	ATA	R	[ACHDA] [equiv.]	ee [%]	Conv. [%]
1	Cv-ATA W60C		1	n.d. ^a	98
2	Cv-ATA W60C		1.05	>99 (S)	>99
3	Cv-ATA wild-type		1	n.d. ^a	97
4	Cv-ATA wild-type	H-	1.05	>99 (S)	>99
5	Cv-ATA F88A/A231F	H-	1.05	>99 (S)	>99
6	ATA-113		1.05	>99 (S)	>99
7	ATA-117		1.05	>99 (R)	>99
8	ω-TA-001		1.05	62 (S)	>99
9	Cv-ATA wild-type	Cl-	1.05	>99 (S)	>99
10	Cv-ATA W60C	Cl-	1.05	>99 (S)	>99
11	Cv-ATA wild-type	Me-	1.05	26 (S)	>99
12	Cv-ATA W60C	Me-	1.05	41 (S)	98
13	Cv-ATA wild-type	MeO-	1.05	>99 (S)	>99
14	Cv-ATA W60C	MeO-	1.05	>99 (S)	>99
15	Cv-ATA wild-type	O ₂ N-	1.05	>99 (S)	>99
16	Cv-ATA W60C	O ₂ N-	1.05	>99 (S)	>99

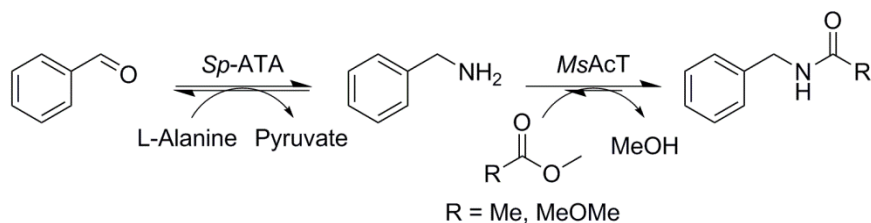
^a Not determined.

After the lowest concentration of ACHDA to achieve full conversion of acetophenone was determined, asymmetric synthesis of a set of 4'-substituted acetophenones applying the developed system was performed (Table 3, entries 9-16). Cv-ATA wild-type and W60C were employed as catalysts with excellent conversions toward all the tested substrates.

In conclusion, an efficient method for equilibrium displacement of the thermodynamically challenging asymmetric synthesis of chiral amines from ketones has been developed. The amino donor ACHDA was successfully applied in the asymmetric synthesis of a set of chiral amines with excellent conversions using only a slight excess (1.05 equiv.). The developed method is with regards to simplicity superior to previously published methods for ATA equilibrium displacements as no additional enzymes are needed. In addition, the reported product inhibition by 3-HBA was only investigated for *Cv*-ATA W60C and it might be different for other ATAs. This was the first study utilizing this kind of “smart” substrate for ATAs and even though drawbacks include high cost and low availability of the amino donor, it was an important proof of concept that has inspired several publications on the same theme.¹²¹⁻¹²⁴

8.2.2. Enzymatic cascade for equilibrium displacement (Paper III)

In **Paper III**, focus was shifted from co-product removal for equilibrium displacement to removal of the product amine. The employed strategy involves an amidation step where the formed amine is transformed to its corresponding amide. Several synthetic cascades involving ATAs have been published^{97,168} but cascades where the product amine is amidated are rare¹⁶⁹. An ATA/acyl transferase cascade for the aqueous formation of amides (Scheme 8) is hereby described. It is also the first reported enzyme cascade combining an ATA and a hydrolase in a one-pot one-step fashion.



Scheme 8. ATA/acyl transferase cascade for the transformation of benzaldehyde into its corresponding amide using either methyl acetate or methyl methoxyacetate as acyl donor.

Hydrolases that can perform trans-acylations in aqueous solutions are rare¹⁷⁰⁻¹⁷⁴ and the acyl transferase from *Mycobacterium smegmatis* (*MsAcT*) is one of the few hydrolases that can¹⁷⁵⁻¹⁷⁶. *MsAcT* have previously been shown to catalyze trans-acylations of alcohols to their

corresponding esters in aqueous solution and we therefore hypothesized that it could also perform amidation under similar conditions. The higher nucleophilicity of amines compared to alcohols in combination with formation of a highly stable and thermodynamically favored amide bond means that the proposed cascade could be an effective method for ATA equilibrium displacement.

The main challenge of combining an ATA and a hydrolase in a one-pot one-step cascade is to avoid competition between the enzymes. The amino donor of the transamination reaction can in theory also be used as a nucleophile by the hydrolase which means that the amino donor needs to be readily accepted by the ATA while not being accepted by the hydrolase to avoid unwanted side products. *MsAcT* does not accept amino acids as substrates (unpublished results) which make it well suited for the proposed cascade as alanine is very well accepted by most ATAs.

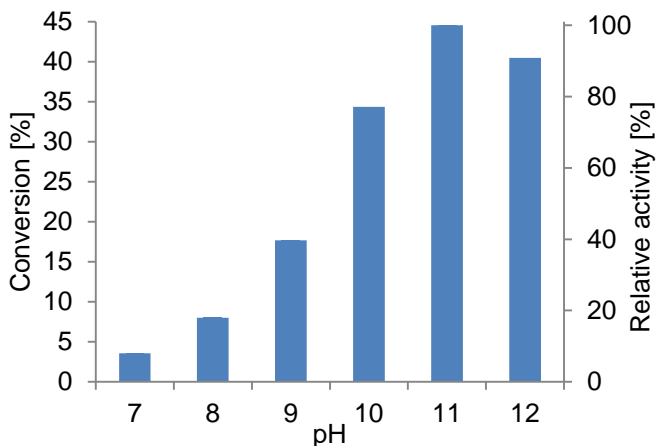


Figure 12. pH-profile for the *MsAcT* catalyzed amidation of benzylamine using methyl acetate as acyl donor. Conversions were determined after 5 min.

Due to the large amount of available ATAs with different optimal conditions the optimization with regards to pH was focused on *MsAcT*. A model reaction using benzylamine and methyl acetate as substrates was designed (Scheme 8, *MsAcT* catalyzed step) and applied at different pH-values to obtain the optimal pH for *MsAcT* catalyzed amidation. Conversion to the product *N*-benzylacetamide was measured after a set amount of time and the optimal pH was found to be around 11 (Figure

12). As no available ATA had such a high pH-optimum, the ATA from *Silicibacter pomeroyi* (*Sp*-ATA) with a pH-optimum of approximately 9.5¹³² was chosen to catalyze the first reaction step and the pH of the cascade was set to 10 as a compromise.

Using the previously determined pH, the *MsAcT* catalyzed amidation of benzylamine could now be followed over time with the purpose of determining how far the reaction could proceed. The reaction between benzylamine (20 mM) and either methyl acetate (1% vol vol⁻¹) or methyl methoxyacetate (1% vol vol⁻¹) reached conversions of 86% in 90 min and 93% in 270 min respectively (Figure 13). Methyl methoxyacetate is a commonly applied acyl donor in biocatalytic amidation.^{90,177-178} The advantage of this acyl donor is the ability of the methoxy oxygen to form a hydrogen bond with the amine hydrogen of the substrate in the transition state.¹⁷⁹ Even though *MsAcT* can catalyze trans-acylations in water, hydrolysis still occur to some extent and methyl methoxyacetate makes the nucleophilic amine a better competitor against water, thereby the higher conversion.

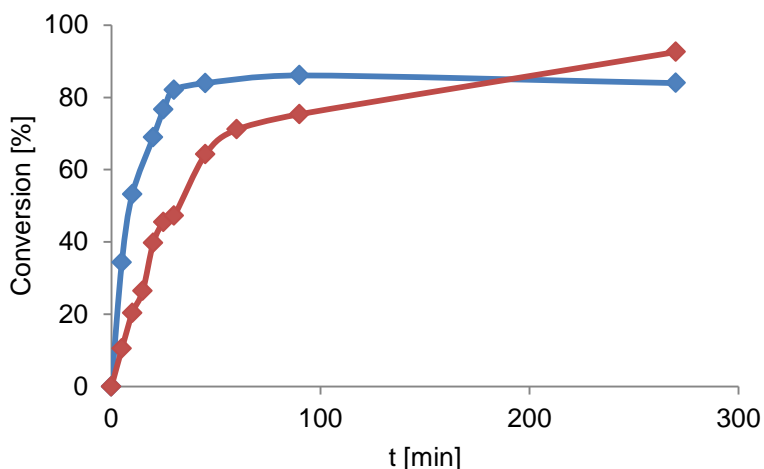


Figure 13. Reaction profile for the reaction between benzylamine and the acyl donors methyl acetate (blue) and methyl methoxyacetate (red) catalyzed by *MsAcT*.

The complete cascade was now optimized by varying the concentrations of both enzymes simultaneously. It was hypothesized that the ATA/acyl transferase ratio is important since too much *MsAcT* will hydrolyze the

acyl donor before enough amine is formed to compete with water. The reaction system depicted in scheme 8 with benzaldehyde, L-alanine and either methyl acetate or methyl methoxyacetate as substrates was employed with varying enzyme concentrations (Figure 14).

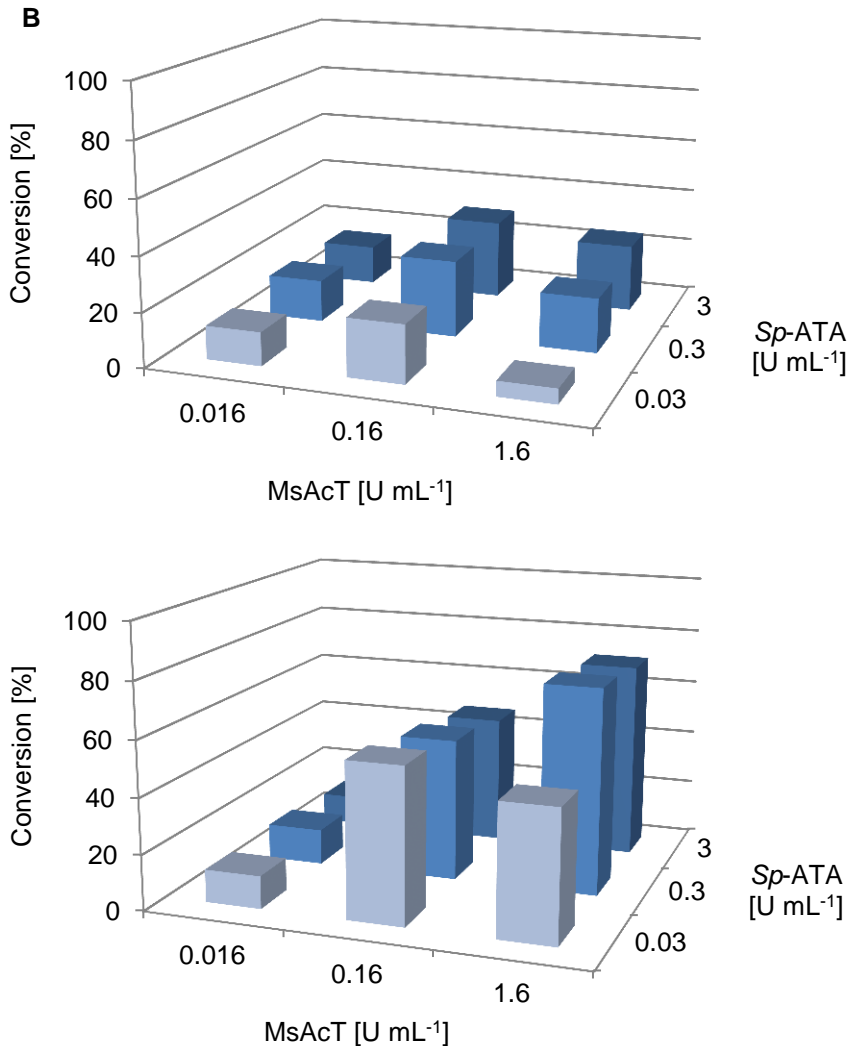


Figure 14. Results from optimization of *MsAcT* and *Sp-ATA* concentrations using methyl acetate (A) or methyl methoxyacetate (B) as acyl donor. Conversions were determined after 18h.

Results indicate that methyl methoxyacetate is the superior acyl donor of the two tested ones in this system. While the highest conversion with methyl acetate was 29%, the highest conversions with methyl methoxyacetate were 74% (1.6 U mL⁻¹ *MsAcT* and 0.3 U mL⁻¹ *Sp-ATA*) and 71% (1.6 U mL⁻¹ *MsAcT* and 3 U mL⁻¹ *Sp-ATA*) (Figure 14). The ability of methyl methoxyacetate to make the amine a better competitor against water is probably the key factor to the performance of methyl methoxyacetate compared to methyl acetate. When the amidation reaction was employed without the transamination step, conversions using methyl acetate and methyl methoxyacetate were similar to each other (Figure 13) but that was at the maximum possible concentration of amine. In the cascade the amine is continuously produced by the transamination reaction and the concentration is therefore lower which means that methyl methoxyacetate can resist hydrolysis at lower amine concentrations than methyl acetate can.

By looking at the conversion patterns in figure 14, one can conclude that the highest conversion using methyl acetate is at a lower concentration of *MsAcT* than in the case of methyl methoxyacetate. This strengthens the hypothesis that methyl methoxyacetate makes the amine a better competitor against water since that system can reach higher conversions even when the rate of competing hydrolysis is higher.

After the optimization of enzyme concentrations methyl acetate was excluded from the following experiments and methyl methoxyacetate was hereby used in all reactions. The cascade was further optimized by increasing the concentration of methyl methoxyacetate. However, the tolerance of *Sp-ATA* against methyl methoxyacetate was first investigated by measuring activity with increasing concentrations of methyl methoxyacetate (Figure 15).

Sp-ATA was heavily inhibited by increasing concentrations of methyl methoxyacetate and the only higher concentration tested in the cascade was therefore 2% vol vol⁻¹. Due to the decreased *Sp-ATA* activity when the concentration of methyl methoxyacetate is increased, the higher concentration of *Sp-ATA* (3 U mL⁻¹) was used even though it was only the second best cascade condition (Figure 14). The concentration of buffer also had to be increased to correct for the increased amount of acid formed from hydrolysis of the acyl donor. When 2% vol vol⁻¹ methyl

methoxyacetate was applied in the cascade, conversion increased from 84% to 97% (Table 4, entries 2 and 3). Interestingly, increasing the buffer concentration also increased conversion using only 1% methyl methoxyacetate from 71% to 84% (Table 4, entries 1 and 2). This is probably due to the fact that the enzymes are allowed to operate at a higher pH for a longer time and it shows that pH control is very important in the proposed cascade.

