Enantioselective biocatalysis for the preparation of optically pure tertiary alcohols

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Table of contents

List of abbreviations and symbols

%	percent
$\Delta\Delta G^{\#}$	differences in Gibbs free energy of activation
ΔG	Gibbs free energy
$\Delta G^{\#}$	Gibbs free energy of activation
°C	degree Celsius
3D	three-dimensional
ABHDH	the α/β-Hydrolase Fold 3DM Database (3DM in short)
BS2	esterase BS2 from *Bacillus subtilis*
c	conversion
DMAP	4-Dimethylaminopyridine
DMF	dimethyl formamide
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
E	enantioselectivity; E-value; enantiomeric ratio
E.coli	*Escherichia coli*
E.C.	Enzyme Commission
ee	enantiomeric excess
ee_P	enantiomeric excess of the product
ee_S	enantiomeric excess of the substrate
g	gram
GC	gas chromatography
h	hour
k_{cat}	"turnover number"
kg	kilogram
K_M	Michaelis-Menten constant
l	liter
m	meter
MCR	multicomponent reaction
mol	$6.022*10^{23}$
NMR	nuclear magnetic resonance
pdb	Brookhaven protein database
PestE	esterase from *Pyrobaculum calidifontis*
PFE	esterase from *Psueudomonas fluorescens*
PLE	pig liver esterase
pNP	*p*-nitro phenol
pNPA	*p*-nitro phenyl acetate
R	gas constant [8.31 J mol^{-1} K^{-1}]
RT	room temperature
S_N1	nucleophilic substitution proceeding by the first-order kinetics
S_N2	nucleophilic substitution proceeding by the second-order kinetics
TBS	t*ert*-butyldimethylsilyl
THF	tetrahydrofuran
TI	tetrahedral intermediate
U	unit [µmolmin^{-1}]
UV	ultra violet
v	reaction rate
v_{max}	maximal reaction rate in the Michaelis-Menten equation

List of articles

Article I. Nguyen, G.S, Thompson, M.L, Grogan, G., Bornscheuer, U.T, Kourist, R. Identification of novel esterases for the synthesis of sterically demanding chiral alcohols by sequence-structure guided genome mining. *Manuscript in preparation.*

Article II. Bassegoda, A., Nguyen, G.S., Kourist, R., Schmidt, M., Diaz, P., Bornscheuer, U.T. (2010), Rational protein design of *Paenibacillus barcinonensis* esterase EstA for kinetic resolution of tertiary alcohols, *ChemCatChem*, 2, 962-967

Article III. Kourist, R., Nguyen, G.S., Strübing, S., Böttcher, D., Liebeton, E., Eck, J., Naumer, C., Bornscheuer, U.T. (2008), Hydrolase-catalyzed stereoselective preparation of protected α,α-dialkyl-α-hydroxycarboxylic acids, *Tetrahedron: Asymmetry*, 19, 1839-1843

Article IV. Nguyen, G.S., Kourist, R., Paravidino, M., Hummel, A., Rehdorf, J., Orru, R.V.A., Hanefeld, U., Bornscheuer, U.T. (2010), An enzymatic toolbox for the kinetic resolution of 2-(pyridin-x-yl)but-3-yn-2-ols and tertiary cyanohydrins, *Eur. J. Org. Chem.*, 2753-2758.

Article V. Theurer, M., Fischer, P., Baro, A., Nguyen, G.S., Kourist, R., Bornscheuer, U.T., Laschat, S. (2010), Formation of chiral tertiary homoallylic alcohols via Evans aldol reaction or enzymatic resolution and their influence on the Sharpless asymmetric dihydroxylation, *Tetrahedron*, 66, 3814-3823.

1. Introduction

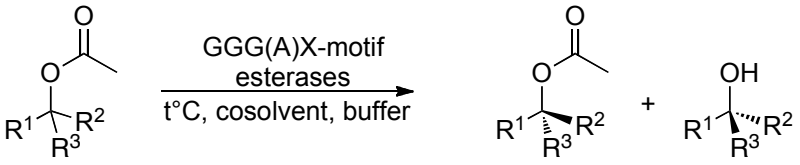
The first applied biocatalysis stemmed from ancient China, Japan, and Mesopotamia in the production of food and alcoholic drinks using isolated enzymes or whole-cell biocatalysts.[1, 2] Later, the acquisition of more knowledge about proteins and enzymes extended their applications, not only in traditional fermentation, but also in the chemical and pharmaceutical industries. One of the first examples of applying enzymes in large-scale chemical production was using penicillin amidase to synthesize penicillins and their derivatives.[2] Enzyme applications nowadays are found in several sectors of chemical industry such as food additives, fine chemicals, drugs, and agricultural chemicals.[3, 4] Many fine chemicals have been produced in multi-ton quantities by using enzymatic processes.[5] The application of enzymes in fine chemical and drugs synthesis will become more important in the near future.[3]

Moreover, enzymes play an important role in the development of a more sustainable chemical production. In many cases, the production processes in which enzymes act as catalysts do not require high temperature, pressure or organic solvents. This helps to reduce energy costs and avoid environmental impacts. Another advantage of enzymes over chemical catalysts is their high chemo-, regio- and enantioselectivity. This has made enzymes more attractive for the pharmaceutical industry, in which more than 50% of the compounds are chiral.[6]

Nevertheless, in many cases, enzymes have a narrow substrate scope, which limits their application in the industrial production. The demand for extending the substrate scope of enzymes and the discovery of new biocatalysts has led to several directions in enzyme research. One approach is to focus on the investigation of the activity and enantioselectivity of enzymes towards different types of compounds, which have potential applications. The other direction is to improve the activity of available enzymes by protein engineering and discovery of new enzymes through functional screening, metagenome derived sources and genome database mining.[7]

Tertiary alcohols have become interesting targets for organic synthesis themselves or as building blocks for valuable pharmaceutical compounds. However the synthesis of optically pure tertiary alcohols is still a challenge when compared with secondary alcohols both by chemical and enzymatic means.[8, 9] Enzymes containing the GGG(A)X motif in the active site region have been known to show activity towards these sterically demanding substrates.[10] Several tertiary alcohols have been resolved with high enantioselectivity by using this biocatalytic synthetic route.[11, 12]

This thesis deals with the discovery of new biocatalysts for the GGG(A)X-motif enzyme toolbox using different approaches and the application of the toolbox for the kinetic resolution of diverse types of tertiary alcohols (Scheme 1). Moreover, it focuses on a better understanding of factors involved in the enzymatic reaction and their effects on enantioselectivity of the biocatalysts.



Scheme 1: Kinetic resolution of optically pure tertiary alcohols **2** from tertiary alcohol acetates

1.1. Scope and outline of this thesis

In this thesis, diverse type of tertiary alcohols have been resolved in the kinetic resolution with GGG(A)X-motif enzymes in the catalytic platform established from previous studies. In complement the available enzymes, new biocatalysts have been found by different approaches: function-based screening, genome database mining and rational protein design.

In **Article I**, new biocatalysts were found by genome database mining with the help of the α/β-Hydrolase Fold 3DM Database (ABHDB). The database provides a high-quality, structure-based multiple-sequence alignment based on almost all available α/β-hydrolase fold enzymes composed of separate subfamily sequence alignments of subfamilies for which a structure is available.[13] These enzymes were cloned, characterized together with other enzymes isolated by functional screening approach and applied for the kinetic resolution of tertiary alcohols.

Article II describes an alignment-inspired method for the identification of key residues in a rational protein design of an esterase. New useful enzyme variants with increased activity and enantioselectivity were created from EstA, an enzyme from *Paenibacillus barcinonensis* isolated from a rice field in the Ebro River delta, Spain.[14] This project is based on cooperation with the group of Prof. Pilar Diaz (Department of Microbiology, University of Barcelona).

Articles III and **IV** present the application of GGG(A)X motif enzymes in the synthesis of enantiomerically enriched tertiary alcohols. In **Article III**, a combination of the Passerini multicomponent reaction (MCR) and a subsequent enzymatic kinetic resolution in the preparation of enantiomerically pure protected α,α-dialkyl-α-hydrocarboxylic acids, important building blocks in organic synthesis, is presented. **Article IV** covers a chemoenzymatic synthesis of diverse optically pure tertiary alcohols bearing a nitrogen substituent. These compounds belong to pyridine-derived tertiary alcohols and tertiary cyanohydrins. The substrate recognition of the enzymes and the effects of reaction conditions on enantioselectivity are discussed.

A comparison between chemical (performed by the group of Prof. Sabine Laschat, University of Stuttgart) and chemoenzymatic approaches to synthesize optically pure homoallylic tertiary alcohols is given in **Article V**.

As GGG(A)X motif enzymes are the main subject of this thesis, the role of GGG(A)X motif in the enantiorecoginition of tertiary alcohols as well as the importance of

tertiary alcohols as building blocks for organic synthesis will be discussed. Preparation of substrates through the Passerini multi-component reaction will be presented. A short introduction to the ABHDB (or 3DM) database as a basis for protein design and genome database mining will be given.

1.2. Enzymes as catalysts for sustainable chemistry

Concerns about environmental impacts of chemicals and pharmaceuticals production such as the employment of heavy metal catalysts, intensive use of organic solvents and energy consumption have led to the demand for more sustainable processes. Green chemistry is a concept aimed at satisfying this demand.