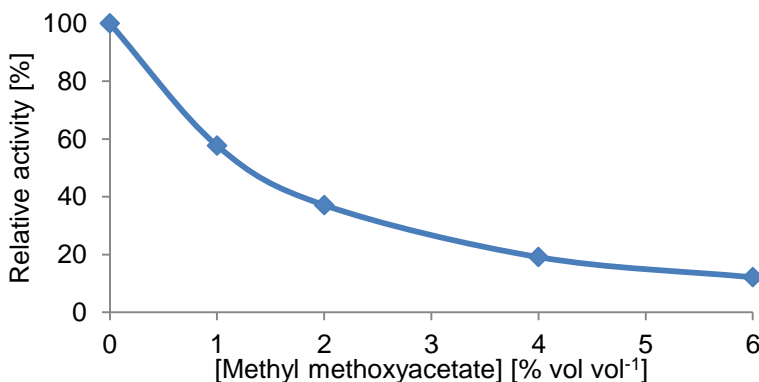


Figure 15. *Sp*-ATA tolerance toward increasing concentrations of methyl methoxyacetate.

As the cascade conditions had been optimized for the synthesis of *N*-benzyl-2-methoxyacetamide, a set of aromatic and aliphatic substrates were now tested. The synthesis of *N*-phenylethyl-2-methoxyacetamide, *N*-butyl-2-methoxyacetamide and *N*-heptyl-2-methoxyacetamide were performed and as can be seen in Table 4 (entries 5-7), conversion are lower than for the synthesis of *N*-benzyl-2-methoxyacetamide (Table 4, entry 3). This is however not that surprising as both enzymes do not have the same relative activity against the substrates and to optimize the synthesis of a certain amide, the enzyme concentration ratio will have to be optimized for that particular case.

It was previously hypothesized that the amidation step of the cascade would displace the equilibrium of the transamination step. This was shown by running the first step of the cascade without addition of *MsAct* or methyl methoxyacetate with benzaldehyde as a substrate and this yielded a conversion of 40% to benzylamine. By comparing this to the conversion of the complete cascade using the same conditions (Table 4,

entry 3) it is clear that the amidation by *MsAcT* readily shifts the equilibrium of the ATA catalyzed reaction.

Table 4. Results of the continued cascade optimization after optimal enzyme concentrations and choice of acyl donor had been established.

Entry	R	[Methyl methoxyacetate] [% vol vol ⁻¹]	[Buffer] [M]	Conv. [%]
1 ^a		1	0.2	71
2		1	0.4	84
3		2	0.4	97
4 ^b		2	0.4	92
5		2	0.4	59
6		2	0.4	31
7		2	0.4	55

^a Previous condition (Figure 14) that was chosen for continuation. ^b Preparative synthesis.

The ATA/acyl transferase cascade was further evaluated toward the synthesis of enantiomerically pure chiral amides. The enantioselectivity and substrate scope of ATAs is well documented and the initial evaluation was therefore focused on *MsAcT*. The chiral substrate analog of benzylamine, 1-PEA, was first tested as a substrate for *MsAcT* catalyzed amidation. *MsAcT* was however completely inactive toward this substrate and focus was shifted toward aliphatic chiral amines as *MsAcT* previously has been reported to catalyze esterification of 2-octanol¹⁷⁶. *MsAcT* showed activity toward 2-aminohexane as it was able to convert both (*S*)-2-aminohexane and (*R*)-2-aminohexane into their corresponding methoxyacetamides at 18% and 5% conversion respectively (Figure 16).

Conversions and reaction rates were lower than for benzylamine and the fact that both enantiomers were converted indicates a low enantioselectivity which is consistent with the enantioselectivity toward 2-octanol¹⁷⁶. In fact, when the racemic 2-aminoheptane was employed in *MsAcT* catalyzed kinetic resolution a conversion of 13% was reached with an *ee* of the product amide of 56% (*S*). As *Sp*-ATA is an (*S*)-selective ATA¹³², synthesis of (*S*)-*N*-(hexan-2-yl)-2-methoxyacetamide was investigated by the full cascade but no product was detected.

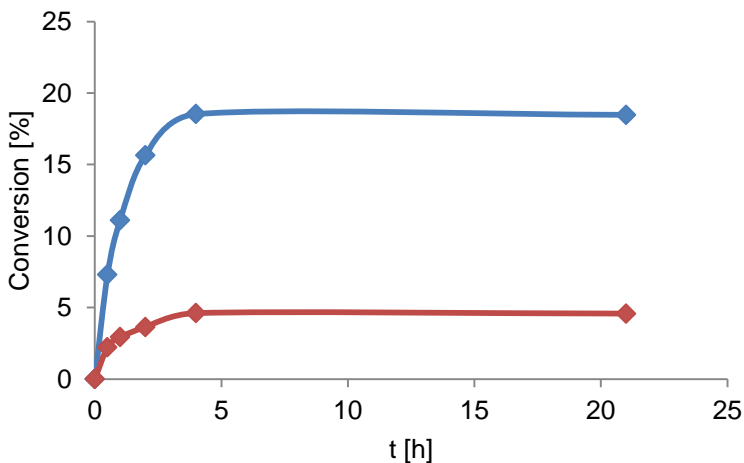


Figure 16. Reaction profile for the *MsAcT* catalyzed amidation of (*S*)-2-aminoheptane (blue) and (*R*)-2-aminoheptane.

To show the applicability of the ATA/acyl transferase cascade, a preparative synthesis of *N*-benzyl-2-methoxyacetamide at a 100 mg scale was performed. After 24 h of reaction, a conversion of 92% (Table 4, entry 4) and 75% isolated yield (134 mg) was achieved using the optimized reaction conditions (2% vol vol⁻¹ methyl methoxyacetate, 0.5 M L-alanine, 3 U mL⁻¹ *Sp*-ATA and 1.6 U mL⁻¹ *MsAcT*).

In conclusion, a successful ATA/acyl transferase cascade for the synthesis of amides from aldehydes in aqueous solution has been developed. It works well for aldehydes and it is believed that optimization of enzyme concentrations can yield high conversion for a large set of aldehydes. When *MsAcT* activity toward a certain amine is low, it might also be desirable to increase the acyl donor concentration. This will however lead

to more acid being formed and when a certain acyl donor concentration is reached the use of buffer to control pH might not be sufficient. Continuous pH correction will then be needed by the use of a pH stat or similar equipment. Increased acyl donor concentrations will also lead to higher solubility of hydrophobic substrates.

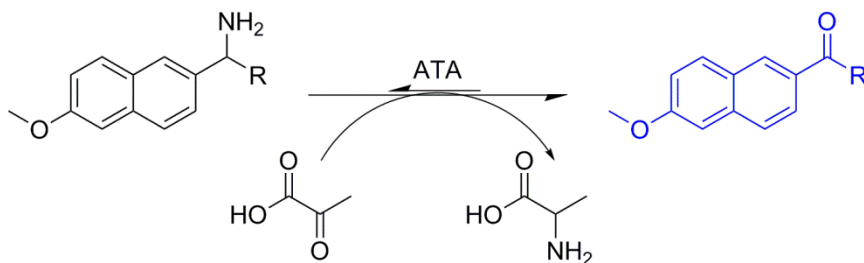
A drawback of this system is the inability of the ATA/acyl transferase cascade to synthesize chiral amides. This was however not fully investigated and in the case of 2-hexanone an ATA with higher activity toward aliphatic ketones and a higher tolerance toward methyl methoxyacetate could probably be used to obtain a decent conversion. *Sp*-ATA has previously been shown to display low activity toward 2-hexanone¹³² and by investigating the use of more ATAs in the cascade a larger spectrum of products could probably be synthesized. The main reason for this drawback is however the low activity of *MsAcT* toward chiral primary amines. The low enantioselectivity is not necessarily an issue as enantioselectivity is introduced in the first step of the cascade by the highly enantioselective ATAs. This means that if an amidation catalyst with low enantioselectivity is used, both (*S*)- and (*R*)-amides can be synthesized depending on which ATA is employed. If an ATA/acyl transferase cascade is to be successful in the synthesis of chiral amides, *MsAcT* either has to be engineered for the desired activity or other acyl transferases with activity toward chiral primary amines has to be obtained.

8.3. Fluorescence-based assay for screening of amine transaminases (Paper IV)

The discovery and engineering of ATAs require efficient, sensitive and reliable kinetic assays and high-throughput screening methods. As previously mentioned, many methods for this purpose have been developed with the most prominent one being the acetophenone assay¹⁵⁹. It is based on measurement of the strong UV-absorbance of acetophenone that is formed from 1-PEA and it is widely used due to its simplicity and generality. However, previous to this study, no fluorescence-based ATA assays were available. The sensitivity of a method based on fluorescence emission is expected to be higher than one based on UV-absorbance and we therefore developed an assay based on the strong blue emission of 6-methoxy-2-acetonaphthone and derivatives thereof. A general synthesis

route for screening substrates that enable screening of ATAs with extended substrate scope was also developed.

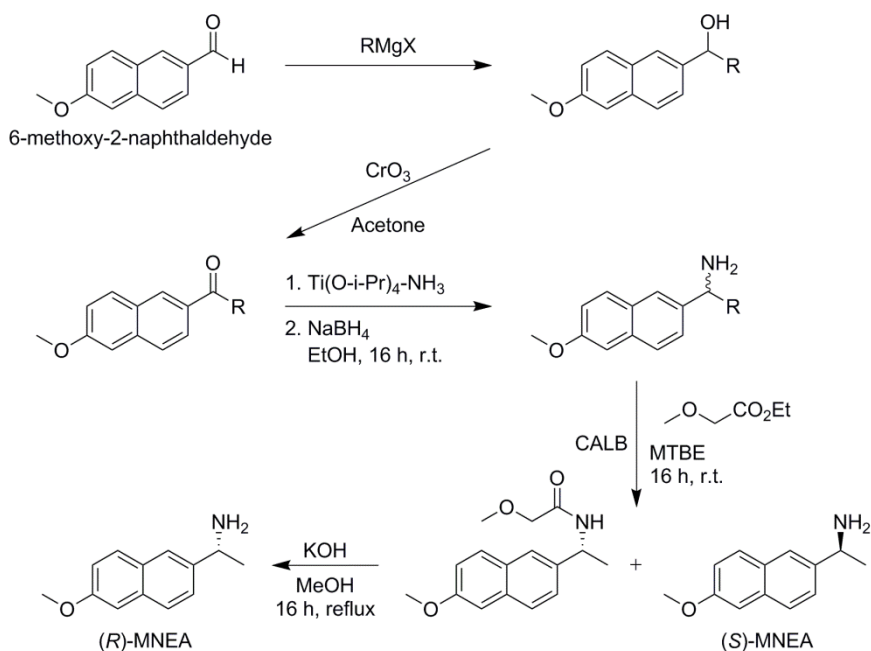
The developed assay is based on transamination of the amine 1-(6-methoxynaphth-2-yl)ethylamine (MNEA) and derivatives thereof to form their corresponding ketones that show fluorescence emission at 450 nm¹⁸⁰⁻¹⁸¹ (Scheme 9). It can be easily detected using either a cuvette based fluorometer or a fluorescence plate reader. An initial evaluation of the assay was performed with the ATA from *Neosartorya fischeri* using commercially available racemic MNEA and pyruvate as substrates. Upon addition of enzyme, a steady increase of fluorescence was observed, thus verifying the assay concept.



Scheme 9. Assay principle for ATA catalyzed transamination of the substrate amine to form its corresponding ketone that shows a strong blue fluorescence.

To make the method feasible, the substrates needed must be readily available, either commercially or via simple synthesis. The substrate amines are not commercially available, except for the racemic MNEA and therefore a synthesis route needed to be developed. Reductive amination from the corresponding ketones is one option but if variations in the R group (Scheme 9) are desired this is also not a viable option as most of these ketones are not commercially available either. A general synthesis method starting from a common starting substrate was needed and therefore a synthesis route starting from the commercially available 6-methoxy-2-naphthaldehyde was designed (Scheme 10). The structural variation in the R group was introduced in the first step via Grignard addition¹⁸² followed by Jones oxidation¹⁸³ of the resulting carbinol, yielding its corresponding ketone. Titanium(IV) isopropoxide-mediated reductive amination using ammonia with subsequent borohydride reduction¹⁸⁴ was performed to obtain the final racemic amines. In the

case of MNEA, lipase catalyzed kinetic resolution⁸⁸⁻⁹⁰ was performed to obtain both pure enantiomers. Hofmann rearrangement¹⁸⁵ of the commercially available enantiomerically pure (*S*)-naproxen was performed to verify the absolute configurations of the synthesized enantiomers. The product of this reaction must be (*S*)-MNEA since Hofmann rearrangement proceeds stereospecifically with retention of configuration.



Scheme 10. Synthesis route for obtaining amine substrates for the developed fluorescent assay.

Fluorescence measurements were performed at 330 nm excitation and 460 nm emission and a linear relationship between fluorescence intensity and ketone concentration was observed which made it possible to make a standard curve and quantify enzyme activity. The limit of detection (LOD) and the limit of quantification (LOQ)¹⁸⁶ were calculated to 0.35 nmol min⁻¹ and 1.2 nmol min⁻¹ respectively. In addition the Z-factor was calculated at substrate concentrations of 0.7 mM and 0.35 mM to 0.95 and 0.80 respectively which defines the method as excellent for high-throughput screening.¹⁸⁷

Table 5. Kinetic data for a set of ATAs toward both enantiomers of MNEA determined by the developed fluorescent assay.

Enzyme	Enantiomer of MNEA	K_M [mM]	K_I [mM]	V_{max} [$\mu\text{mol min}^{-1} \text{mg}^{-1}$]	V_{max}/K_M	E
<i>Neosartorya fischeri</i> ^a	(S)	1.2	4.9	0.00015	0.00013	30 (R)
	(R)	0.59	3.2	0.0023	0.0039	
<i>Neosartorya fischeri</i>	(S)	0.79	3.3	0.00044	0.00056	36 (R)
	(R)	0.88	0.72	0.018	0.020	
<i>Chromobacterium violaceum</i>	(S)	0.45	1.4	0.76	1.7	19 (S)
	(R)	1.1	5.1	0.099	0.090	
<i>Vibrio fluvialis</i>	(S)	0.77	2.0	22	29	>200 (S)
	(R)	0.65	2.0	0.035	0.054	
<i>Aspergillus fumigatus</i>	(S)	0.77	1.3	0.0020	0.0026	42 (R)
	(R)	0.27	0.73	0.031	0.11	
<i>Aspergillus terreus</i>	(S)	1.1	2.0	0.00011	0.00010	80 (R)
	(R)	0.050	1.1	0.00040	0.0080	
ATA-117	(S)	2.0	0.31	0.044	0.022	50 (R)
	(R)	0.23	0.52	0.25	1.1	
ATA-113	(S)	0.0025	1.1	0.012	4.8	110 (S)
	(R)	0.55	0.68	0.023	0.042	
<i>Geobacillus thermodenitrificans</i> ^a	(S)	0.80	6.1	0.00039	0.00049	38 (S)
	(R)	1.6	5.6	0.000020	0.000013	
<i>Geobacillus thermoleovorans</i> ^a	(S)	0.58	6.1	0.000031	0.000053	- (S)
	(R)			n.d. ^b		

^a Measured in phosphate buffer. All other data was measured in HEPES buffer. ^b Not determined, no activity detected.