According to Roger Sheldon,[15] "green chemistry efficiently utilizes (preferably renewable) raw materials, eliminates waste and avoids the use of toxic and/or hazardous reagents and solvents in the manufacture and application of chemical products". Twelve principles of green chemistry can be summarized in the word PRODUCTIVELY:[15-17]

Prevent waste
Renewable materials
Omit derivatization steps
Degradable chemical products
Use safe synthetic methods
Catalytic reagents
Temperature, pressure ambient
In-process monitoring
Very few auxiliary substances
E-factor, maximize feed in product
Low toxicity of chemical products
Yes, it is safe

The E factor, first introduced by Roger Sheldon in 1992 as the mass ratio of waste to desired product and the atom efficiency,[18] has been used to evaluate the environmental impact of manufacturing processes. Hence, a process with a high E factor will produce more waste than the one with a lower E factor. While some processes like oil refining and production of bulk chemicals have an E factor from one to five, the E factors of fine chemicals and pharmaceuticals production are usually very high (50-100).[19] Together with the rising concerns about environmental impacts, the pressure from consumers has led pharmaceutical companies to develop safer and more environmentally friendly processes.[20]

The ability to catalyze a reaction with high chemo-, regio- and stereoselectivity in water under mild conditions makes enzymes attractive for green chemistry.[20-22] Biocatalysis can help to reduce the number of process steps due to the high selectivity of enzymes and therefore the use of hazardous reagents and waste generation are reduced or avoided. Furthermore, enzymes can often catalyze the

reaction under mild conditions, which leads to a higher energy efficiency and safer processes. Because of their high selectivity, unnecessary protection and deprotection steps, in many cases, can be avoided in enzymatic reactions; hence the atom economy is increased.[20, 21] In the pharmaceutical industry, solvents are the largest contributor on a mass basis and therefore, become the greatest problem. Organic solvents like dichloromethane and toluene are still being used widely in the production of pharmaceuticals. For example, dichloromethane is the largest mass contributor (80%) to materials of concern in GlaxoSmithKline.[23] Though the major use of dichloromethane obviously raised some concerns about health and environmental impacts,[24] the alternatives are still not ready and common. Water, in which biocatalysis usually occur, is safe and a benign "universal solvent".[25]

1.3. Tertiary alcohols in natural products and their roles as building blocks in organic chemistry

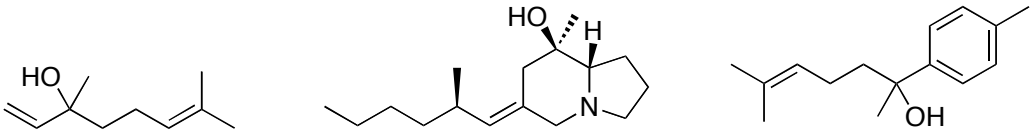
1.3.1. Tertiary alcohols in natural products

In nature, tertiary alcohols can be found as flavour compounds in plants such as α-terpineol in tea plants,[26] rosemary, anises and linalool in lavender. Linalool **3** is a target of organic synthesis and biocatalysis because of its importance to the flavour industry.[27] In Table 1, the annual industrial usage from of some flavour compounds which are tertiary alcohols is shown.[28]

Table 1: Annual demand for some of the flavour compounds which are tertiary alcohols[28]

Compounds	Odour	Approximate annual usage (tons) - 2003
α-terpineol	Pine	3000
Dihydromyrcenol	Citrus, floral	2500
Linalool	Floral	4000

Another natural tertiary alcohol, gossonorol **5**, which is found in *Chamomilla recutita*, a medicinal plant, is applied to synthesize boivinianin B and yingzhaosu C, a remedy for malaria that has been used in China for centuries.[29] Enantioselective derivatives from pumiliotoxins **4**, poisons found in frogs,[30] contain a tertiary alcohol functional group in their structures.



Linalool
3

Pumiliotoxin 251D
4

Gossonorol
5

Figure 1: Tertiary alcohols found in nature. From left to right: linalool in lavender, pumiliotoxin 251D in toxic frog *Epipedobates tricolor*, gossonorol in *Hieracium cymosum*.

1.3.2. Tertiary alcohols as building blocks in organic chemistry

As compounds that show versatile biological activities in nature, tertiary alcohols have become interesting targets for organic synthesis themselves or as building blocks for valuable pharmaceutical compounds. Pyridine-derived tertiary alcohols can be used as building blocks for the synthesis of A_{2A} receptor antagonists such as **6**, promising compounds for the therapy of Parkinson's disease.[31] Ekegren et al.[32] have described a new class of HIV-1 protease inhibitors of type **7** containing a tertiary alcohol in the transition-state mimicking scaffold (Figure 2). In the search for a therapy for Alzheimer's disease, a new class of tertiary alcohols based on BACE-1 inhibitors has been investigated.[33] The chiral tertiary alcohol *(S)*-2-hydroxy-2-methylbutyric acid **8**, which occurs naturally in clerodendrin-A,[34] is used for the synthesis of cyclooxygenase inhibitor.[35]

In the field of organic synthesis, tertiary allyl alcohols have been used as starting materials for a novel method of epoxide synthesis by palladium-catalyzed reactions.[36] Tertiary cyanohydrins, also tertiary alcohols themselves, are versatile precursors for the synthesis of α-hydroxy acids, β-amino alcohols and β-hydroxy amides.[37]

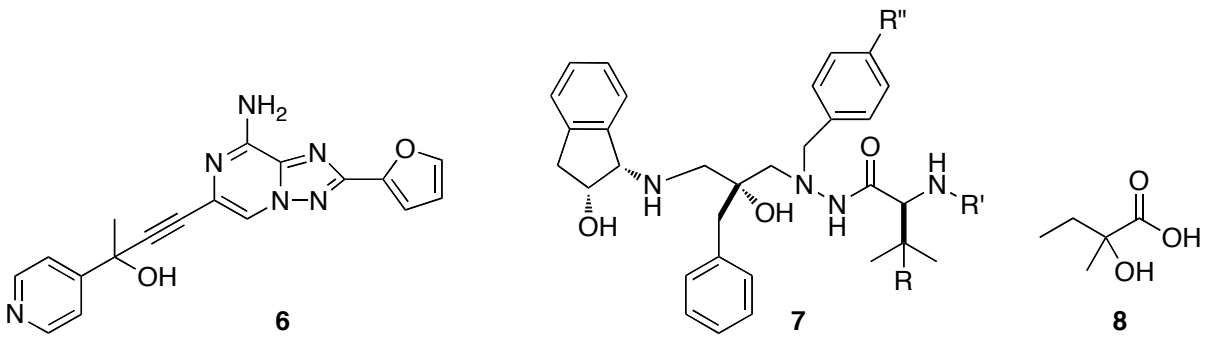


Figure 2: Tertiary alcohols in pharmaceuticals: (**6**) A_{2A} adenosine receptor antagonists,[31] (**7**) HIV-1 protease inhibitor,[32] (**8**) 2-hydroxy-2-methylbutyric acid.

1.4. Chemical synthesis of optically pure tertiary alcohols

A few examples will be given to outline scope and limitations of catalytic methods. The most common approach is to add organometallic reagents (Grignard reagents) to carbonyl compounds, ketones in this case, in the presence of chiral ligands.[8, 38] Leuser et al. proposed a pathway to synthesize tertiary alcohols through chiral precursors prepared by copper-catalyzed reaction, followed by oxidation and rearrangement to yield tertiary alcohols (Figure 3).[39]

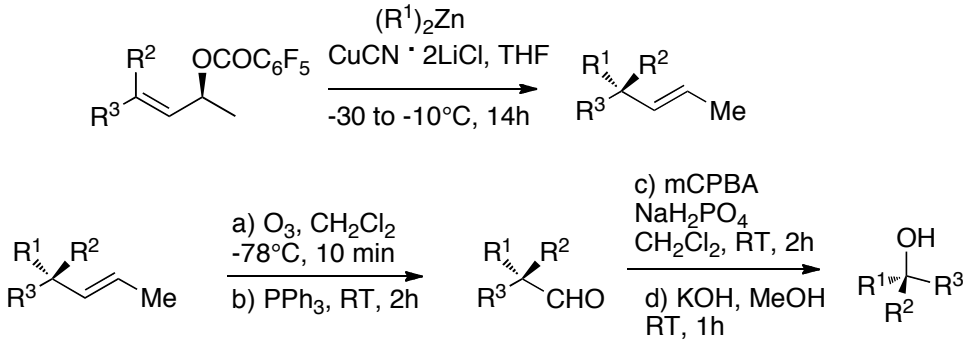


Figure 3: Copper-catalyzed substitution to prepare chiral precursors for the synthesis of chiral tertiary alcohols by rearrangement.[39]

Stymiest et al. have reported a new method for producing chiral tertiary alcohols starting from chiral carbenoids (Cb) derived from secondary alcohols (Figure 4).[40]

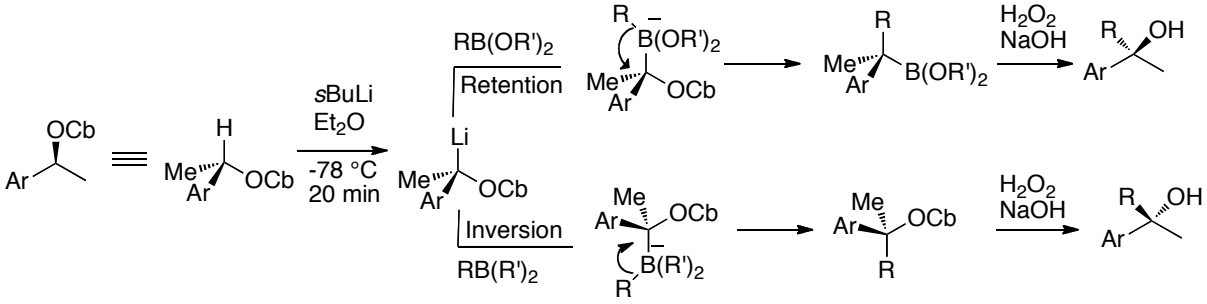


Figure 4: Lithiation–borylation of chiral secondary carbamates leading to tertiary alcohols pathway proposed by Stymiest et al.[40]

Another approach from Abecassis et al. is to synthesize an enantiopure allylic tertiary alcohol and gossonorol **1y** starting from allyl ethers of the tricarbonylchromium(0) complex of benzylic alcohols (Figure 5).[29, 41] The two enantiomers of **1y** could be obtained by this method with high yield and enantiomeric purity.

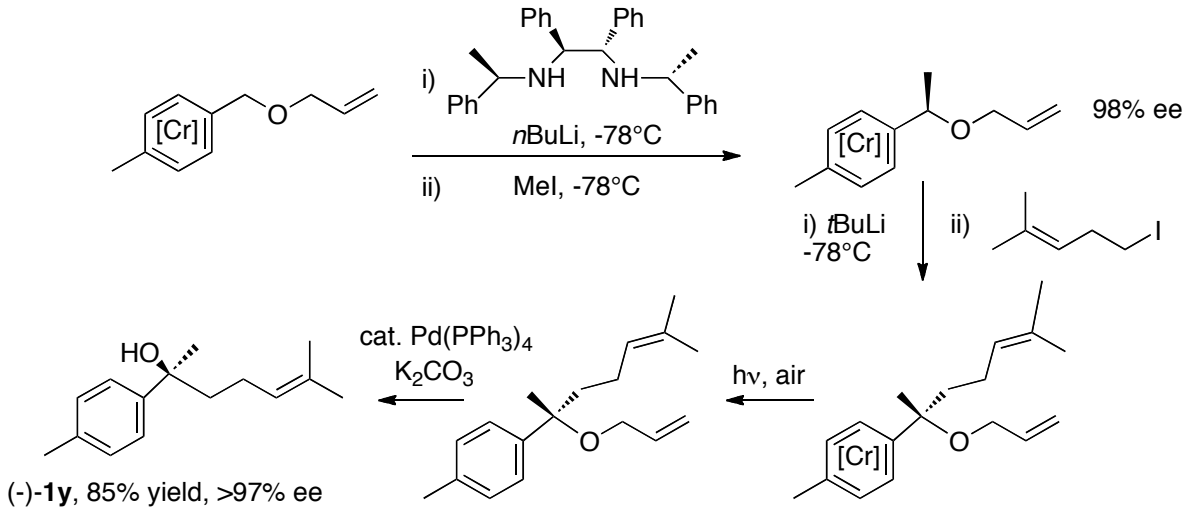


Figure 5: Asymmetric synthesis of gossonorol.[29]

The presented examples show that the chemical synthesis of chiral tertiary alcohols still raises some concerns about toxicity of heavy metal catalysts involved in the reactions. Especially in the pharmaceutical production, the amount of heavy metal traces in the final product is strictly regulated.[42] In addition, reaction conditions in some cases require a high demand of energy as well as the employment of high amounts of organic solvents.[40, 43] Therefore, biocatalytic pathways to synthesize optically pure tertiary alcohols present themselves as a sustainable alternative.

1.5. Different strategies for enzymatic synthesis of optically pure compounds

Different biocatalytic strategies applied to synthesize chiral compounds are summarized in Figure 6. With asymmetric synthesis, the starting materials can be prochiral compounds and the product yield is up to 100%. In the case of kinetic resolution, with the assumption that the enzyme has a high enantioselectivity towards one substrate enantiomer, one enantiomer is converted to the corresponding product enantiomer much faster than the other enantiomer. The maximum yield of the kinetic resolution is only 50% with a selective enzyme. In dynamic kinetic resolution, aside from the kinetic resolution, the simultaneous racemization occurs to transform a slow-reacting enantiomer to a fast-reacting one. In this case, a theoretical 100% yield can be reached.

Recently, a new concept for the enantiopure compounds synthesis was presented as an enantio-convergent process.[44] In this method, one substrate enantiomer is converted to the corresponding product enantiomer by a retaining enzyme, which maintains the stereo configuration of the substrate. At the same time, an inverting enzyme, which inverts the stereo configuration of the substrate, will transform the other enantiomer of the substrate. The stereo configuration of the product, in the

latter case, will be inverted with the starting substrate enantiomer. Consequently, the sole product will be formed with the theoretical 100% yield. The advantage of this approach is that no racemization process is required compared with dynamic kinetic resolution. Nevertheless, a prerequisite requirement is that both the retaining and inverting enzymes are needed for the enantio-convergent process to be carried out, which is not always easily fulfilled.

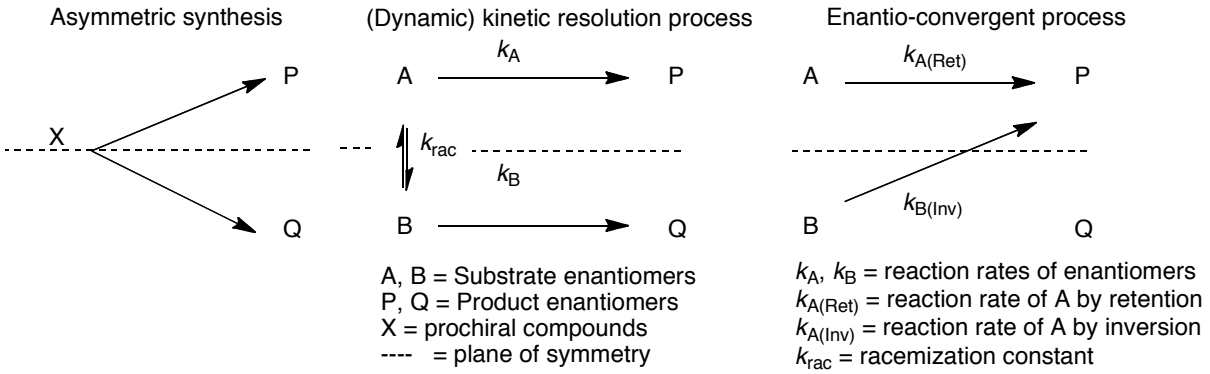


Figure 6: Strategies for the synthesis of chiral compounds (adapted from Gadler et al., 2007).[45]

Despite the limitation of 50% maximum yield, kinetic resolution is still being widely employed for the synthesis of chiral compounds using a racemate as the starting compound. In most cases, the prochiral compounds used for asymmetric synthesis have only three variations of functional elements (R_1, R_2, X) while the racemic mixture has four different functional groups (R_1-R_4) on a sp^3 carbon of the chiral center (Figure 7).[45] Hence, the racemic mixture can provide more possibilities for the design of synthesis process.

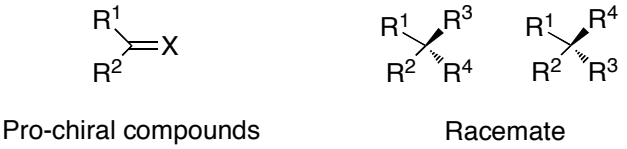


Figure 7: Prochiral compounds and racemates.

Asymmetric synthesis of chiral tertiary alcohols by biocatalysts is still limited and no racemization process that can be applied in the combination with enzymes has been reported so far. Therefore, the kinetic resolution pathway is still the main approach to synthesize enantiopure tertiary alcohols.

1.6. Biocatalytic routes for the synthesis of chiral tertiary alcohols

The search for more efficient and environmentally friendly methods to synthesize chiral tertiary alcohols has led to enzymatic synthesis approaches. Unfortunately, not all efficient enzymatic pathways to synthesize enantiopure secondary alcohols can be applied for the synthesis of chiral tertiary alcohols. In the case of carbonyl reductases, the asymmetric synthesis of secondary alcohols could be carried out with a wide range of substrates and at high enantioselectivity.[46] However, it is not

possible to synthesize tertiary alcohols by carbonyl reductases since there are no applicable ketones that can be used as starting compounds.