To show the applicability of the developed assay, kinetic constants toward both enantiomers of MNEA were determined for a set of ATAs (Table 5). The amine concentration was varied between 0.00043-7.0 mM and kinetic constants were calculated by assuming pseudo one-substrate kinetics. However, substrate inhibition was observed for all applied enzymes and the modified Michaelis-Menten equation for substrate inhibition (equation 5) was therefore used to fit the data. The ATAs incorporated in this experiment include (*R*)-selective ATAs from *Neosartorya fischeri*, *Aspergillus fumigatus* and *Aspergillus terreus*, (*S*)-selective *Cv*-ATA and *Vf*-ATA, commercial ATAs ATA-117 and ATA-113 as well as two novel thermostable taurine-pyruvate transaminases from *Geobacillus thermodenitrificans*¹⁸⁸ and *Geobacillus thermoleovorans*. By fitting the data to equation 5, kinetic constants K_M , K_I and V_{max} could be determined and from these values the apparent specificity constant V_{max}/K_M could be derived (Table 5). This also means that E-values (equation 9) could be determined (Table 5).

The engineering of ATAs for acceptance of bulkier substrates, especially in the S pocket (Scheme 6), than the ones naturally accepted is of high interest.^{13,131,189-191} The simplicity of our designed screening substrate synthesis makes the developed screening method suitable for this kind of engineering project since almost any variation in the R group (Scheme 10) can be synthesized and screened for. This was visualized by synthesizing the substrates 1-(6-methoxynaphth-2-yl)propylamine (MNPA) and 1-(6-methoxynaphth-2-yl)butylamine (MNBA) (Figure 17) and measuring the activity of *Cv*-ATA toward these substrates as well as toward MNEA. As can be seen in figure 17, MNPA has a relative activity of 14% compared to MNEA and MNBA has a relative activity of less than 1%.

In conclusion, a novel fluorescence-based assay that is suitable for kinetic measurements and high-throughput screening of ATAs has been developed. A large set of ATAs including two novel thermostable taurine-pyruvate transaminases were kinetically evaluated using the developed method. The two latter enzymes displayed a very low activity toward the applied amine but this is not surprising as their natural substrate (taurine) is structurally very different. Additionally, as they both originate from thermostable organisms, their temperature optimum is much higher than the temperature applied in this study (the taurine-pyruvate

transaminase from *Geobacillus thermodenitrificans* only displays around 20% activity at 37°C)¹⁸⁸.

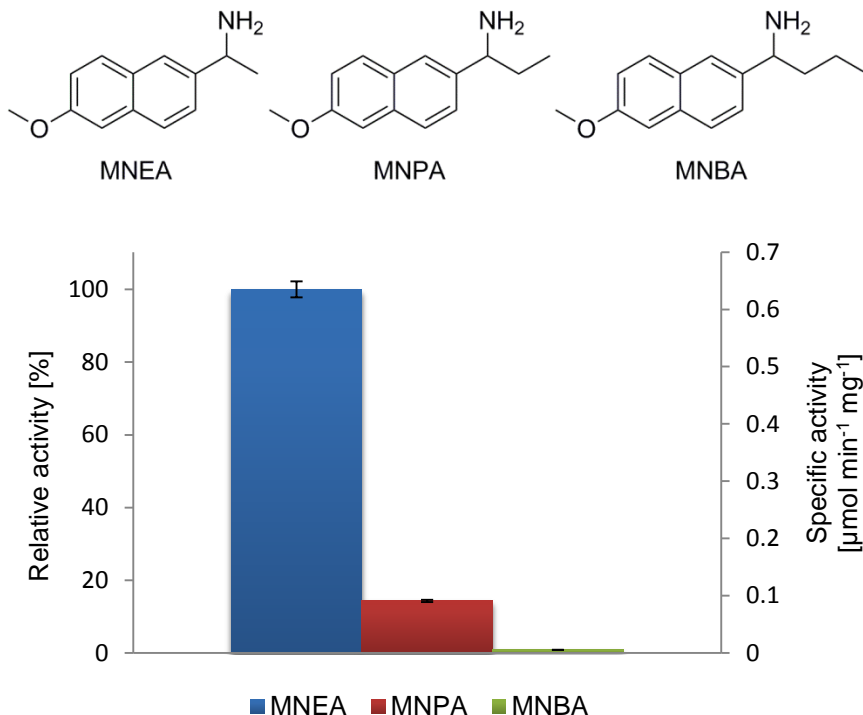


Figure 17. Relative activity of Cv-ATA toward amine substrates MNEA (blue), MNPA (red) and MNBA (green).

The developed assay is also a promising high-throughput screening method for directed evolution of ATAs due to excellent Z-factors and low substrate consumption (4.5 mg MNEA/96-well plate when applied at 1 mM). The assay was not evaluated in different varied reaction conditions such as pH, temperature and addition of co-solvents but as long as they do not interfere with the fluorescence measurement, no problems should arise. Screening of amino donor spectrum is not suitable as the substrate amine needs to contain the fluorophore, but screening of amino acceptor spectrum should be straightforward as long as the amino acceptor is non-fluorescent.

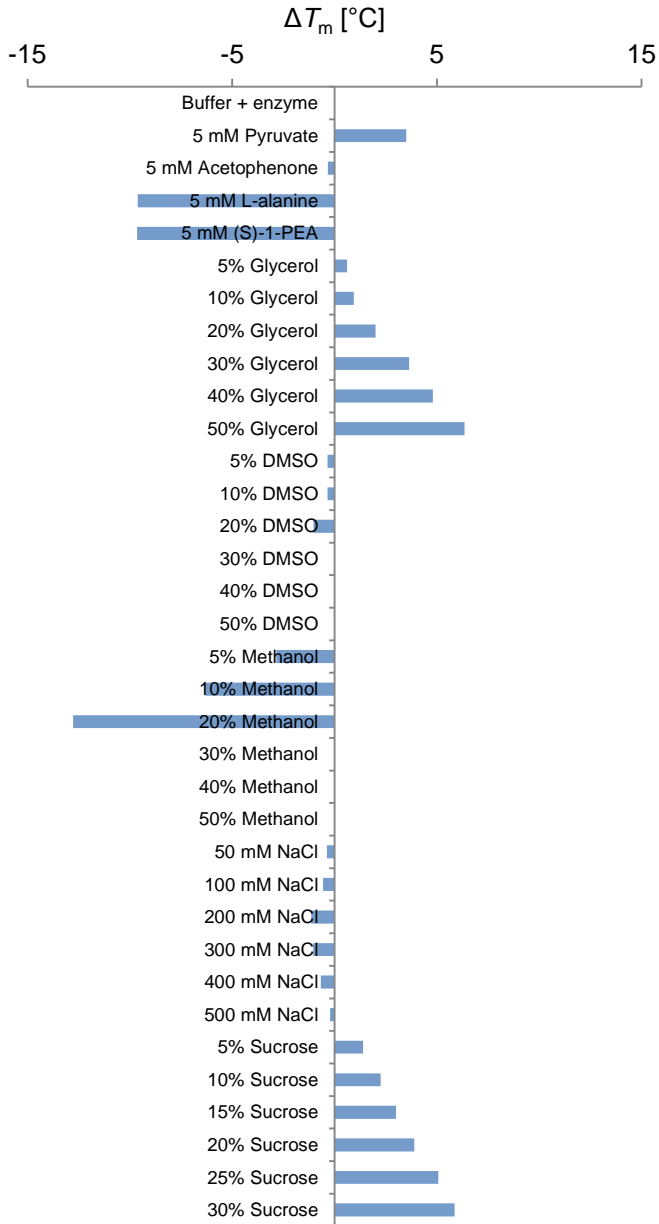
Finally, due to the versatility of the designed substrate synthesis route, structural variations can easily be introduced in the screening substrate. This enables engineering of ATAs toward acceptance of bulkier substrates using a substrate walking approach.

8.4. Investigation of amine transaminase stability (Paper V)

In the previous chapters on enzyme engineering and enzyme stability it was discussed how wild-type enzymes rarely can be applied in industrial processes due to low operational stability. This is the case for ATAs as well which is evident from the directed evolution of *Ac*-ATA for production of substituted 2-aminotetralin⁴⁸ and an (*R*)-selective ATA for the production of Sitagliptin¹³. In both these cases major engineering was required to obtain variants stable enough for the desired industrial conditions.

Cv-ATA is no exception. In a previous study by our research group¹³⁰ the melting point (T_m) of *Cv*-ATA was determined in the presence of different buffers and additives in order to find stabilizing conditions for crystallization. As an example, in the presence of HEPES buffer (100 mM, pH 7.4) and NaCl (100 mM) *Cv*-ATA displayed a T_m of 78°C which is considered to be rather high. However, unpublished results from our group show that *Cv*-ATA is unable to perform catalysis at elevated temperatures as well as other harsh conditions such as high concentrations of co-solvents. This knowledge was used as a starting point in this study where the stability of *Cv*-ATA was investigated. In addition, this study includes the use of a co-lyophilization strategy for *Cv*-ATA and its subsequent application in organic solvents.

Firstly, the melting point differences (ΔT_m) of *Cv*-ATA compared to the T_m with only buffer (HEPES 50 mM, pH 8.2) in the presence of different co-solvents and additives were investigated using differential scanning fluorimetry¹⁹². As can be seen in figure 18, glycerol and sucrose has a positive impact on ΔT_m . Methanol however, has a negative impact while DMSO and NaCl does not have a significant influence on ΔT_m . A set of common ATA substrates were also investigated and while pyruvate and acetophenone both increased and had no impact on ΔT_m , respectively, the addition of amines L-alanine and (*S*)-1-PEA had a significant negative impact on ΔT_m .

Figure 18. ΔT_m of Cv-ATA in the presence of substrates, co-solvents and additives.

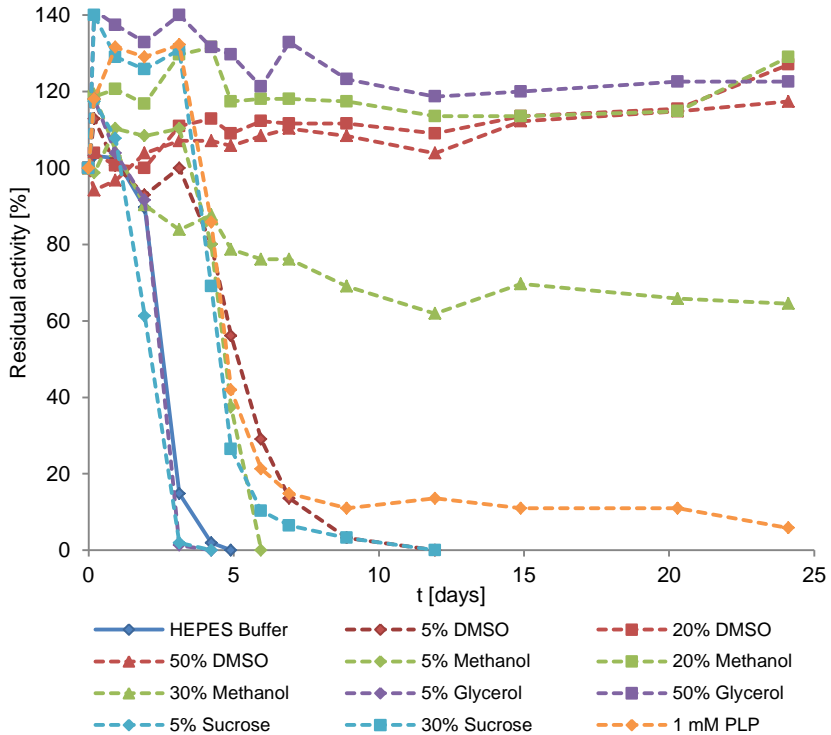


Figure 19. Stability over time of Cv-ATA at room temperature in the presence of HEPES buffer, DMSO, methanol, glycerol, sucrose and PLP. Only a fraction of the investigated conditions are shown for clarity. For a full display of all investigated conditions, see Appendix V, figure 2.

The effects on enzyme storage time when adding different amounts of co-solvents (DMSO, methanol and glycerol) and sucrose as well as PLP, (*S*)-1-PEA and surfactants were investigated. The different enzyme solutions were stored at room temperature (20–25°C, not controlled but temperature is assumed to have been the same over the course of the experiment) and the remaining activity of the samples was measured regularly over the course of 24 days. As can be seen in figure 19, the enzyme that was only stored in buffer lost all activity after 5 days of incubation. Low concentrations of DMSO and glycerol however yield a slight increase in stability while higher concentrations (10–50% DMSO and 20–50% glycerol) helps to maintain full activity for 24 days. The enzyme solution containing 50% glycerol was stored for an even longer

time at room temperature and -20°C and 77% as well as 114% activity was maintained after 6 months, respectively. Methanol displays the same behavior as 10-20% induces the same stability as high concentrations of DMSO and glycerol. However, 30% methanol partially reduces activity but keeps it stable while 40-50% completely and almost instantly denatures all enzyme in the solution. Precipitated enzyme was also visible in 30% methanol but it seems as it was only denatured to a certain extent. Sucrose, PLP and the surfactants only yielded a slight increase in stability while (S)-1-PEA decreased it.