Recently, Faber et al. have reported a synthetic pathway for chiral secondary alcohols using alkyl sulfatase with alkyl sulfate esters as the starting compounds (Figure 8).[45, 47]

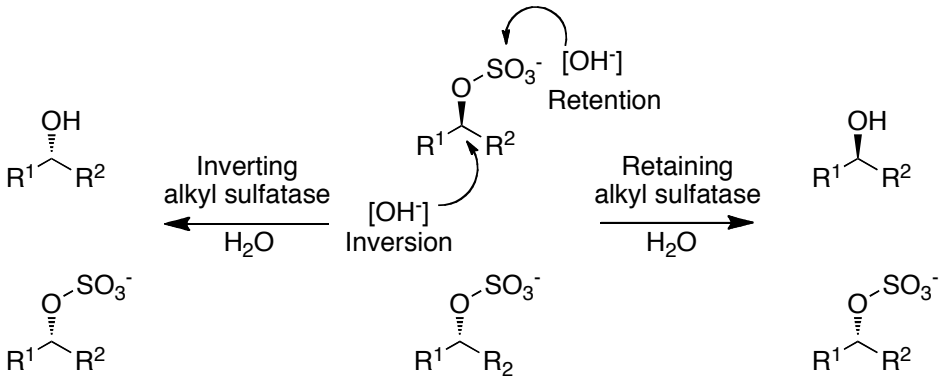


Figure 8: Synthesis of chiral secondary alcohols by kinetic resolution using alkyl sulfatases.[45]

In the kinetic resolution of *sec*-alkyl sulfate esters, two reactions can occur depending on which type of alkyl sulfatase is applied. An inverting alkyl sulfatase, which cleaves the C-O bond, will produce a product in the homo chiral form. On the contrary, hetero chiral products will be formed in a reaction catalyzed by a retaining alkyl sulfatase, in which the S-O bond is cleaved. In the first case, a tertiary alkyl sulfate ester would not be accepted as a substrate for the inverting alkyl sulfatase. Whether chiral tertiary alcohols can be synthesized by the retaining alkyl sulfatase is a tempting thought but is still unknown.

In a chemoenzymatic approach, March-Cortijos et al. have established a method for the asymmetric synthesis of β-amino tertiary alcohols using a two-step reaction: desymmetrisation of a prochiral diol by using esterases to yield *(S)*-(+)-(2-(hydroxymethyl)oxiran-2-yl)methyl acetate, which is later applied for the epoxide ring-opening reaction to obtain β-amino tertiary alcohols (Figure 9).[48, 49]

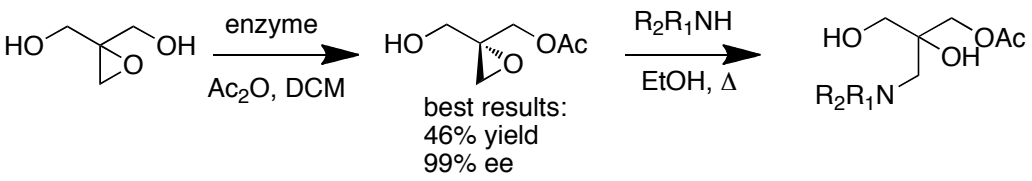


Figure 9: Asymmetric synthesis of β-amino tertiary alcohols.[49]

Other types of enzyme have been investigated using different approaches in accepted substrates and reaction mechanisms. Elenkov et al. proposed a method for the preparation of enantiopure tertiary alcohols by epoxide ring opening catalyzed by a halohydrin dehalogenase (Figure 10a).[50] In the search for an alternative pathway

for the enzymatic synthesis of tertiary cyanohydrins, which are also tertiary alcohols, Holt et al. proposed the pathway of applying the kinetic resolution of carboxylic acids bearing a cyano functional group with the help of proteases and esterases.[51] Brodkorb et al. have discovered a novel asymmetric synthesis pathway to synthesize chiral linalool from myrcene using linalool dehydratase with high enantioselectivity and 100% theoretical yield (Figure 10b).[52] Nevertheless, the reported case is very limited and not yet fully investigated.

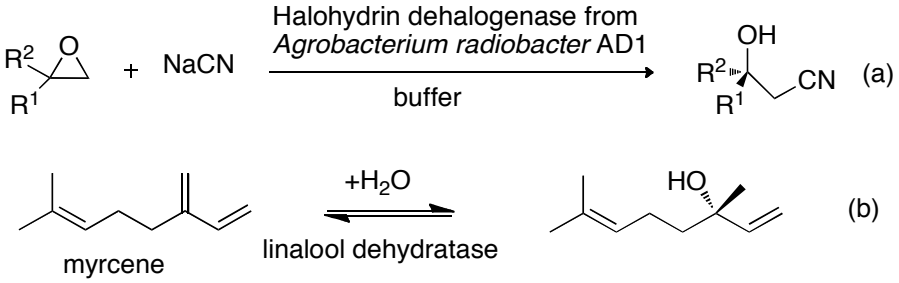


Figure 10: Selected pathways for enzymatic synthesis of enantiopure tertiary alcohols (a) kinetic resolution[50] (b) asymmetric synthesis[52].

Hydrolases, including lipases and esterases, have been used as biocatalysts for the enzymatic synthesis of tertiary alcohols due to their abundance, mild reaction conditions and easy-to-prepare starting materials.[53] Moreover, with hydrolases as biocatalysts in the kinetic resolution of racemic tertiary alcohol esters, the number of substitutions on the chiral center is more diverse compared to asymmetric synthesis with prochiral compounds.[45]

Racemic tertiary alcohols can be synthesized using Grignard reactions with ketones and organomagnesiumbromide reagents (Figure 11). This method can be used to synthesize a wide range of tertiary alcohols with moderate to good yields. The alcohols can be later transformed to esters by an acetylation reaction. Results from the kinetic resolution of tertiary alcohol by this chemoenzymatic pathway are presented in **Article IV**.

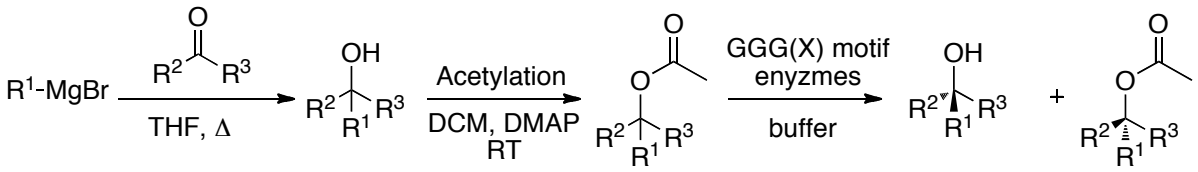


Figure 11: Chemoenzymatic pathway for the synthesis of optically pure tertiary alcohols with the starting compounds prepared by Grignard reaction.

Another type of compound that can be considered as a substrate in the kinetic resolution of GGG(X) motif enzymes is the ester of α,α-dialkyl-α-hydroxycarboxylic acids. Enantiomerically pure α,α-dialkyl-α-hydroxycarboxylic acids are important building blocks in organic synthesis. (*S*)-2-hydroxy-2-methylbutyric acid is present in the natural product clerodendrin-A[34] and has been applied in the synthesis of a

cyclooxygenase inhibitor.[35] A biocatalytic route using oxynitrilases to synthesize (*S*)-2-hydroxy-2-methylbutyric acid **8** with high enantiomeric excess (*ee* >99%) has been reported by Fechter et al. (2007).[54] Nevertheless, the approach requires HCN as a substrate. Another approach from Sugai et al. (1990) is to apply a kinetic resolution of benzyloxy-protected esters of 2-hydroxy-2-methylbutyric acid **8** and several other derivatives. Whilst the enantioselectivities for several substrates were up to E=52, *(S)*-2-hydroxy-2-methylbutyric acid **8** was resolved with an enantioselectivity of only E=10.[55]

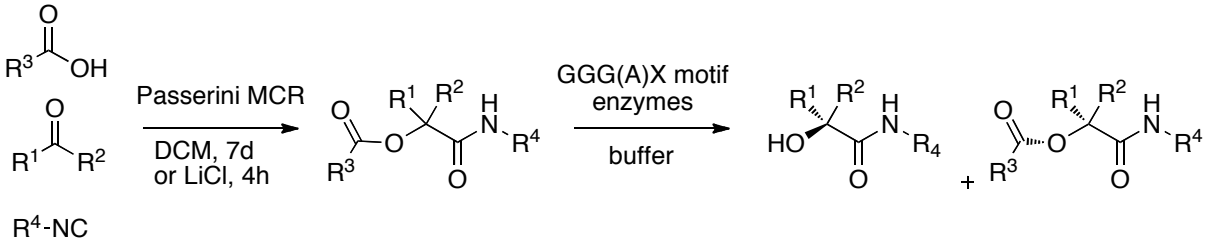


Figure 12: Chemoenzymatic synthetic route for the synthesis of protected α,α-dialkyl-α-hydroxycarboxylic acids.

Our approach for the synthesis of enantiomerically pure α,α-dialkyl-α-hydroxycarboxylic acids is to resolve the tertiary alcohol of tertiary hydroxyesters, in which the acid group is protected with an amide, by using GGG(A)X motif enzymes. Despite the similarity of reaction mechanism between enzymatic hydrolysis of amides and esters, promiscuous amidase activity of esterases is usually very low.[56] Protected hydroxyesters can be easily synthesized in one step by the Passerini reaction, a multi-component reaction (MCR) widely applied in combinatorial chemistry.[57] A subsequent enzymatic resolution and deprotection would yield the desired α,α-dialkyl-α-hydroxycarboxylic acids. The results from this chemoenzymatic route for the synthesis of protected α,α-dialkyl-α-hydroxycarboxylic acids (Figure 12) are presented in **Article III**. The Passerini reaction has been efficiently applied in a combined method with enzymatic resolution to synthesize enantiomerically enriched α-amino acids[58] and α-hydroxyamides.[59] Our study aims to provide a two-step synthetic route to access these bulky protected α,α-dialkyl-α-hydroxycarboxylic acids with high optical purity.