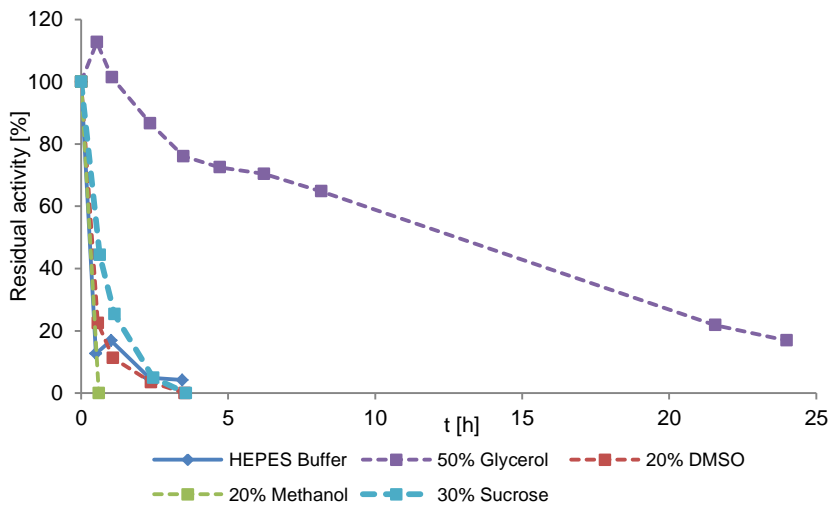


Figure 20. Stability over time of Cv-ATA at 65°C in the presence of a set of chosen stabilizing conditions.

Some of the conditions showing increased stability in the previous experiment were chosen for a similar experiment at elevated temperature (65°C) (Figure 20). None of the conditions showed a significant increase in stability except for 50% glycerol that drastically increased the stability at 65°C as activity still remained at 17% after 24 h. The other conditions had lost all activity within 5 h and 30% methanol decreased stability as all activity was lost after approximately 30 min.

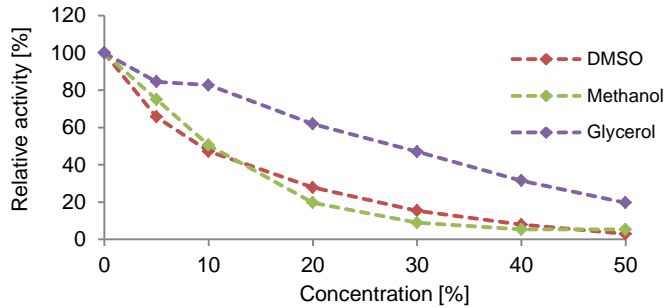


Figure 21. Initial activity of Cv-ATA in increasing concentrations of co-solvents DMSO, methanol and glycerol.

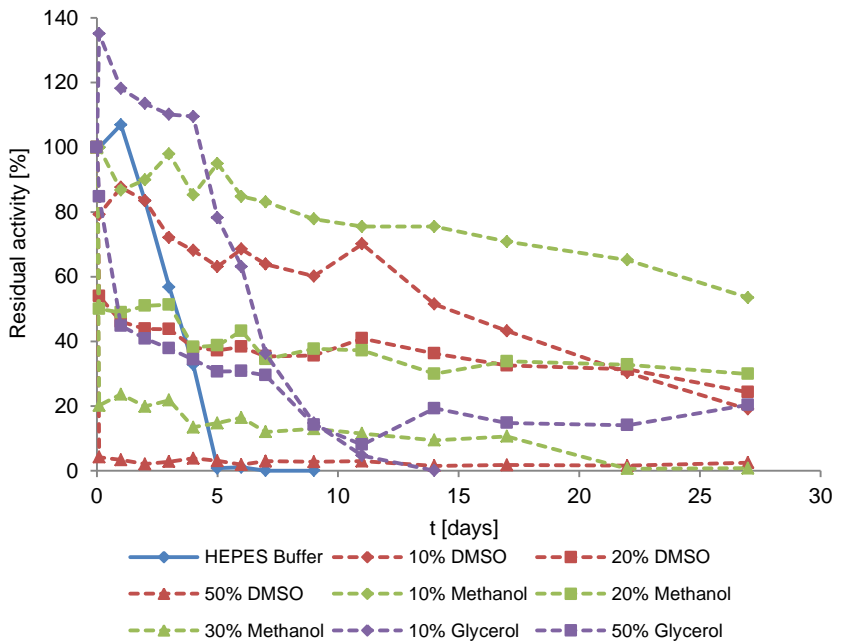


Figure 22. Stability and activity over time of Cv-ATA at room temperature in the presence of HEPES buffer, DMSO, methanol and glycerol. Only a fraction of the investigated conditions are shown for clarity. For a full display of all investigated conditions, see Appendix V, figure 5.

In an industrial application, the stability of an enzyme in different co-solvents is not the only concern. Activity in co-solvents also plays an important role and the initial activity of Cv-ATA in different amounts of

the co-solvents DMSO, methanol and glycerol was therefore investigated (Figure 21). DMSO and methanol display a similar effect on activity as it decreases with increasing concentration of co-solvent and at 50% co-solvent nearly all activity is lost. Glycerol shows a similar behavior but the decrease in activity is not as significant.

To simulate an industrial setting, a combination of the previous experiments was performed as the enzyme was stored at different concentrations of co-solvents at room temperature but when the samples were collected, residual activity was measured in the same concentration of co-solvent as the enzyme was stored in. Results show that even though the activity is lower in most cases, it is maintained for a longer time than when the enzyme is stored in buffer (Figure 22).

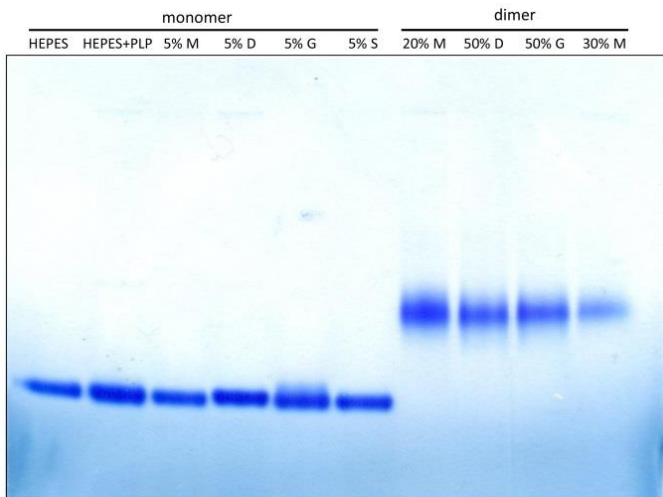


Figure 23. BN-PAGE of a chosen set of samples from the storage stability experiment in room temperature (Figure 19). Samples were collected after 20 days of incubation.

As previously mentioned, an enzyme can lose activity due to reversible unfolding or by irreversible denaturation. In the previous storage stability experiment (Figure 19), precipitation was observed in the cases of 30-50% methanol. This observation is consistent with irreversible denaturation of the enzyme. However, in all other enzyme solutions that lost activity, there was no visible precipitation. To investigate this phenomenon, Blue Native PAGE (BN-PAGE) was performed on some of

the samples that had lost all activity as well as some of the samples that still displayed full activity (Figure 23). *Cv*-ATA is a homodimeric protein and both subunits contribute with active site residues to the active site of each monomer.¹³⁰ *Cv*-ATA is therefore not active in its monomeric form and as can be seen in figure 23, all inactive samples are in their monomeric form while all active samples are in their dimeric form.

The *Cv*-ATA dimer dissociation was further investigated by storing the enzyme at different enzyme concentrations. Enzyme concentration is known to affect the monomer-dimer equilibrium¹⁹³ and residual activity measurements (Figure 24) as well as BN-PAGE analysis (Figure 25) was therefore simultaneously performed to show a correlation between loss of activity and dimer dissociation. By comparing figure 24 and 25, one can clearly see that as activity decreases, monomers start forming. At the last measurement (7 days), the 0.2 mg mL⁻¹ and 0.5 mg mL⁻¹ solutions show very little residual activity and most of them are also in the monomeric form. 1 mg mL⁻¹ has slightly higher residual activity and some dimer is still visible. In the case of 5 mg mL⁻¹, which is clearly the most stable concentration, approximately 80% activity remains and most of the enzyme is also in its dimeric form.

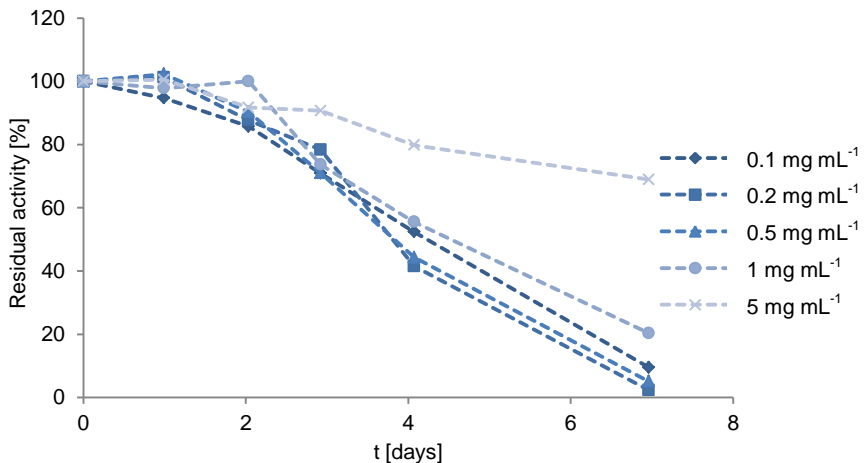


Figure 24. Residual activity of *Cv*-ATA when stored in different concentrations over time in room temperature.

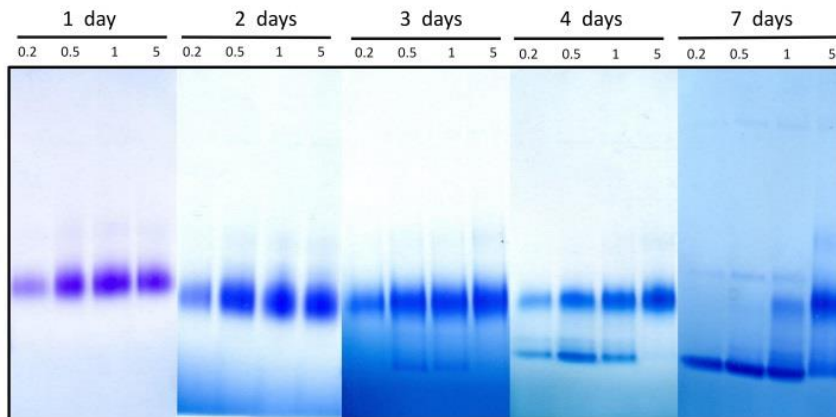


Figure 25. BN-PAGE of *Cv*-ATA stored in different concentrations (0.2, 0.5, 1 and 5 mg ml⁻¹) over time in room temperature.

The storage stability experiment also showed that an excess of PLP increases stability of *Cv*-ATA. This was further investigated by treating the enzyme different with regards to PLP after purification and running BN-PAGE after 5 days of storage. The enzyme was either treated with an excess of PLP (Figure 26, wells 1 and 2) or had no excess PLP added (Figure 26, well 3). Well 2 was also desalted after incubation overnight to remove any excess PLP. The samples were stored at room temperature. Wells 4-6 are the same as wells 1-3 but they were stored at 4°C. As can be seen in figure 26, the sample without any extra PLP added is less stable as more monomer is formed.

The use of lyophilized ATAs in organic solvents has been previously reported¹⁹⁴⁻¹⁹⁵ but lyophilization has also been shown to influence enzyme activity negatively¹⁹⁶⁻¹⁹⁷. Addition of surfactants have however been reported to shield enzymes from damage during lyophilization¹⁹⁸⁻¹⁹⁹ and the surfactants Brij® C10 and octyl β-D-glycopyranoside which previously have been shown to have a positive effect on application of subtilisin Carlsberg in organic solvents²⁰⁰ were therefore added to *Cv*-ATA before lyophilization. Reactions in different organic solvents (MTBE, isooctane and toluene) between (*S*)-1-PEA and methoxyacetone were performed by adding lyophilized *Cv*-ATA with or without surfactants. As can be seen in Table 6, co-lyophilization with surfactants improved conversion 5-fold in all applied solvents. In isooctane, conversion to acetophenone reached

84% which shows that co-lyophilization with surfactants clearly improves activity in organic solvents.

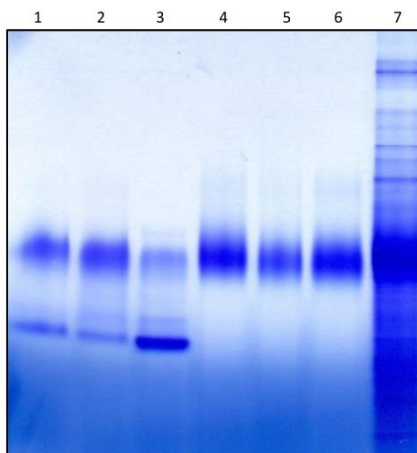
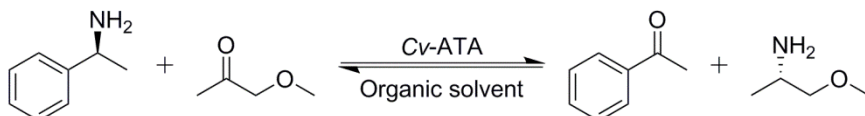


Figure 26. BN-PAGE of Cv-ATA treated different with regards to PLP addition at room temperature (wells 1-3) and 4°C (wells 4-6). Well 7 contains the supernatant before purification and it was also stored at 4°C.

Table 6. Transamination of (*S*)-1-PEA and methoxyacetone in organic solvents catalyzed by Cv-ATA lyophilized with (+) or without (-) surfactants (Brij® C10 and octyl β -D-glycopyranoside).



Organic solvent	Surfactants	Conversion [%]	
		24 h	48 h
MTBE	-	5.9	9.6
MTBE	+	32	45
Isooctane	-	13	17
Isooctane	+	65	84
Toluene	-	9.1	12
Toluene	+	36	54

In conclusion, the thermostability and stability over time of *Cv*-ATA has been investigated. The thermostability was investigated in the presence of different co-solvents and additives with T_m measurements and by storing the enzyme solutions in 65°C. The results of the two experiments are consistent as glycerol increases thermostability, DMSO has no significant effect and methanol decreases thermostability. The results for sucrose are however not consistent as the T_m measurements show an increase in thermostability while the storage at 65°C does not. This indicates that sucrose has a positive effect on kinetic stability but not on thermodynamic stability as there are no significant effects on dimer stabilization (Figure 19).

Experiments related to enzyme stability over time and activity in co-solvents in room temperature show that all the tested co-solvents induce stability but they simultaneously decrease activity. This means that if a *Cv*-ATA catalyzed process with high concentrations of co-solvents is to be developed, stability is not an issue but activity is. Stability might of course need to be improved against other factors but it does not need to be improved with regards to the application of co-solvents. However, even though activity is decreased when co-solvents are applied, the activity is maintained for a longer time so the TTN²³ (Total Turnover Number, moles of substrate that a mole of catalyst can convert before becoming inactivated, not to be confused with k_{cat}) is actually increased.

The reason for loss of activity over time was found to be dimer dissociation into the inactive monomers which is common for multimeric enzymes²⁰¹⁻²⁰². The tested co-solvents seem to stabilize the dimer and the fact that activity is not only maintained but also increased in most stabilizing conditions (Figure 19) points to formation of more dimers in the presence of these stabilizing co-solvents. In addition, the hypothesis that high concentrations of methanol do not induce dimer dissociation but instead irreversibly denature the enzyme is strengthened by the results in figure 23 (30% methanol) where the dimer band is weaker than the others while no monomer band is visible. This confirms that the reduced activity (Figure 19) is not a result of dimer dissociation.

PLP seem to be important for dimer stability as dimer dissociation occurred faster when no extra PLP was added after purification (Figure 26). As *Cv*-ATA production is over-expressed in *E. coli* while PLP

production is not, there is probably not enough PLP produced by the cell to occupy all the produced enzyme active sites. PLP is known to be coordinated by several amino acid residues in the active site and one important site is the “phosphate group binding cup”^{130,203} that coordinates the phosphate group of PLP and consists of amino acid residues from both subunits. The dissociation of the dimer structure opens the “phosphate group binding cup”, thereby promoting the release of PLP. The release of PLP from the monomers might also be the reason for their inactivity. The importance of PLP for stability is further illustrated by the negative impact that amine substrates (*S*)-1-PEA and L-alanine have on stability. By adding amines to the enzyme solution, PLP is transformed to PMP which is not as strongly bound to the enzyme as PLP. This might also promote dimer dissociation.

Finally, *Cv*-ATA was co-lyophilized in the presence of surfactants and successfully applied in dry organic solvents. This is the first study in which this has been done with an ATA.

9. Concluding remarks and future outlook

ATAs provide an option for the challenging synthesis of amines and the breakthroughs reported over the last two decades have shown that this option can be an improvement compared to other methods. As an example, three steps in the production of Sitagliptin that included metal catalyzed asymmetric hydrogenation followed by carbon treatment for complete removal of the catalyst were replaced by a single ATA catalyzed step. The superior enantioselectivity of the ATA catalyzed step (>99.95% *ee*) also made it possible to skip an additional crystallization that was performed to improve the *ee* (95%) of the previous process. The final process had a 13% improved total yield, 53% improved productivity and 19% less waste was produced compared to the previous process.²⁰⁴ This case shows that it is possible to apply ATAs in industrial processes for the synthesis of amines.

This thesis has been focused on improvement of ATA catalyzed synthesis of amines by increasing substrate specificity and enantioselectivity using rational design (**Paper I**), displacing the thermodynamically challenging equilibrium of amine synthesis by applying ATAs in two different cascades (**Paper II** and **III**) and investigating enzyme stability to enhance the life-time of ATAs, both in storage and synthesis (**Paper V**). A sensitive fluorescence-based assay for the measurement of ATA activity which can be applied as a screening method to make the evolution of ATAs with extended substrate scopes straightforward has also been developed (**Paper IV**).

An efficient enzyme catalyzed process requires high substrate specificity to keep reaction times and costs down. If the enzyme is already active toward the substrate in question, a simple rational design approach is usually feasible, as was shown in **Paper I** where the specific activity of *Cv*-ATA toward (*S*)-1-PEA was increased 29 times by a single amino acid substitution. The variant (W60C) was also evaluated using Swain-Lupton

plots to explore if there was a difference in the variant's mechanism of action. This method¹⁸ and other variants²⁰⁵ of the Hammett equation¹⁹ are rarely used in biocatalysis but their use in other fields of chemistry is more common²⁰⁶⁻²⁰⁷.

Equilibrium displacement of ATA catalyzed reactions has been one of the major focuses of ATA research. As previously mentioned, most research has been focused on systems containing several enzymes which lead to complexity and high cost in large-scale transformations. These systems can be very useful in lab-scale synthesis but in an industrial setting simplicity and low cost is prioritized. That is why most industrial processes so far have been employing the cheap bulk chemical IPA as amino donor.^{13,48} It can be added in large excess and the formed co-product acetone is easily evaporated which leads to equilibrium displacement. IPA is not an ideal amino donor for ATAs and systems involving better amino donors that can be added in smaller excess are therefore needed. Therefore, **Paper II** describes a method where the amino donor forms a co-product that spontaneously tautomerizes, thus heavily shifting the equilibrium. This system is simple as no additional enzymes are needed and the amino donor can be used in equimolar amounts and still reach good conversions. However, this method has a drawback as the amino donor is hard to obtain and it is also expensive but other similar methods utilizing “smart” amino donors have subsequently been published with the most recent ones employing simple aliphatic diamines as amino donors¹²³⁻¹²⁴. The formed co-products spontaneously cyclize and the equilibrium is therefore shifted toward product formation. These simple amino donors are relatively cheap and as they can be added in much smaller excess than IPA and still reach the same conversions they might be interesting for industrial applications.

Another way to shift the equilibrium is to remove the product amine from the reaction by further transforming it and if the ATA catalyzed reaction is only part of a longer reaction sequence this can be the favored method. An example of this was demonstrated in **Paper III** where the product amine was amidated by an acyl transferase in a one-pot one-step fashion in aqueous solution. The reported method has its drawbacks as it is not able to synthesize chiral amides but more research into the method can probably solve this issue. Also, even if an amide is not the desired product the amine often needs to be protected due to its reactivity if the

compound is to react further. This is commonly achieved with amidation and can also be an application of the designed cascade.

Amine-containing compounds of interest are seldom natural substrates of ATAs as they are usually bulky on both sides of the amine functional group and engineering is therefore required. The screening method described in **Paper IV** is a straightforward and simple method to screen ATA libraries for the acceptance of almost any bulky sidechain. As it is based on fluorescence, it is also sensitive and can measure very low activities that can be improved by engineering. The simple synthesis route developed for the screening substrates also makes it possible to obtain substrates with basically any desired sidechain. The developed screening method should be general for almost any project where acceptance of bulkier sidechains is desired which means that new screening methods do not need to be developed for every unique project.

The stability of a biocatalyst is a key factor to its application in an industrial process. Enzymes usually perform catalysis in aqueous solution but many substrates are not very soluble in that environment and co-solvents like DMSO, methanol and other water-miscible organic solvents are therefore often used to improve solubility. Wild-type ATAs usually do not perform well in the presence of these co-solvents and it has generally been assumed that this is due to stability issues. The investigation in **Paper V** however shows that these co-solvents act as stabilizers for *Cv*-ATA and the poor performance is actually due to low activity. This shows that it is important to investigate the reasons for non-optimal performance in the conditions that are demanded by the process before the enzyme is engineered for improvement.

The future of ATA chemistry and biocatalysis in general looks promising. The massive research effort in the ATA field over the last two decades has led to a good understanding of how these enzymes work and many methods for their application have been developed. The ATA field is now entering a phase where most basic research has been performed and there is now enough knowledge available for straightforward application of ATAs in any given process. The limitation that biocatalysts used to pose on industrial processes as conditions had to be modified to fit the enzyme is now gone. All the recent breakthroughs in biotechnology have now instead made it possible to engineer enzymes to fit the desired process

conditions¹¹ and this has turned biocatalysis into a much more attractive option for the industry. As discussed above, the successful Sitagliptin case¹³ shows that switching chemical processes to biocatalytic ones can have positive impacts and hopefully more and more companies will realize this. The successful application of enzymes in large scale industrial processes require large amounts of basic research like investigations and method developments and the work presented in this thesis contribute to this progress.

Acknowledgements

Thank you **Per** for accepting me as a PhD student in your group. You have been an excellent supervisor and by giving me lots of freedom to work on my own and taking my own decisions you have turned me into an independent scientist.

Thank you **Maria** for good supervision and collaboration. You have basically been my unofficial co-supervisor and your presence has been invaluable to me.

Thank you **Mats** and **Kalle** for always answering questions when my supervisor was not available. Also, special thanks to Kalle for reviewing my thesis.

Thank you **Karim** for being an excellent supervisor during my Master's thesis and for being my colleague at the beginning of my PhD.

A big thanks to all my fellow PhD students and postdocs in the Biocatalysis group, both past and present. **Peter**, for being here with me from the beginning. **Mattias**, for always being critical towards my ideas and making me think twice. **Stefan**, for discussing football with me when no one else wants to. **Maja**, for always challenging how things are done and for making the group feel alive. **Shan**, for good collaborations in the lab. **Fabian**, thank you for rewarding collaborations with you and your "other group" and for always showing me a better (German) way of doing things in the lab. Thank you **Wang** for teaching me organic chemistry and how to work safely in the lab. Finally, thank you **Lisa** and **Fei** for being my colleagues during the last part of my PhD.

Thank you **Wolf-Dieter Fessner**, **Uwe Bornscheuer**, **Matthias Höhne** and **Vahak Abedi** for rewarding collaborations that resulted in very good publications.

Thank you **Jennifer Littlechild** for inviting me to your lab in Exeter for two weeks so I could learn about crystallography.

Thank you to all other co-workers at the divisions of Industrial Biotechnology and Glycoscience. A special thanks to **Ela** for making life in the lab easier.

Thank you to my former students who I have gladly supervised: **Mattias, Andreas, Anders, Erik, Numan** and **Karolina**.

Till sist vill jag tacka min kära familj: **Erica, Saga** och **Liv**. Tack för att ni har stått ut med att bo tillsammans med mig när jag varit "tvungen" att åka in på kvällar och helger för att starta bakterieodlingar. Utan er skulle livet vara bra mycket tråkigare! Stort tack även till **Mamma** och **Pappa** för att ni alltid hjälper mig när det krisar.

KTH Royal Institute of Technology is greatly acknowledged for funding my PhD studies. **EU COST Action CM0701 Cascade Chemoenzymatic Processes** is acknowledged for funding my Short Term Scientific Mission to Exeter (United Kingdom) and **EU COST Action CM1303 Systems Biocatalysis** is acknowledged for funding my participation in the SysBiocat training school in Siena (Italy) as well as for funding my participation in the Transam 2.0 conference in Greifswald (Germany). **Roos stiftelse** and **Björns stiftelse** are acknowledged for funding my participation in the Biocat2012 conference in Hamburg (Germany) and the Pacificchem 2015 conference in Honolulu (USA). Finally, the **organizing committee of BIOPROSP_15** is acknowledged for funding my participation in the BIOPROSP_15 conference in Tromsø (Norway).

Bibliography

1. Brasier M, McLoughlin N, Green O and Wacey D. A fresh look at the fossil evidence for early Archaean cellular life. *Philos. Trans. R. Soc. Lond. B: Biol. Sci.* **2006**, *361*, 887-902.
2. Radzicka A and Wolfenden R. A Proficient Enzyme. *Science* **1995**, *267*, 90-93.
3. Walsh C. Enabling the chemistry of life. *Nature* **2001**, *409*, 226-231.
4. Anastas P T and Warner J C. Green Chemistry: Theory and Practice. Oxford University Press, **1998**.
5. Smith H O and Welcox K W. A restriction enzyme from *Hemophilus influenzae*. I. Purification and general properties. *J. Mol. Biol.* **1970**, *51*, 379-391.
6. Kelly T J and Smith H O. A restriction enzyme from *Hemophilus influenzae*. II. Base sequence of the recognition site. *J. Mol. Biol.* **1970**, *51*, 393-409.
7. Padmanabhan R, Jay E and Wu R. Chemical Synthesis of a Primer and Its Use in the Sequence Analysis of the Lysozyme Gene of Bacteriophage T4. *Proc. Natl. Acad. Sci. USA.* **1974**, *71*, 2510-2514.
8. Sanger F, Nicklen S and Coulson A R. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA.* **1977**, *74*, 5463-5467.
9. Maxam A M and Gilbert W. A new method for sequencing DNA. *Proc. Natl. Acad. Sci. USA.* **1977**, *74*, 560-564.
10. Saiki R K, Scharf S, Faloona F, Mullis K B, Horn G T, Erlich H A and Arnheim N. Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* **1985**, *230*, 1350-1354.
11. Bornscheuer U T, Huisman G W, Kazlauskas R J, Lutz S, Moore J C and Robins K. Engineering the third wave of biocatalysis. *Nature* **2012**, *485*, 185-194.
12. Rosenthaler L. Durch Enzyme bewirkte asymmetrische Synthese. *Biochem. Z.* **1908**, *14*, 238-253.
13. Savile C K, Janey J M, Mundorff E C, Moore J C, Tam S, Jarvis W R, Colbeck J C, Krebber A, Fleitz F J, Brands J, Devine P N, Huisman G W and Hughes G J. Biocatalytic Asymmetric Synthesis of Chiral Amines from Ketones Applied to Sitagliptin Manufacture. *Science* **2010**, *329*, 305-309.
14. Henri V. Lois Générales de l'action des diastases. Librairie Scientifique, A. Hermann, **1903**.
15. Michaelis L and Menten M L. Die kinetik der invertinwirkung. *Biochem. Z.* **1913**, *49*, 333-369.
16. Briggs G E and Haldane J B S. A Note on the Kinetics of Enzyme Action. *Biochem. J.* **1925**, *19*, 338-339.
17. Copeland R A. Enzymes: A Practical Introduction to Structure, Mechanism, and Data Analysis. John Wiley & Sons, Inc., **2000**.
18. Swain C G and Lupton E C. Field and resonance components of substituent effects. *J. Am. Chem. Soc.* **1968**, *90*, 4328-4337.
19. Hammett L P. The Effect of Structure upon the Reactions of Organic Compounds. Benzene Derivatives. *J. Am. Chem. Soc.* **1937**, *59*, 96-103.
20. Swain C G, Unger S H, Rosenquist N R and Swain M S. Substituent effects on chemical reactivity. Improved evaluation of field and resonance components. *J. Am. Chem. Soc.* **1983**, *105*, 492-502.