1.7. GGG(A)X-motif enzymes as biocatalysts for optically pure tertiary alcohols synthesis

1.7.1. Esterases and lipases

Belonging to the enzymatic group of hydrolases (EC 3.1), lipases (EC 3.1.1.3) and esterases (EC 3.1.1.1), catalyze the reaction of cleavage and formation of ester bonds.[1, 60] While lipases can readily accept water-insoluble compounds, esterases prefer water-soluble substrates.[1] Both enzyme classes show the characteristic of α/β-hydrolase fold in the 3D structures (Figure 13).[61]

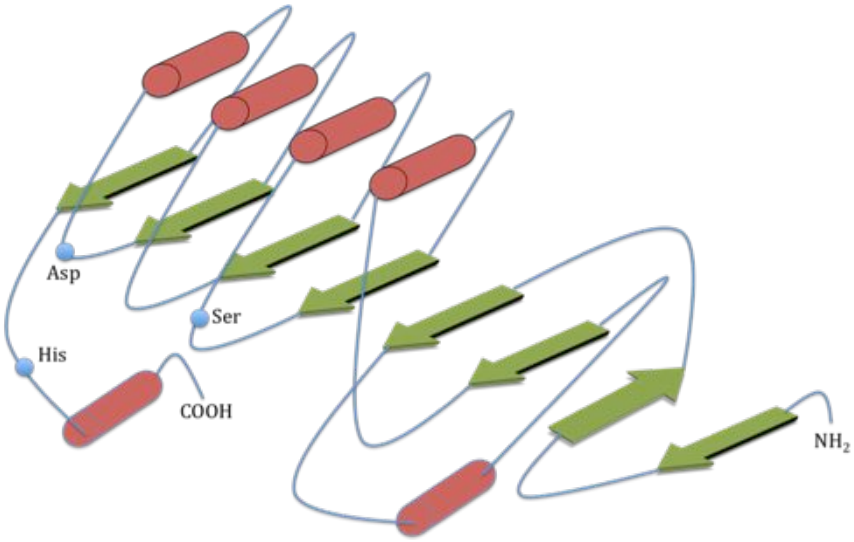


Figure 13: Schematic presentation of the α/β-hydrolase fold (adapted from Ollis et al.)[61]. α-helices are show in as red columns, β-sheets as green arrows. The relative positions of catalytic triad residues are indicated as blue circles.

The catalytic triad composed of Ser-Asp-His (Glu instead of Asp in some cases) and a sequence motif Gly-X-Ser-X-Gly are found to be conserved in these enzyme classes.[60] Although two enzyme classes share similarity in structure and catalytic mechanism, lipases are known for their unique interfacial activation phenomenon which comes from a hydrophobic domain covering the active site of lipases.[53, 60]

1.7.2. Mechanism of serine-esterase catalysis

The ester hydrolysis mechanism of serine hydrolases is presented in Figure 14. In the first step, the ester interacts with the enzyme in the active site. The catalytic serine residue attacks the carbonyl group leading to formation of the first tetrahedral intermediate (**T1**). The alcohol is released and the acyl enzyme is formed when **T1** collapses. In the hydrolysis reaction, the water can attack to the acyl enzyme and then the second tetrahedral intermediate **T2** is formed. When **T2** collapses, the acid is released and the active site of the enzyme is ready for the new catalysis. Since only **T1** includes the alcohol moiety, the enantioselectivity of the enzyme comes solely from this first tetrahedral intermediate.

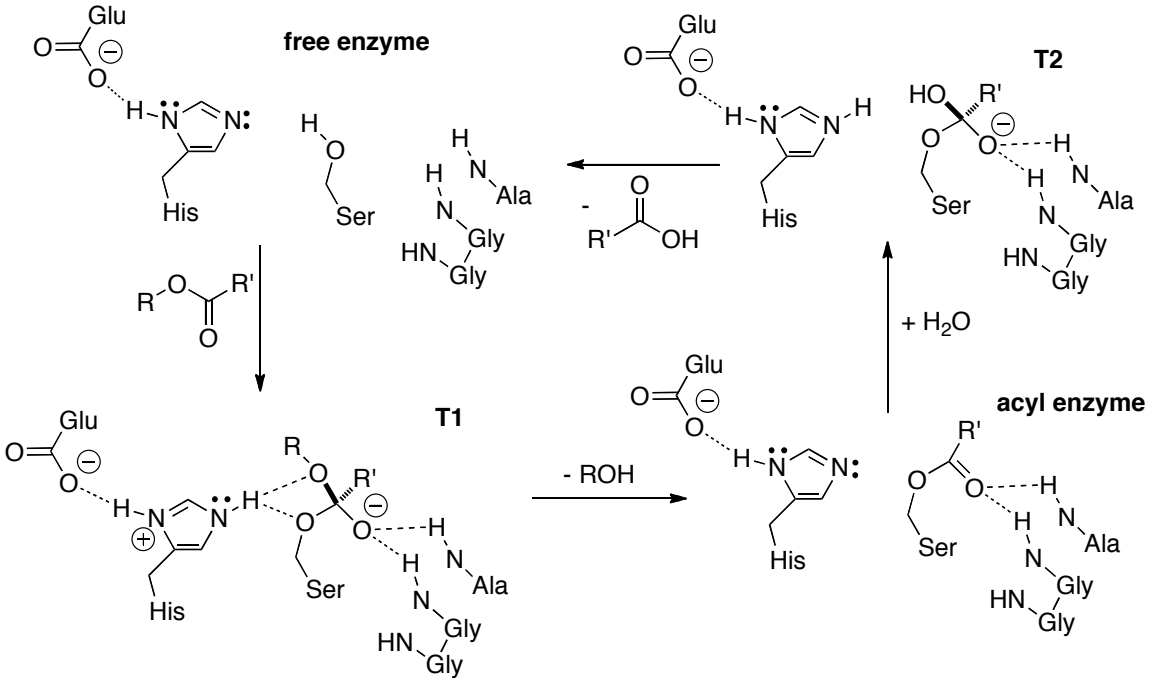


Figure 14: The mechanism of serine-hydrolases (adapted from Bornscheuer and Kazlauskas, 2006).[53] **T1**, **T2**: tetrahedral intermediates.

The catalytic serine acts as a nucleophile that attacks the carbonyl group of an ester. In a catalytic triad, serine is activated by the imidazole group on the catalytic histidine making it a better nucleophile. The carbonyl group of the substrate is activated as an electrophile by the NH groups of the residues in the oxyanion hole (shown as glycine and alanine residues in Figure 14). This leads to a build up of positive charge on the carbon atom of the carbonyl group. Consequently, the attack of catalytic serine on the carbonyl group is facile. As the unprotonated hydroxy group of the alcohol is a poor leaving group, a proton donation from catalytic histidine will permit the alcohol to leave more easily.[62] Therefore, the catalytic triad in the active site of serine hydrolases facilitates the hydrolysis of an ester by creating a good nucleophile, a good electrophile, and a good leaving group. It is fascinating how elegantly nature combines acid/base catalysis.

1.7.3. Enantiodiscrimination in lipases and esterases

The enantiomeric ratio, or enantioselectivity or E value, measures the ability of an enzyme to distinguish between enantiomers. An enzyme with an E value of 1 is non-selective. While resolutions with E values above 20 are useful for synthesis, a high selective enzyme has an E value of more than 100.[53] The E value, with the assumption that the reaction is virtually irreversible and without product inhibition, can be calculated from two of three variables: enantiomeric excess of the substrate (ee_S), enantiomeric excess of the product (ee_P), and extent of conversion (c) that can be applied to one of three below equations:[63]

$$E = \frac{\ln[1 - c(1 + ee_P)]}{\ln[1 - c(1 - ee_P)]}; \; E = \frac{\ln[(1 - c)(1 - ee_S)]}{\ln[(1 - c)(1 + ee_S)]}; \; E = \frac{\left[\frac{1 - ee_S}{1 + (ee_S / ee_P)}\right]}{\left[\frac{1 + ee_S}{1 + (ee_S / ee_P)}\right]} \quad (1)$$

In many cases, enantiomeric excess can be measured more accurately than conversion. Therefore, the third equation is preferred for the calculation when both of ee_S and ee_P can be determined.[1]

Two enantiomers of a racemic compounds share nearly identical physical properties. Nevertheless, when an enantiomer interacts with another chiral molecule, in this case, an enzyme, to form a enzyme-compound complex, the difference between two enantiomers becomes apparent.[64] Consequently, the transition-state free energy $\Delta G^{\#}$ of the preferred enantiomer is assumed to be lower than that of the slow-reacting enantiomer. The energy profile of the enantioselective conversion of two enantiomers is shown in Figure 15.