21. Yoon T P and Jacobsen E N. Privileged Chiral Catalysts. *Science* **2003**, 299, 1691-1693.
22. Dalko P I and Moisan L. Enantioselective Organocatalysis. *Angew. Chem. Int. Ed.* **2001**, 40, 3726-3748.
23. Faber K. Biotransformations in Organic Chemistry: A Textbook. Springer, **2011**.
24. Breuer M, Ditrich K, Habicher T, Hauer B, Keßeler M, Stürmer R and Zelinski T. Industrial Methods for the Production of Optically Active Intermediates. *Angew. Chem. Int. Ed.* **2004**, 43, 788-824.
25. El Gihani M T and Williams J M J. Dynamic kinetic resolution. *Curr. Opin. Chem. Biol.* **1999**, 3, 11-15.
26. Martin V S, Woodard S S, Katsuki T, Yamada Y, Ikeda M and Sharpless K B. Kinetic resolution of racemic allylic alcohols by enantioselective epoxidation. A route to substances of absolute enantiomeric purity? *J. Am. Chem. Soc.* **1981**, 103, 6237-6240.
27. Chen C S, Fujimoto Y, Girdaukas G and Sih C J. Quantitative analyses of biochemical kinetic resolutions of enantiomers. *J. Am. Chem. Soc.* **1982**, 104, 7294-7299.
28. Clayden J, Greeves N, Warren S and Wothers P. Organic Chemistry. Oxford University Press, **2001**.
29. Hult K and Berglund P. Enzyme promiscuity: mechanism and applications. *Trends Biotechnol.* **2007**, 25, 231-238.
30. Humble M S and Berglund P. Biocatalytic Promiscuity. *Eur. J. Org. Chem.* **2011**, 2011, 3391-3401.
31. Newman D J and Cragg G M. Natural Products as Sources of New Drugs from 1981 to 2014. *J. Nat. Prod.* **2016**, 79, 629-661.
32. Polizzi K M, Bommarius A S, Broering J M and Chaparro-Riggers J F. Stability of biocatalysts. *Curr. Opin. Chem. Biol.* **2007**, 11, 220-225.
33. Kendrew J C, Bodo G, Dintzis H M, Parrish R G, Wyckoff H and Phillips D C. A Three-Dimensional Model of the Myoglobin Molecule Obtained by X-Ray Analysis. *Nature* **1958**, 181, 662-666.
34. Shi Y. A Glimpse of Structural Biology through X-Ray Crystallography. *Cell* **2014**, 159, 995-1014.
35. Clore G M and Gronenborn A M. Structures of Larger Proteins in Solution: Three- and Four-Dimensional Heteronuclear NMR Spectroscopy. *Science* **1991**, 252, 1390-1399.
36. Markwick P R L, Malliavin T and Nilges M. Structural Biology by NMR: Structure, Dynamics, and Interactions. *PLoS Comput. Biol.* **2008**, 4, e1000168.
37. Venselaar H, Joosten R P, Vrolijk B, Baakman C A B, Hekkelman M L, Krieger E and Vriend G. Homology modelling and spectroscopy, a never-ending love story. *Eur. Biophys. J.* **2010**, 39, 551-563.
38. Cho B-K, Park H-Y, Seo J-H, Kim J, Kang T-J, Lee B-S and Kim B-G. Redesigning the substrate specificity of ω -aminotransferase for the kinetic resolution of aliphatic chiral amines. *Biotechnol. Bioeng.* **2008**, 99, 275-284.
39. Cassimjee K E, Humble M S, Land H, Abedi V and Berglund P. *Chromobacterium violaceum* ω -transaminase variant Trp60Cys shows increased specificity for (S)-1-phenylethylamine and 4'-substituted acetophenones, and follows Swain-Lupton parameterisation. *Org. Biomol. Chem.* **2012**, 10, 5466-5470.
40. Terao Y, Iijima Y, Miyamoto K and Ohta H. Inversion of enantioselectivity of arylmalonate decarboxylase via site-directed mutation based on the proposed reaction mechanism. *J. Mol. Catal. B: Enzym.* **2007**, 45, 15-20.
41. Svedendahl M, Branneby C, Lindberg L and Berglund P. Reversed Enantiopreference of an ω -Transaminase by a Single-Point Mutation. *ChemCatChem* **2010**, 2, 976-980.

42. Humble M S, Cassimjee K E, Abedi V, Federsel H-J and Berglund P. Key Amino Acid Residues for Reversed or Improved Enantiospecificity of an ω -Transaminase. *ChemCatChem* **2012**, *4*, 1167-1172.
43. Yow G-Y, Watanabe A, Yoshimura T and Esaki N. Conversion of the catalytic specificity of alanine racemase to a D-amino acid aminotransferase activity by a double active-site mutation. *J. Mol. Catal. B: Enzym.* **2003**, *23*, 311-319.
44. Branneby C, Carlqvist P, Magnusson A, Hult K, Brinck T and Berglund P. Carbon-Carbon Bonds by Hydrolytic Enzymes. *J. Am. Chem. Soc.* **2003**, *125*, 874-875.
45. Hutchison C A, 3rd, Phillips S, Edgell M H, Gillam S, Jahnke P and Smith M. Mutagenesis at a specific position in a DNA sequence. *J. Biol. Chem.* **1978**, *253*, 6551-6560.
46. Papworth C, Bauer J, Braman J and Wright D. Site-directed mutagenesis in one day with > 80% efficiency. *Strategies* **1996**, *9*, 3-4.
47. Turner N J. Directed evolution of enzymes for applied biocatalysis. *Trends Biotechnol.* **2003**, *21*, 474-478.
48. Martin A R, DiSanto R, Plotnikov I, Kamat S, Shonnard D and Pannuri S. Improved activity and thermostability of (S)-aminotransferase by error-prone polymerase chain reaction for the production of a chiral amine. *Biochem. Eng. J.* **2007**, *37*, 246-255.
49. Cadwell R C and Joyce G F. Randomization of genes by PCR mutagenesis. *PCR Methods Appl.* **1992**, *2*, 28-33.
50. Chen K and Arnold F H. Enzyme Engineering for Nonaqueous Solvents: Random Mutagenesis to Enhance Activity of Subtilisin E in Polar Organic Media. *Nat. Biotechnol.* **1991**, *9*, 1073-1077.
51. Chen K and Arnold F H. Tuning the activity of an enzyme for unusual environments: sequential random mutagenesis of subtilisin E for catalysis in dimethylformamide. *Proc. Natl. Acad. Sci. USA.* **1993**, *90*, 5618-5622.
52. Stemmer W P. Rapid evolution of a protein in vitro by DNA shuffling. *Nature* **1994**, *370*, 389-391.
53. Steffens D L and Williams J G K. Efficient Site-Directed Saturation Mutagenesis Using Degenerate Oligonucleotides. *J. Biomol. Tech.* **2007**, *18*, 147-149.
54. Miyazaki K and Takenouchi M. Creating random mutagenesis libraries using megaprimer PCR of whole plasmid. *BioTechniques* **2002**, *33*, 1033-1034, 1036-1038.
55. Turner N J. Directed evolution drives the next generation of biocatalysts. *Nat. Chem. Biol.* **2009**, *5*, 567-573.
56. Astle T W. Recollections of Early Microplate Automation. *J. Assoc. Lab. Autom.* **2000**, *5*, 30-31.
57. Xiao H, Bao Z and Zhao H. High Throughput Screening and Selection Methods for Directed Enzyme Evolution. *Ind. Eng. Chem. Res.* **2015**, *54*, 4011-4020.
58. Willies S C, White J L and Turner N J. Development of a high-throughput screening method for racemase activity and its application to the identification of alanine racemase variants with activity towards L-arginine. *Tetrahedron* **2012**, *68*, 7564-7567.
59. Weiß M S, Pavlidis I V, Vickers C, Höhne M and Bornscheuer U T. Glycine Oxidase Based High-Throughput Solid-Phase Assay for Substrate Profiling and Directed Evolution of (R)- and (S)-Selective Amine Transaminases. *Anal. Chem.* **2014**, *86*, 11847-11853.
60. Rothschild L J and Mancinelli R L. Life in extreme environments. *Nature* **2001**, *409*, 1092-1101.
61. Iyer P V and Ananthanarayan L. Enzyme stability and stabilization—Aqueous and non-aqueous environment. *Process Biochem.* **2008**, *43*, 1019-1032.

62. Stepankova V, Bidmanova S, Koudelakova T, Prokop Z, Chaloupkova R and Damborsky J. Strategies for Stabilization of Enzymes in Organic Solvents. *ACS Catal.* **2013**, *3*, 2823-2836.
63. Klibanov A M. Improving enzymes by using them in organic solvents. *Nature* **2001**, *409*, 241-246.
64. Manning M C, Chou D K, Murphy B M, Payne R W and Katayama D S. Stability of protein pharmaceuticals: An update. *Pharm. Res.* **2010**, *27*, 544-575.
65. Ó'Fágáin C. Engineering Protein Stability. In Protein Chromatography: Methods and Protocols, Walls, D.; Loughran, T. S., Eds. Humana Press, **2011**, 103-136.
66. Dombkowski A A, Sultana K Z and Craig D B. Protein disulfide engineering. *FEBS Lett.* **2014**, *588*, 206-212.
67. Sheldon R A. Enzyme Immobilization: The Quest for Optimum Performance. *Adv. Synth. Catal.* **2007**, *349*, 1289-1307.
68. Mohamad N R, Marzuki N H C, Buang N A, Huyop F and Wahab R A. An overview of technologies for immobilization of enzymes and surface analysis techniques for immobilized enzymes. *Biotechnol. Biotechnol. Equip.* **2015**, *29*, 205-220.
69. Langer L J and Engel L L. Human Placental Estradiol-17 β Dehydrogenase: I. Concentration, Characterization and Assay. *J. Biol. Chem.* **1958**, *233*, 583-588.
70. Chen S, Land H, Berglund P and Humble M S. Stabilization of an amine transaminase for biocatalysis. *J. Mol. Catal. B: Enzym.* **2016**, *124*, 20-28.
71. Ghislieri D and Turner N J. Biocatalytic Approaches to the Synthesis of Enantiomerically Pure Chiral Amines. *Top. Catal.* **2014**, *57*, 284-300.
72. Kohls H, Steffen-Munsberg F and Höhne M. Recent achievements in developing the biocatalytic toolbox for chiral amine synthesis. *Curr. Opin. Chem. Biol.* **2014**, *19*, 180-192.
73. Kurihara N. Chirality and Chiral Pesticides. In Encyclopedia of Agrochemicals, John Wiley & Sons, Inc., **2003**.
74. Nugent T C. Chiral Amine Synthesis: Methods, Developments and Applications. John Wiley & Sons, Inc., **2010**.
75. Nguyen L A, He H and Pham-Huy C. Chiral Drugs: An Overview. *Int. J. Biomed. Sci.* **2006**, *2*, 85-100.
76. Tombo G M R and Belluš D. Chirality and Crop Protection. *Angew. Chem. Int. Ed.* **1991**, *30*, 1193-1215.
77. Torres P U. Cinacalcet HCl: A Novel Treatment for Secondary Hyperparathyroidism Caused by Chronic Kidney Disease. *J. Renal Nutr.* **2006**, *16*, 253-258.
78. Wilde M I and McTavish D. Tamsulosin. *Drugs* **1996**, *52*, 883-898.
79. Jann M W. Rivastigmine, a New-Generation Cholinesterase Inhibitor for the Treatment of Alzheimer's Disease. *Pharmacotherapy* **2000**, *20*, 1-12.
80. Murdoch D and McTavish D. Sertraline. *Drugs* **1992**, *44*, 604-624.
81. Herman G A, Stevens C, Van Dyck K, Bergman A, Yi B, De Smet M, Snyder K, Hilliard D, Tanen M, Tanaka W, Wang A Q, Zeng W, Musson D, Winchell G, Davies M J, Ramael S, Gottesdiener K M and Wagner J A. Pharmacokinetics and pharmacodynamics of sitagliptin, an inhibitor of dipeptidyl peptidase IV, in healthy subjects: Results from two randomized, double-blind, placebo-controlled studies with single oral doses. *Clin. Pharmacol. Ther.* **2005**, *78*, 675-688.
82. Steer C, Froelich J, Soutullo C A, Johnson M and Shaw M. Lisdexamfetamine Dimesylate. *CNS Drugs* **2012**, *26*, 691-705.
83. Herbert J M, Frehel D, Vallee E, Kieffer G, Gouy D, Berger Y, Necciari J, Defreyn G and Maffrand J P. Clopidogrel, A Novel Antiplatelet and Antithrombotic Agent. *Cardiovasc. Drug Rev.* **1993**, *11*, 180-198.
84. Simon R C, Grischek B, Zepeck F, Steinreiber A, Belaj F and Kroutil W. Regio- and Stereoselective Monoamination of Diketones without Protecting Groups. *Angew. Chem. Int. Ed.* **2012**, *51*, 6713-6716.

85. Duke S O and Dayan F E. Modes of Action of Microbially-Produced Phytotoxins. *Toxins* **2011**, 3, 1038.
86. Morohashi M, Nagasawa S, Enya N, Suzuki K, Kose T and Kawata K. Behavior of Bromobutide in Paddy Water and Soil After Application. *B. Environ. Contam. Tox.* **2012**, 88, 521-525.
87. Frost E H, Shutler D and Hillier N K. Effects of fluvalinate on honey bee learning, memory, responsiveness to sucrose, and survival. *J. Exp. Biol.* **2013**, 216, 2931-2938.
88. Reetz M T and Dreisbach C. Highly Efficient Lipase-Catalyzed Kinetic Resolution of Chiral Amines. *Chimia* **1994**, 48, 570-570.
89. Öhrner N, Orrenius C, Mattson A, Norin T and Hult K. Kinetic resolutions of amine and thiol analogues of secondary alcohols catalyzed by the *Candida antarctica* lipase B. *Enzyme Microb. Tech.* **1996**, 19, 328-331.
90. Balkenhohl F, Ditrich K, Hauer B and Ladner W. Optisch aktive Amine durch Lipase-katalysierte Methoxyacetylierung. *J. prakt. Chem.* **1997**, 339, 381-384.
91. Reetz M T and Schimossek K. Lipase-Catalyzed Dynamic Kinetic Resolution of Chiral Amines: Use of Palladium as the Racemization Catalyst. *Chimia* **1996**, 50, 668-669.
92. Paetzold J and Bäckvall J E. Chemoenzymatic Dynamic Kinetic Resolution of Primary Amines. *J. Am. Chem. Soc.* **2005**, 127, 17620-17621.
93. Shin J-S and Kim B-G. Optical Resolution of Racemic 1-Phenylethylamine Catalyzed by Aminotransferase and Dehydrogenase. *Ann. NY Acad. Sci.* **1996**, 799, 717-724.
94. Alexeeva M, Enright A, Dawson M J, Mahmoudian M and Turner N J. Deracemization of α -methylbenzylamine using an enzyme obtained by in vitro evolution. *Angew. Chem. Int. Ed.* **2002**, 41, 3177-3180.
95. Pesnot T, Gershater M C, Ward J M and Hailes H C. The Catalytic Potential of *Coptis japonica* NCS2 Revealed – Development and Utilisation of a Fluorescamine-Based Assay. *Adv. Synth. Catal.* **2012**, 354, 2997-3008.
96. Shin J-S and Kim B-G. Asymmetric synthesis of chiral amines with ω -transaminase. *Biotechnol. Bioeng.* **1999**, 65, 206-211.
97. Fuchs M, Farnberger J E and Kroutil W. The Industrial Age of Biocatalytic Transamination. *Eur. J. Org. Chem.* **2015**, 2015, 6965-6982.
98. Abrahamson M J, Vazquez-Figueroa E, Woodall N B, Moore J C and Bommarius A S. Development of an Amine Dehydrogenase for Synthesis of Chiral Amines. *Angew. Chem. Int. Ed.* **2012**, 51, 3969-3972, S3969/3961-S3969/3967.
99. Abrahamson M J, Wong J W and Bommarius A S. The Evolution of an Amine Dehydrogenase Biocatalyst for the Asymmetric Production of Chiral Amines. *Adv. Synth. Catal.* **2013**, 355, 1780-1786.
100. Mitsukura K, Suzuki M, Shinoda S, Kuramoto T, Yoshida T and Nagasawa T. Purification and Characterization of a Novel (*R*)-Imine Reductase from *Streptomyces* sp. GF3587. *Biosci. Biotechnol. Biochem.* **2011**, 75, 1778-1782.
101. Mitsukura K, Kuramoto T, Yoshida T, Kimoto N, Yamamoto H and Nagasawa T. A NADPH-dependent (*S*)-imine reductase (SIR) from *Streptomyces* sp. GF3546 for asymmetric synthesis of optically active amines: purification, characterization, gene cloning, and expression. *Appl. Microbiol. Biotechnol.* **2013**, 97, 8079-8086.
102. McIntosh J A, Coelho P S, Farwell C C, Wang Z J, Lewis J C, Brown T R and Arnold F H. Enantioselective Intramolecular C-H Amination Catalyzed by Engineered Cytochrome P450 Enzymes In Vitro and In Vivo. *Angew. Chem. Int. Ed.* **2013**, 52, 9309-9312.
103. Farwell C C, Zhang R K, McIntosh J A, Hyster T K and Arnold F H. Enantioselective Enzyme-Catalyzed Aziridination Enabled by Active-Site Evolution of a Cytochrome P450. *ACS Cent. Sci.* **2015**, 1, 89-93.
104. Mathew S and Yun H. ω -Transaminases for the Production of Optically Pure Amines and Unnatural Amino Acids. *ACS Catal.* **2012**, 2, 993-1001.

105. Guo F and Berglund P. Transaminase Biocatalysis: Optimization and Application. *Green Chem.* **2016**, DOI: 10.1039/C1036GC02328B.
106. Steffen-Munsberg F, Vickers C, Kohls H, Land H, Mallin H, Nobili A, Skalden L, van den Bergh T, Joosten H J, Berglund P, Höhne M and Bornscheuer U T. Bioinformatic analysis of a PLP-dependent enzyme superfamily suitable for biocatalytic applications. *Biotechnol. Adv.* **2015**, *33*, 566-604.
107. Eliot A C and Kirsch J F. Pyridoxal phosphate enzymes: mechanistic, structural, and evolutionary considerations. *Annu. Rev. Biochem.* **2004**, *73*, 383-415.
108. Cassimjee K E, Humble M S, Miceli V, Colomina C G and Berglund P. Active Site Quantification of an ω -Transaminase by Performing a Half Transamination Reaction. *ACS Catal.* **2011**, *1*, 1051-1055.
109. Kirsch J F, Eichele G, Ford G C, Vincent M G, Jansonius J N, Gehring H and Christen P. Mechanism of action of aspartate aminotransferase proposed on the basis of its spatial structure. *J. Mol. Biol.* **1984**, *174*, 497-525.
110. Cassimjee K E, Manta B and Himo F. A quantum chemical study of the ω -transaminase reaction mechanism. *Org. Biomol. Chem.* **2015**, *13*, 8453-8464.
111. van Oosterwijk N, Willies S, Hekelaar J, Terwisscha van Scheltinga A C, Turner N J and Dijkstra B W. Structural Basis of the Substrate Range and Enantioselectivity of Two (S)-Selective ω -Transaminases. *Biochemistry* **2016**, *55*, 4422-4431.
112. Shin J-S and Kim B-G. Exploring the Active Site of Amine:Pyruvate Aminotransferase on the Basis of the Substrate Structure-Reactivity Relationship: How the Enzyme Controls Substrate Specificity and Stereoselectivity. *J. Org. Chem.* **2002**, *67*, 2848-2853.
113. Höhne M, Schätzle S, Jochens H, Robins K and Bornscheuer U T. Rational assignment of key motifs for function guides in silico enzyme identification. *Nat. Chem. Biol.* **2010**, *6*, 807-813.
114. Yoshimura T, Jhee K-H and Soda K. Stereospecificity for the Hydrogen Transfer and Molecular Evolution of Pyridoxal Enzymes. *Biosci. Biotech. Biochem.* **1996**, *60*, 181-187.
115. Shin J S and Kim B G. Kinetic modeling of ω -transamination for enzymatic kinetic resolution of α -methylbenzylamine. *Biotechnol. Bioeng.* **1998**, *60*, 534-540.
116. Koszelewski D, Lavandera I, Clay D, Rozzell D and Kroutil W. Asymmetric Synthesis of Optically Pure Pharmacologically Relevant Amines Employing ω -Transaminases. *Adv. Synth. Catal.* **2008**, *350*, 2761-2766.
117. Koszelewski D, Lavandera I, Clay D, Guebitz G M, Rozzell D and Kroutil W. Formal asymmetric biocatalytic reductive amination. *Angew. Chem. Int. Ed.* **2008**, *47*, 9337-9340.
118. Höhne M, Kühl S, Robins K and Bornscheuer U T. Efficient Asymmetric Synthesis of Chiral Amines by Combining Transaminase and Pyruvate Decarboxylase. *ChemBioChem* **2008**, *9*, 363-365.
119. Cassimjee K E, Branneby C, Abedi V, Wells A and Berglund P. Transaminations with isopropyl amine: equilibrium displacement with yeast alcohol dehydrogenase coupled to in situ cofactor regeneration. *Chem. Commun.* **2010**, *46*, 5569-5571.
120. Wang B, Land H and Berglund P. An efficient single-enzymatic cascade for asymmetric synthesis of chiral amines catalyzed by ω -transaminase. *Chem. Commun.* **2013**, *49*, 161-163.
121. Green A P, Turner N J and O'Reilly E. Chiral amine synthesis using ω -transaminases: an amine donor that displaces equilibria and enables high-throughput screening. *Angew. Chem. Int. Ed.* **2014**, *53*, 10714-10717.
122. Martínez-Montero L, Gotor V, Gotor-Fernández V and Lavandera I. But-2-ene-1,4-diamine and But-2-ene-1,4-diol as Donors for Thermodynamically Favored Transaminase- and Alcohol Dehydrogenase-Catalyzed Processes. *Adv. Synth. Catal.* **2016**, *358*, 1618-1624.

123. Gomm A, Lewis W, Green A P and O'Reilly E. A New Generation of Smart Amine Donors for Transaminase-Mediated Biotransformations. *Chemistry* **2016**, *22*, 12692-12695.
124. Galman J L, Slabu I, Weise N J, Iglesias C, Parmeggiani F, Lloyd R C and Turner N J. Biocatalytic transamination with near-stoichiometric inexpensive amine donors mediated by bifunctional mono- and di-amine transaminases. *Green Chem.* **2016**, DOI: 10.1039/C1036GC02102F.
125. Matcham G, Bhatia M, Lang W, Lewis C, Nelson R, Wang A and Wu W. Enzyme and Reaction Engineering in Biocatalysis: Synthesis of (S)-Methoxyisopropylamine (= (S)-1-Methoxypropan-2-amine). *Chimia* **1999**, *53*, 584-589.
126. Yun H and Kim B G. Asymmetric synthesis of (S)- α -methylbenzylamine by recombinant *Escherichia coli* co-expressing omega-transaminase and acetolactate synthase. *Biosci. Biotechnol. Biochem.* **2008**, *72*, 3030-3033.
127. Truppo M D, Rozzell J D and Turner N J. Efficient Production of Enantiomerically Pure Chiral Amines at Concentrations of 50 g/L Using Transaminases. *Org. Process Res. Dev.* **2010**, *14*, 234-237.
128. Land H, Hendil-Forsell P, Martinelle M and Berglund P. One-pot biocatalytic amine transaminase/acyl transferase cascade for aqueous formation of amides from aldehydes or ketones. *Catal. Sci. Technol.* **2016**, *6*, 2897-2900.
129. Watanabe N, Sakabe K, Sakabe N, Higashi T, Sasaki K, Aibara S, Morita Y, Yonaha K, Toyama S and Fukutani H. Crystal Structure Analysis of ω -Amino Acid:Pyruvate Aminotransferase with a Newly Developed Weissenberg Camera and an Imaging Plate Using Synchrotron Radiation. *J. Biochem.* **1989**, *105*, 1-3.
130. Humble M S, Cassimjee K E, Håkansson M, Kimbung Y R, Walse B, Abedi V, Federsel H-J, Berglund P and Logan D T. Crystal structures of the *Chromobacterium violaceum* ω -transaminase reveal major structural rearrangements upon binding of coenzyme PLP. *FEBS J.* **2012**, *279*, 779-792.
131. Midelfort K S, Kumar R, Han S, Karmilowicz M J, McConnell K, Gehlhaar D K, Mistry A, Chang J S, Anderson M, Villalobos A, Minshull J, Govindarajan S and Wong J W. Redesigning and characterizing the substrate specificity and activity of *Vibrio fluvialis* aminotransferase for the synthesis of imigabalin. *Protein Eng. Des. Sel.* **2013**, *26*, 25-33.
132. Steffen-Munsberg F, Vickers C, Thontowi A, Schätzle S, Tumlirsch T, Humble M S, Land H, Berglund P, Bornscheuer U T and Höhne M. Connecting Unexplored Protein Crystal Structures to Enzymatic Function. *ChemCatChem* **2013**, *5*, 150-153.
133. Steffen-Munsberg F, Vickers C, Thontowi A, Schätzle S, Meinhardt T, Humble M S, Land H, Berglund P, Bornscheuer U T and Höhne M. Revealing the Structural Basis of Promiscuous Amine Transaminase Activity. *ChemCatChem* **2013**, *5*, 154-157.
134. Rausch C, Lerchner A, Schiefner A and Skerra A. Crystal structure of the ω -aminotransferase from *Paracoccus denitrificans* and its phylogenetic relationship with other class III amino- transferases that have biotechnological potential. *Proteins* **2013**, *81*, 774-787.
135. Sayer C, Isupov M N, Westlake A and Littlechild J A. Structural studies of *Pseudomonas* and *Chromobacterium* ω -aminotransferases provide insights into their differing substrate specificity. *Acta Crystallogr. D Biol. Crystallogr.* **2013**, *69*, 564-576.
136. Lyskowski A, Gruber C, Steinkellner G, Schürmann M, Schwab H, Gruber K and Steiner K. Crystal Structure of an (R)-Selective ω -Transaminase from *Aspergillus terreus*. *PLoS One* **2014**, *9*, e87350.
137. Thomsen M, Skalden L, Palm G J, Höhne M, Bornscheuer U T and Hinrichs W. Crystallographic characterization of the (R)-selective amine transaminase from *Aspergillus fumigatus*. *Acta Crystallogr. D Biol. Crystallogr.* **2014**, *70*, 1086-1093.

138. Sayer C, Martinez-Torres R J, Richter N, Isupov M N, Hailes H C, Littlechild J A and Ward J M. The substrate specificity, enantioselectivity and structure of the (*R*)-selective amine : pyruvate transaminase from *Nectria haematococca*. *FEBS J.* **2014**, *281*, 2240-2253.
139. Skalden L, Thomsen M, Höhne M, Bornscheuer U T and Hinrichs W. Structural and biochemical characterization of the dual substrate recognition of the (*R*)-selective amine transaminase from *Aspergillus fumigatus*. *FEBS J.* **2015**, *282*, 407-415.
140. Guan L-J, Ohtsuka J, Okai M, Miyakawa T, Mase T, Zhi Y, Hou F, Ito N, Iwasaki A, Yasohara Y and Tanokura M. A new target region for changing the substrate specificity of amine transaminases. *Sci. Rep.* **2015**, *5*, 10753.
141. Cuetos A, García-Ramos M, Fischereder E-M, Díaz-Rodríguez A, Grogan G, Gotor V, Kroutil W and Lavandera I. Catalytic Promiscuity of Transaminases: Preparation of Enantioenriched β -Fluoroamines by Formal Tandem Hydrodefluorination/Deamination. *Angew. Chem. Int. Ed.* **2016**, *55*, 3144-3147.
142. Shin J-S, Kim B-G and Shin D-H. Kinetic resolution of chiral amines using packed-bed reactor. *Enzyme Microb. Technol.* **2001**, *29*, 232-239.
143. Yi S-S, Lee C-w, Kim J, Kyung D, Kim B-G and Lee Y-S. Covalent immobilization of ω -transaminase from *Vibrio fluvialis* JS17 on chitosan beads. *Process Biochem.* **2007**, *42*, 895-898.
144. Koszelewski D, Müller N, Schrittwieser J H, Faber K and Kroutil W. Immobilization of ω -transaminases by encapsulation in a sol-gel/celite matrix. *J. Mol. Catal. B: Enzym.* **2010**, *63*, 39-44.
145. Cárdenas-Fernández M, Neto W, López C, Álvaro G, Tufvesson P and Woodley J M. Immobilization of *Escherichia coli* containing ω -transaminase activity in LentiKats®. *Biotechnol. Progress* **2012**, *28*, 693-698.
146. Ni K, Zhou X, Zhao L, Wang H, Ren Y and Wei D. Magnetic Catechol-Chitosan with Bioinspired Adhesive Surface: Preparation and Immobilization of ω -Transaminase. *PLoS One* **2012**, *7*, e41101.
147. Rehn G, Grey C, Branneby C, Lindberg L and Adlercreutz P. Activity and stability of different immobilized preparations of recombinant *E. coli* cells containing ω -transaminase. *Process Biochem.* **2012**, *47*, 1129-1134.
148. Truppo M D, Strotman H and Hughes G. Development of an Immobilized Transaminase Capable of Operating in Organic Solvent. *ChemCatChem* **2012**, *4*, 1071-1074.
149. Mallin H, Menyes U, Vorhaben T, Höhne M and Bornscheuer U T. Immobilization of two (*R*)-Amine Transaminases on an Optimized Chitosan Support for the Enzymatic Synthesis of Optically Pure Amines. *ChemCatChem* **2013**, *5*, 588-593.
150. Halim A A, Szita N and Baganz F. Characterization and multi-step transketolase- ω -transaminase bioconversions in an immobilized enzyme microreactor (IEMR) with packed tube. *J. Biotechnol.* **2013**, *168*, 567-575.
151. Mallin H, Muschiol J, Byström E and Bornscheuer U T. Efficient Biocatalysis with Immobilized Enzymes or Encapsulated Whole Cell Microorganism by Using the SpinChem Reactor System. *ChemCatChem* **2013**, *5*, 3529-3532.
152. Andrade L H, Kroutil W and Jamison T F. Continuous Flow Synthesis of Chiral Amines in Organic Solvents: Immobilization of *E. coli* Cells Containing Both ω -Transaminase and PLP. *Org. Lett.* **2014**, *16*, 6092-6095.
153. Gustavsson M, Muraleedharan M N and Larsson G. Surface Expression of ω -Transaminase in *Escherichia coli*. *Appl. Environ. Microbiol.* **2014**, *80*, 2293-2298.
154. Cassimjee K E, Kadow M, Wikmark Y, Humble M S, Rothstein M L, Rothstein D M and Bäckvall J E. A general protein purification and immobilization method on controlled porosity glass: biocatalytic applications. *Chem. Commun.* **2014**, *50*, 9134-9137.
155. Neto W, Schürmann M, Panella L, Vogel A and Woodley J M. Immobilisation of ω -transaminase for industrial application: Screening and characterisation of