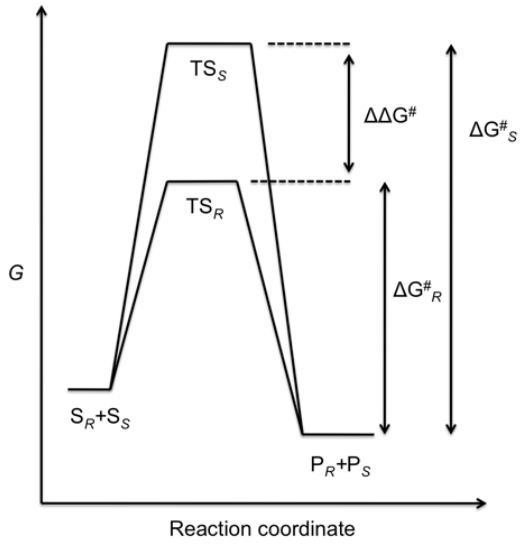


Figure 15: Free energy profile for the conversion of a serine hydrolase acyl enzyme complex and alcohol to the free enzyme and ester (adapted from Raza et al. 2001).[65] In this case, the (*R*)-enantiomer is preferred. S: substrate; P: product; TS: transition state; $\Delta G^{\#}$: transition state enthalpy; $\Delta\Delta G^{\#}$: difference in transition-state enthalpy.

The E value can also be related as the difference in the transition-state free energy between two enantiomers:[65]

$$E = e^{-\left(\frac{\Delta G_R^{\#} - \Delta G_S^{\#}}{RT}\right)} = e^{-\left(\frac{\Delta\Delta G^{\#}}{RT}\right)} \quad (2)$$

According to (2), a small change in transition-state energy can lead to a large corresponding E value. Therefore, the ability of an enzyme to distinguish between two enantiomers of a racemic compound comes from the difference in the transition-state free energy of the enzyme-compound complex during the interaction of the enantiomers with the amino acid resides in the active site.

Holmberg et al. (1991) have observed that the temperature can be a parameter to affect on the enantioselectivity of enzymatic reaction.[66] The enantioselectivity increases when the reaction temperature decreases in most cases. This phenomenon can be explained by the equations related to enantioselectivity. The equation (2) can be rewritten with T_1 and T_2 (two different temperatures):

$$RT_1 \ln E = -\Delta\Delta G^{\#}_{T_1} \quad (3)$$

$$RT_2 \ln E = -\Delta\Delta G^{\#}_{T_2}$$

With the assumption that the conformation of the enzyme is not changed at two different temperatures, therefore $\Delta\Delta G^{\#}_{T_1}$ equals $\Delta\Delta G^{\#}_{T_2}$ and (3) will be:

$$RT_1 \ln E_1 = RT_2 \ln E_2 \Leftrightarrow (E_1)^{T1} = (E_2)^{T2} \quad (4)$$

Equation (4) shows that the temperature is proportional to the logarithm of the enantioselectivity (E value). Therefore, the E value is expected to increase when the reaction temperature decreases in a biocatalysis.

1.7.4. The role of GGG(A)X-motif in the substrate acceptance of enzymatic reaction of tertiary alcohols

Tertiary alcohols are not converted by the majority of hydrolases. In 2002, Henke et al. studied 25 commercially available enzymes for their activity towards tertiary alcohols and only two lipases showed activity, namely the lipases from *Candida rugosa* and *Candida antarctica* A.[10] Moreover, the enantioselectivity is low in most cases. In a further study, only one out of 35 enzymes from metagenome sources showed good enantioselectivity towards arylaliphatic tertiary alcohols in eight investigated compounds.[12]

The reason for the poor performance of α/β fold hydrolases towards tertiary alcohols was proposed by Henke et al.[10] A comparison of 3D structures and sequences of enzymes that show activity towards tertiary alcohols revealed the presence of a wider binding pocket (1.5-2 Å) caused by a special GGG(A)X motif positioned in the active site. On the contrary, GX motif enzymes showed no activity towards tertiary alcohol esters.

Thus, the GGG(A)X motif conferred the acceptance of tertiary alcohol esters as substrates by the formation of a special conformation in the oxyanion hole region (Figure 16). This helps to stabilize the anionic carbonyl oxygen atom of the tetrahedral intermediate during the ester hydrolysis in the active site by forming two hydrogen bonds provided by two amide groups of the protein backbone.[10] Moreover, the loop created by the GGG(A)X motif can provide a more flexible conformation due to a small hydrogen side chain in glycine and the carbonyl oxygen atoms are aligned parallel to the binding pocket. These conditions lead to a broader space for bulky tertiary alcohols that can be accommodated in the active site.[67]

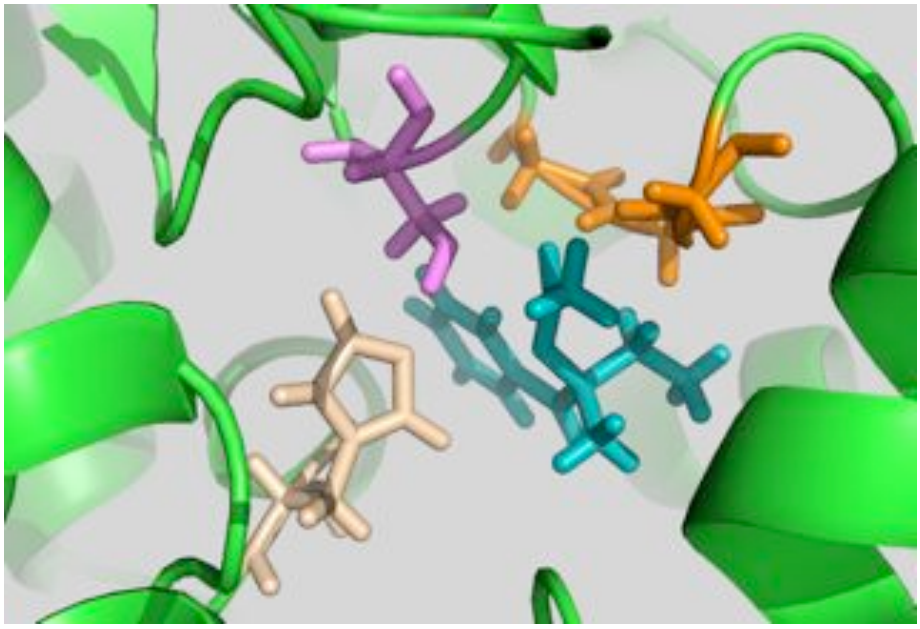


Figure 16: GGG(A)X motif (orange) in the active site of PestE from *Pyrobaculum calidifontis*. **Orange**: GGG(A)X motif, **pink**: catalytic serine, **pale**: a tertiary alcohol acetate docked into the active site, **wheat**: catalytic histidine. (PDB code: 2wir)

Since the findings of Henke et al., this motif has become a guideline for the identification of enzymes that show activity towards tertiary alcohol esters. A recombinant esterase from *Bacillus subtilis* BS2 with the GGG(A)X motif in its active site and its variants showed not only activity but also excellent enantioselectivity towards tertiary alcohol acetates.[11] In a later study of the activity and the enantioselectivity of the enzymes from metagenome libraries identified as containing GGG(A)X motif, 75% of the enzymes showed activity towards the tested substrates.[12] These studies underline the importance of a GGG(A)X motif in the catalysis of tertiary alcohols.

Nevertheless, having a GGG(A)X motif in the active site does not guarantee an enzyme to be a good catalyst for the synthesis of chiral tertiary alcohols. Most of the studied enzymes showed low enantioselectivity in the kinetic resolution of tertiary alcohol esters. One out of 25 tested GGG(A)X enzymes showed good enantioselectivity towards tertiary alcohol acetates.[12]

To increase the performance of the GGG(A)X enzymes towards tertiary alcohols, rational protein design approach have been showed to be successful in case of BS2 esterase. Two variants of BS2, BS2-G105A and BS2-E188D, were created based on predictions from molecular modelling results. While BS2 wild-type could resolve **1g** with a good enantioselectivity (E=42), excellent enantioselectivity (E>100) was obtained in the kinetic resolution of **1g**[11] and other tertiary alcohol homologues (**1h**, **1i**).[68] Furthermore, in a study by Bartsch et al. (2008) that employed a focused directed evolution approach based on CASTing method, the enantiopreference of BS2 was inverted by the double mutant E188W/M193C.[69]

To complement the protein engineering approach, identification of biocatalysts that show activity towards tertiary alcohols stemming from metagenomic sources, functional screening, and database mining can be employed to search for new useful enzymes to synthesize enantiomerically enriched sterically demanding substrates.