- commercial ready to use enzyme carriers. *J. Mol. Catal. B: Enzym.* **2015**, *117*, 54-61.
156. Hwang B-Y and Kim B-G. High-throughput screening method for the identification of active and enantioselective ω -transaminases. *Enzyme Microb. Technol.* **2004**, *34*, 429-436.
157. Yun H, Hwang B-Y, Lee J-H and Kim B-G. Use of Enrichment Culture for Directed Evolution of the *Vibrio fluvialis* JS17 ω -Transaminase, Which Is Resistant to Product Inhibition by Aliphatic Ketones. *Appl. Environ. Microbiol.* **2005**, *71*, 4220-4224.
158. Truppo M D, Rozzell J D, Moore J C and Turner N J. Rapid screening and scale-up of transaminase catalysed reactions. *Org. Biomol. Chem.* **2009**, *7*, 395-398.
159. Schätzle S, Höhne M, Redestad E, Robins K and Bornscheuer U T. Rapid and sensitive kinetic assay for characterization of ω -transaminases. *Anal. Chem.* **2009**, *81*, 8244-8248.
160. Schätzle S, Höhne M, Robins K and Bornscheuer U T. Conductometric Method for the Rapid Characterization of the Substrate Specificity of Amine-Transaminases. *Anal. Chem.* **2010**, *82*, 2082-2086.
161. Hopwood J, Truppo M D, Turner N J and Lloyd R C. A fast and sensitive assay for measuring the activity and enantioselectivity of transaminases. *Chem. Commun.* **2011**, *47*, 773-775.
162. Sehl T, Simon Robert C, Hailes Helen C, Ward John M, Schell U, Pohl M and Rother D. TTC-based screening assay for ω -transaminases: a rapid method to detect reduction of 2-hydroxy ketones. *J. Biotechnol.* **2012**, *159*, 188-194.
163. Mathew S, Shin G, Shon M and Yun H. High throughput screening methods for ω -transaminases. *Biotechnol. Bioprocess Eng.* **2013**, *18*, 1-7.
164. Scheidt T, Land H, Anderson M, Chen Y, Berglund P, Yi D and Fessner W-D. Fluorescence-Based Kinetic Assay for High-Throughput Discovery and Engineering of Stereoselective ω -Transaminases. *Adv. Synth. Catal.* **2015**, *357*, 1721-1731.
165. Harding V J and MacLean R M. The Ninhydrin Reaction with Amines and Amides. *J. Biol. Chem.* **1916**, *25*, 337-350.
166. Pocklington T and Jeffery J. Competition of two substrates for a single enzyme. A simple kinetic theorem exemplified by a hydroxy steroid dehydrogenase reaction. *Biochem. J.* **1969**, *112*, 331-334.
167. Seo J H, Kyung D, Joo K, Lee J and Kim B G. Necessary and sufficient conditions for the asymmetric synthesis of chiral amines using ω -aminotransferases. *Biotechnol. Bioeng.* **2011**, *108*, 253-263.
168. Simon R C, Richter N, Busto E and Kroutil W. Recent developments of cascade reactions involving ω -transaminases. *ACS Catal.* **2014**, *4*, 129-143.
169. Anderson M, Afewerki S, Berglund P and Córdova A. Total Synthesis of Capsaicin Analogues from Lignin-Derived Compounds by Combined Heterogeneous Metal, Organocatalytic and Enzymatic Cascades in One Pot. *Adv. Synth. Catal.* **2014**, *356*, 2113-2118.
170. Oyama K, Kihara K-i and Nonaka Y. On the mechanism of the action of thermolysin: kinetic study of the thermolysin-catalysed condensation reaction of N-benzyloxycarbonyl-L-aspartic acid with L-phenylalanine methyl ester. *J. Chem. Soc., Perkin Trans. 2* **1981**, 356-360.
171. Guranda D T, van Langen L M, van Rantwijk F, Sheldon R A and Švedas V K. Highly efficient and enantioselective enzymatic acylation of amines in aqueous medium. *Tetrahedron: Asymmetr.* **2001**, *12*, 1645-1650.
172. Neang P M, Subileau M, Perrier V and Dubreucq E. Peculiar features of four enzymes of the CaLA superfamily in aqueous media: Differences in substrate specificities and abilities to catalyze alcoholysis. *J. Mol. Catal. B: Enzym.* **2013**, *94*, 36-46.

173. Sattler J H, Fuchs M, Mutti F G, Grischek B, Engel P, Pfeffer J, Woodley J M and Kroutil W. Introducing an in situ capping strategy in systems biocatalysis to access 6-aminohexanoic acid. *Angew. Chem. Int. Ed.* **2014**, *53*, 14153-14157.
174. Schmidt S, Scherkus C, Muschiol J, Menyess U, Winkler T, Hummel W, Gröger H, Liese A, Herz H-G and Bornscheuer U T. An Enzyme Cascade Synthesis of ϵ -Caprolactone and its Oligomers. *Angew. Chem. Int. Ed.* **2015**, *54*, 2784-2787.
175. Mathews I, Soltis M, Saldajeno M, Ganshaw G, Sala R, Weyler W, Cervin M A, Whited G and Bott R. Structure of a novel enzyme that catalyzes acyl transfer to alcohols in aqueous conditions. *Biochemistry* **2007**, *46*, 8969-8979.
176. Wiermans L, Hofzumahaus S, Schotten C, Weigand L, Schallmey M, Schallmey A and Domínguez de María P. Transesterifications and Peracid-Assisted Oxidations in Aqueous Media Catalyzed by Mycobacterium smegmatis Acyl Transferase. *ChemCatChem* **2013**, *5*, 3719-3724.
177. Veld M A J, Hult K, Palmans A R A and Meijer E W. Fast DKR of Amines Using Isopropyl 2-Methoxyacetate as Acyl Donor. *Eur. J. Org. Chem.* **2007**, *2007*, 5416-5421.
178. Thalén L K and Bäckvall J-E. Development of dynamic kinetic resolution on large scale for (\pm)-1-phenylethylamine. *Beilstein J. Org. Chem.* **2010**, *6*, 823-829.
179. Cammenberg M, Hult K and Park S. Molecular basis for the enhanced lipase-catalyzed *N*-acylation of 1-phenylethylamine with methoxyacetate. *ChemBioChem* **2006**, *7*, 1745-1749.
180. Wierzychowski J, Dafeldecker W P, Holmquist B and Vallee B L. Fluorometric assays for isozymes of human alcohol dehydrogenase. *Anal. Biochem.* **1989**, *178*, 57-62.
181. List B, Barbas C F and Lerner R A. Aldol sensors for the rapid generation of tunable fluorescence by antibody catalysis. *Proc. Natl. Acad. Sci. USA.* **1998**, *95*, 15351-15355.
182. Grignard V. Sur quelques nouvelles combinaisons organométalliques du magnésium et leur application à des synthèses d'alcools et d'hydrocarbures. *Compt. Rend.* **1900**, *130*, 1322-1325.
183. Bowden K, Heilbron I M, Jones E R H and Weedon B C L. 13. Researches on acetylenic compounds. Part I. The preparation of acetylenic ketones by oxidation of acetylenic carbinols and glycols. *J. Chem. Soc.* **1946**, 39-45.
184. Miriyala B, Bhattacharyya S and Williamson J S. Chemoselective reductive alkylation of ammonia with carbonyl compounds: synthesis of primary and symmetrical secondary amines. *Tetrahedron* **2004**, *60*, 1463-1471.
185. Hofmann A W. Ueber die Einwirkung des Broms in alkalischer Lösung auf Amide. *Ber. Dtsch. Chem. Ges.* **1881**, *14*, 2725-2736.
186. Analytical Methods C. Recommendations for the definition, estimation and use of the detection limit. *Analyst* **1987**, *112*, 199-204.
187. Zhang J-H, Chung T D Y and Oldenburg K R. A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. *J. Biomol. Screen.* **1999**, *4*, 67-73.
188. Chen Y, Yi D, Jiang S and Wei D. Identification of novel thermostable taurine-pyruvate transaminase from *Geobacillus thermodenitrificans* for chiral amine synthesis. *Appl. Microbiol. Biotechnol.* **2016**, *100*, 3101-3111.
189. Nobili A, Steffen-Munsberg F, Kohls H, Trentin I, Schulzke C, Höhne M and Bornscheuer U T. Engineering the Active Site of the Amine Transaminase from *Vibrio fluvialis* for the Asymmetric Synthesis of Aryl-Alkyl Amines and Amino Alcohols. *ChemCatChem* **2015**, *7*, 757-760.
190. Pavlidis I V, Weiß M S, Genz M, Spurr P, Hanlon S P, Wirz B, Iding H and Bornscheuer U T. Identification of (*S*)-selective transaminases for the asymmetric synthesis of bulky chiral amines. *Nat. Chem.* **2016**, DOI: 10.1038/nchem.2578.
191. Genz M, Melse O, Schmidt S, Vickers C, Dörr M, van den Bergh T, Joosten H-J and Bornscheuer U T. Engineering the Amine Transaminase from *Vibrio fluvialis*

- towards Branched-Chain Substrates. *ChemCatChem* **2016**, DOI: 10.1002/cctc.201601007.
192. Niesen F H, Berglund H and Vedadi M. The use of differential scanning fluorimetry to detect ligand interactions that promote protein stability. *Nat. Protocols* **2007**, *2*, 2212-2221.
193. Fernandez-Lafuente R. Stabilization of multimeric enzymes: Strategies to prevent subunit dissociation. *Enzyme Microb. Technol.* **2009**, *45*, 405-418.
194. Yun H, Kim J, Kinnera K and Kim B G. Synthesis of enantiomerically pure trans-(1R,2R)- and cis-(1S,2R)-1-amino-2-indanol by lipase and ω -transaminase. *Biotechnol. Bioeng.* **2006**, *93*, 391-395.
195. Mutti F G and Kroutil W. Asymmetric Bio-amination of Ketones in Organic Solvents. *Adv. Synth. Catal.* **2012**, *354*, 3409-3413.
196. Crowe J H, Carpenter J F, Crowe L M and Anchordoguy T J. Are freezing and dehydration similar stress vectors? A comparison of modes of interaction of stabilizing solutes with biomolecules. *Cryobiology* **1990**, *27*, 219-231.
197. Carpenter J F, Prestrelski S J and Arakawa T. Separation of Freezing- and Drying-Induced Denaturation of Lyophilized Proteins Using Stress-Specific Stabilization. *Arch. Biochem. Biophys.* **1993**, *303*, 456-464.
198. Bordusa F. Proteases in organic synthesis. *Chem. Rev.* **2002**, *102*, 4817-4867.
199. Imamura K, Murai K, Korehisa T, Shimizu N, Yamahira R, Matsuura T, Tada H, Imanaka H, Ishida N and Nakanishi K. Characteristics of Sugar Surfactants in Stabilizing Proteins During Freeze-Thawing and Freeze-Drying. *J. Pharm. Sci.* **2014**, *103*, 1628-1637.
200. Borén L, Martín-Matute B, Xu Y, Córdova A and Bäckvall J-E. (S)-Selective Kinetic Resolution and Chemoenzymatic Dynamic Kinetic Resolution of Secondary Alcohols. *Chemistry* **2006**, *12*, 225-232.
201. Neet K E and Timm D E. Conformational stability of dimeric proteins: Quantitative studies by equilibrium denaturation. *Protein Sci.* **1994**, *3*, 2167-2174.
202. Sakurai K, Oobatake M and Goto Y. Salt-dependent monomer-dimer equilibrium of bovine β -lactoglobulin at pH 3. *Protein Sci.* **2001**, *10*, 2325-2335.
203. Denesyuk A I, Denessiouk K A, Korpela T and Johnson M S. Functional attributes of the phosphate group binding cup of pyridoxal phosphate-dependent enzymes. *J. Mol. Biol.* **2002**, *316*, 155-172.
204. Desai A A. Sitagliptin Manufacture: A Compelling Tale of Green Chemistry, Process Intensification, and Industrial Asymmetric Catalysis. *Angew. Chem. Int. Ed.* **2011**, *50*, 1974-1976.
205. Taft R W. Polar and Steric Substituent Constants for Aliphatic and o-Benzoate Groups from Rates of Esterification and Hydrolysis of Esters. *J. Am. Chem. Soc.* **1952**, *74*, 3120-3128.
206. Lehmann J and Lloyd-Jones G C. Regiocontrol and Stereoselectivity in Tungsten-Bipyridine Catalysed Allylic Alkylation. *Tetrahedron* **1995**, *51*, 8863-8874.
207. Armstrong P B, Dembicer E A, DesBois A J, Fitzgerald J T, Gehrman J K, Nelson N C, Noble A L and Bunt R C. Investigation of the Electronic Origin of Asymmetric Induction in Palladium-Catalyzed Allylic Substitutions with Phosphinooxazoline (PHOX) Ligands by Hammett and Swain-Lupton Analysis of the ^{13}C NMR Chemical Shifts of the (π -Allyl)palladium Intermediates. *Organometallics* **2012**, *31*, 6933-6946.