1.8. Searching for potential biocatalysts by functional screening and genome database mining.

1.8.1. Functional screening in strain libraries and isolated strains from enrichment cultures

New biocatalysts can be discovered by functional screening approach. Strains from strain library are screened for their activity towards targeted compounds. Several strain libraries have been established such as ATCC (American Type Culture Collection), DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen or German Collection of Microorganisms and Cell Cultures) or proprietary libraries. The active strains will be later characterized and identified by phylogenetic methods. Although this approach can be used to identify new biocatalysts, the enzymes in many cases need to be cloned for a better expression.[7] Another strategy for screening is using enrichment procedures to isolate desired microorganisms under specific selection pressures. This approach is usually applied for samples taken directly from the environment, such as soil or water samples.[70]

A screening assay is required to identify an active strain in a strain library or in an enrichment culture. There are established colorimetric assays for screening hydrolase libraries based on detectable pH changes during the screening procedure. Some pH indicators such as bromothymol blue and phenol red are widely used.[71] Not only was the activity of screened enzymes detected but also the enantioselectivity could be estimated.[72]

1.8.2. Discovering new biocatalysts by genome databases mining.

Genome database mining with the help of bioinformatics has been widely employed to identify new biocatalysts.[7] One approach is to identify specific motifs in genome databases, to predict the relationship between gene sequences and functions of encoded proteins.[73] Fraaije et al. have discovered the sequence motif: (FXGXXXHXXXW(P/D)) which can be used as an amino acid fingerprint for Baeyer-Villiger monooxygenase (BVMO) identification.[74] The Lipase Engineering Database (LED) (www.led.uni-stuttgart.de) is a collection of more than 20,000 sequence entries of proteins with the α/β hydrolase fold which share conserved active site signatures, the GxSxG and GxDxG motifs.[75-77] The database led to the discovery of GGG(A)X motif. By using the GGG(A)X motif which is located in the oxyanion hole near the active site as a guideline, metagenome-derived esterases have been identified to show activity and high enantioselectivity towards tertiary alcohol esters.[12] Using the amino acid sequence motif described by Kertesz et al.[78] C/S-X-P-X-R- X_4-TG, which was conserved in retaining sulfatases, a high number of genes encoding sulfatases

were found in *Rhodopirellula baltica* DSM 10527. Further studies showed that whole resting cells could hydrolyse *sec*-alkyl sulfate esters with high enantioselectivity.[79] Recently, using a combined method of rational protein design and database mining, Höhne et al. have discovered 17 (*R*)-selective amine transaminases which show excellent optical purity (up to >99%) in the asymmetric synthesis of (*R*)-amines.[80]

The α/β-hydrolase fold enzyme family 3DM database (ABHDB), is a high-quality structure-based multiple-sequence alignment that is based on almost all available α/β-hydrolase fold enzymes. The application of ABHDB to find a new biocatalyst relies on the sequence alignment of enzymes that are known to show interesting activity or high enantioselectivity in the kinetic resolution of tertiary alcohols with other protein sequences in the database. Based on the alignment results, candidate sequences were cloned, expressed and characterized. The results of enzymes obtained by this approach is covered in **Article I**.

1.9. Protein engineering – on the way to create better biocatalysts

1.9.1. Directed evolution and protein design

In spite of the high chemo, regio- and stereoselectivity, enzymes still have limitations when applied to biocatalysis for example, a narrow substrate scope or instability in organic solvents or at high temperature. Isolated enzymes from nature do not always fulfil the requirements for the application in organic synthesis. Adapted from the evolution of nature, a number of methods for performing *in vitro* evolution have been developed, which aim to improve the ability and characteristics of enzymes.[81] These methods are mostly based on molecular biology techniques to create a library of mutants from the wild-type enzyme. The mutant library is then screened using an efficient high-throughput screening system for any improved biocatalysts.[81] In directed evolution, the 3D structure of an enzyme is not required. Moreover, large libraries of mutants can be easily created by using several well-established mutagenesis methods and the mutant libraries can be used for different screening purposes. Nevertheless, in directed evolution, a high-throughput screening assay is the prerequisite factor, which in some cases cannot be easily fulfilled. Additionally, dealing with the large size of mutant libraries can be difficult.

Protein design uses the information of protein structure and enzyme mechanism as guidelines for mutagenesis. Molecular modelling and simulation softwares can be used to identify key residues for mutagenesis by creating and evaluating mutants *in silico*. The best *in silico* mutants will be made *in vitro* to study their characteristics, activity and enantioselectivity towards the targeted compounds.[82] With protein design, the amount of libraries for mutagenesis can be largely reduced. Nevertheless, limitations in molecular modelling, in many cases, can affect the success of protein design.[82]

A combined method including rational and random mutagenesis with the aim to reduce the library size “Iterative Saturation Mutagenesis” (ISM) has been suggested

by Reetz.[83] In ISM, a hit in one library will be used as a template for randomization at other sites (Figure 17).

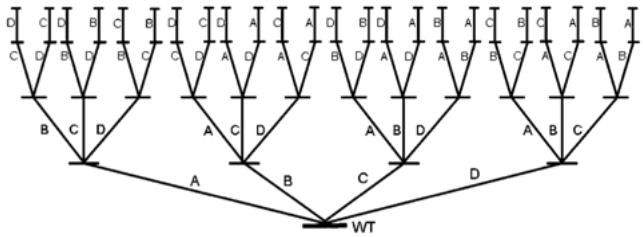


Figure 17: Iterative Saturation Mutagenesis (ISM) method involving (as an example) four randomization sites A, B. C, and D. Each site is comprised of one or more amino acid positions.[84]

The application of degenerated codons like NDT can also further reduce the library size.[85, 86] While the NNK codon encodes all 20 proteinogenic amino acids, NDT degeneracy codon encodes for 12 amino acids: Phe, Leu, Ile, Val, Tyr, His, Asn, Asp, Cys, Arg, Ser, and Gly.[86] However, the compromise between size and quality of the library should be taken in account into the screening process. Recently, an alternative database-oriented strategy to create a “small, but smart” mutant library for site-saturation mutagenesis has been suggested and successfully applied to improve the enantioselectivity[87] or thermostability[88] of the targeted enzymes.

1.9.2. Database-oriented protein design

The α/β-hydrolase fold enzymes can be found in different protein and enzyme databases such as RCSB Protein Databank (www.rcsb.org) or ExPASy (expasy.org/sprot). However, most of these databases are only based on annotated submitted sequences, and in many cases there is no information about the sequence-structure correlation. This limitation makes it difficult to clearly understand the relationship between the sequence and the structure of an enzyme as well as among enzymes of the same group.

The α/β-hydrolase fold enzyme family 3DM database (ABHDB), is a high-quality structure-based multiple-sequence alignment that is based on almost all available α/β-hydrolase fold enzymes.[13] The database is created and maintained by the company Bio-Prodict, Wageningen, Netherlands. The significance of this database is a multiple-sequence alignment based on the structures of included enzymes. From this type of alignment, consensus sequences can be created in the whole superfamily of α/β-hydrolase fold enzymes or in different categorized subfamilies. The database covers two large groups of esterases and lipases that have been shown to convert tertiary alcohols. Enzymes from the hormone-sensitive lipase-like family share the GGG(A)X motif in the oxyanion hole and a highly conserved GDSAGG motif close to the catalytic serine. The second family, which has a high level of similarity to acetylcholine esterases and mammalian liver esterases, includes enzymes containing the GESAGA consensus motif on the catalytic elbow.[13]

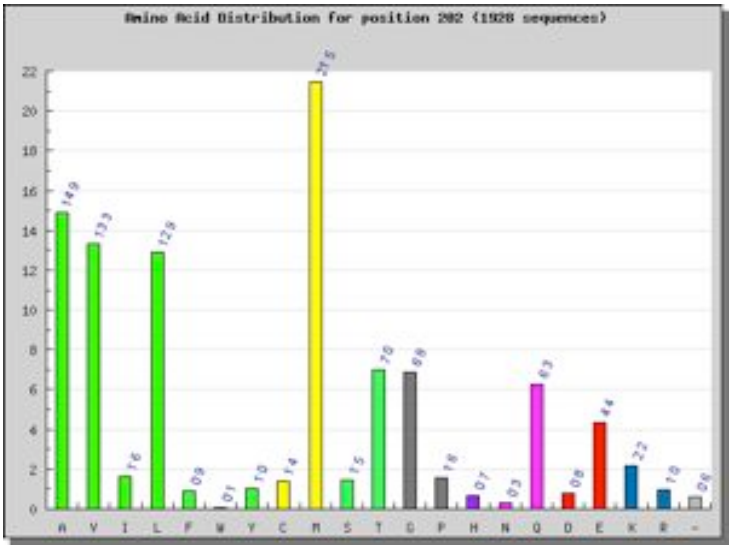


Figure 18: Amino acid abundance of position 202 in 1928 sequences of the hormone-sensitive lipase family in ABHDB.

From ABHDB, amino acid abundance at one specific position in an enzyme can be analyzed. Figure 18 is an example showing the amino acid frequencies at the position 202 in 1,928 sequences of the hormone-sensitive lipase family (methionine is the most frequent residue with 21.5% abundance). This information can be helpful for the identification of potential residues for the mutagenesis. Therefore, the focused library size can be reduced.

The concept for alignment-inspired rational protein design based on the amino acid abundance analyzed from ABHDB has been developed and applied to create a "small, but smart library".[87] The application of ABHDB database for a database-oriented rational protein design of EstA, an enzyme isolated from *Paenibacillus barcinonensis*, is given in **Article II**.

2. Discussion

This thesis aims at providing a better understanding of enantiorecognition of GGG(A)X motif-hydrolases in the enzymatic synthesis of enantiomerically enriched tertiary alcohols.

Kinetic resolution of a wide range of tertiary alcohols (Figure 19) using hydrolases provided insights on factors that can influence the enantioselectivity of GGG(A)X motif-enzymes. Additionally, a new chemoenzymatic pathway to synthesize protected α,α-dialkyl-α-hydroxycarboxylic acids has broadened the application of these enzymes towards sterically demanding tertiary alcohols.

Newly discovered biocatalysts through sequence-structure genome mining and rational protein design approaches provided an enzyme platform for enantiomerically enriched tertiary alcohol resolution.

Finally, a comparison of chemical and chemoenzymatic pathways for the synthesis of a homoallylic tertiary alcohol is described to evaluate the advantages and disadvantages of both approaches.

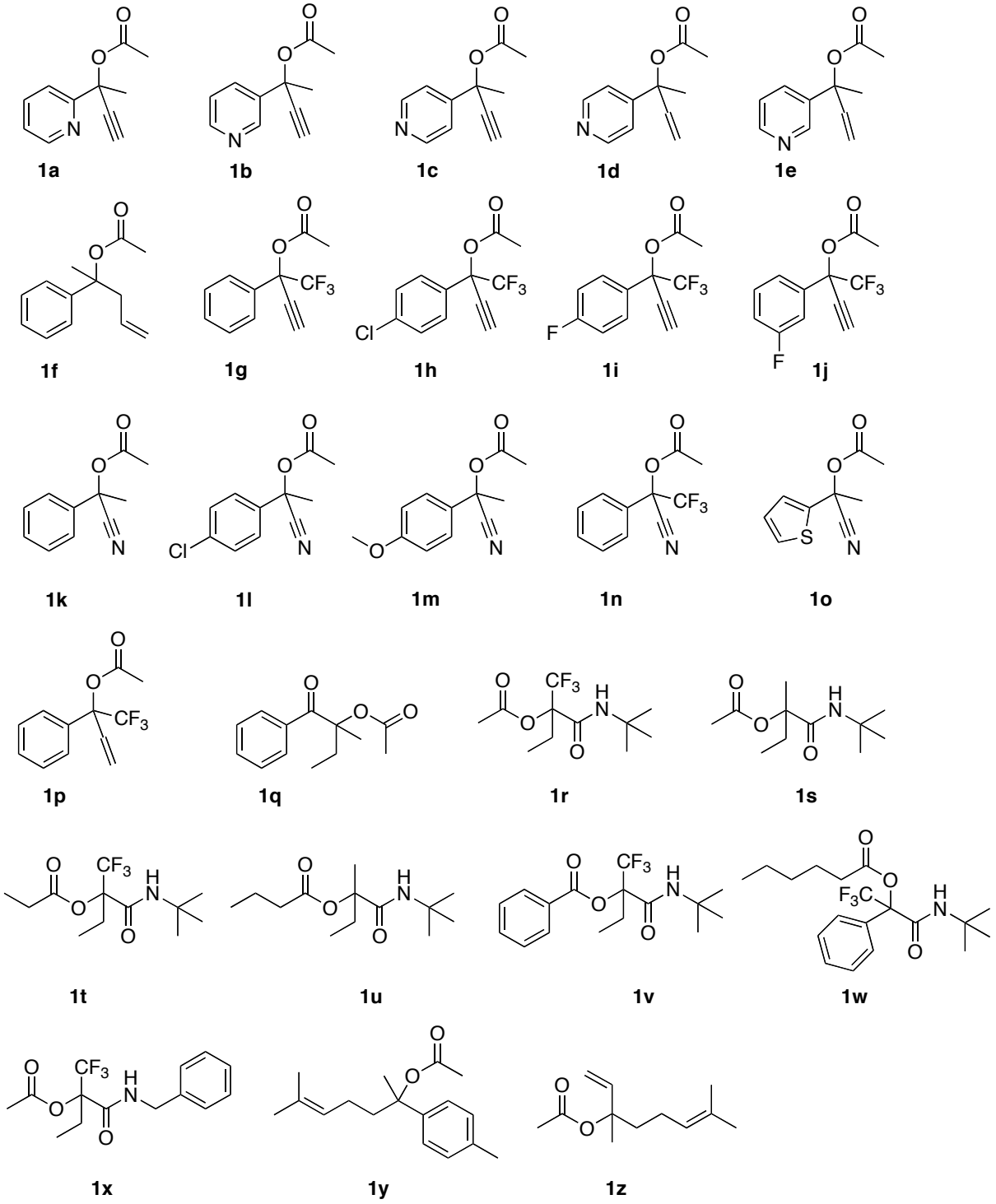


Figure 19: Tertiary alcohol esters studied in this thesis.

2.1. Chemoenzymatic route for the synthesis of enantiopure protected α,α-dialkyl-α-hydroxycarboxylic acids

Kinetic resolution in combination with MCR is a new method for the straightforward access to novel tertiary alcohols. The combined method will provide a two-step synthesis route to synthesize enantiopure protected α,α-dialkyl-α-hydroxycarboxylic acids (Figure 20).

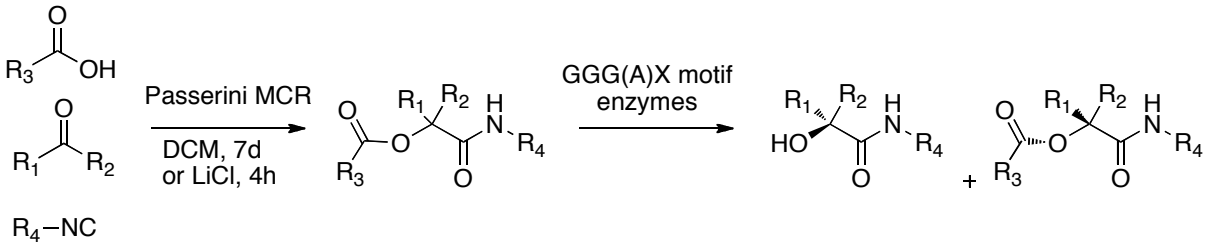


Figure 20: Chemoenzymatic route for the synthesis of optically pure protected α,α-dialkyl-α-hydroxycarboxylic acids

The Passerini reaction has been known to proceed considerably faster in aqueous media than in organic solvents.[57] Compound **1s** was synthesized in aqueous LiCl-solution in 4 hours (31% yield). An extended reaction time did not help to increase the yield of the final product. Moreover, a certain amount of hydrolysis of the substrate to the alcohol was observed and hence, a purification step by column chromatography was required. The synthesis of **1r**, **1t**, and **1v** was performed in dichloromethane (DCM). The products were obtained after seven days with high yields (84% - 93%) and excellent purity. Therefore, no purification step is required in spite of a longer reaction time (**Article III**).

For the kinetic resolution of the products from the Passerini reaction, 40 carboxyl hydrolases were screened for activity towards **1r** and **1s**. These enzymes stemmed from several different sources including metagenome-derived enzymes, recombinant esterases from *Bacillus subtilis* (BS2)[67] and from a thermophilic organism *Pyrobaculum calidifontis* (PestE),[89] and commercially available hydrolases such as *Candida rugosa* lipase, pig liver esterase (PLE), *Candida antarctica* lipase A and Chirazyme P1. Only three out of 40 investigated hydrolases showed activity towards these sterically demanding substrates, namely Est8 (a metagenome-derived enzyme), PestE, and Chirazyme P1. Chirazyme P1 displayed a low enantioselectivity towards **1r** and **1s** while PestE showed no activity towards **1r**. Interestingly, in the kinetic resolution of **1s**, despite the low enantioselectivity (E=10), the enantiopreference of PestE was inversed to that of Est8. Est8 could resolve **1r** and **1s** with moderate (E=22) and good (E=42) enantioselectivity, respectively.

A combined synthetic route involving the Passerini reaction and a subsequent enzymatic resolution with Est8 and PestE has been employed to synthesize optically pure protected α,α-dialkyl-α-hydroxycarboxylic acids. Nevertheless, only a small number of enzymes showed activity towards these sterically demanding substrates. A search for enzymes that are structurally related with Est8 and PestE can reveal new biocatalysts that can be applied for the kinetic resolution of protected α,α-dialkyl-α-hydroxycarboxylic acids and other tertiary alcohols.

2.2. Discovery of new biocatalysts through genome database mining and functional screening approaches

From the previous study of chemoenzymatic synthesis of enantiopure α,α-dialkyl-α-hydroxycarboxylic acids (**Article III**), Est8 and PestE showed activity

towards these sterically demanding compounds with E values up to 42. In another study of GGG(A)X enzymes activity towards several tertiary cyanohydrin acetates (**Article IV**), an excellent enantioselectivity was obtained with Est8 in the kinetic resolution of **1n**. PestE has been known to be stable in organic solvents and at high temperature.[89] These findings encouraged us to search for enzymes which resemble Est8 and PestE, and may therefore show potential for improved enantioselectivity in the kinetic resolution of tertiary alcohols. This approach was initiated through the use of the new α/β-Hydrolase Fold Enzyme Family 3DM Database (ABHDB), which is a structure-based classification of 12,431 available sequences of α/β-hydrolase fold enzymes (section 1.9.2). The database facilitates a deeper analysis of structure-function relationships within this diverse class of enzymes.

A sequence alignment was performed in ABHDB and three candidates, those with the highest sequence identity were chosen (Table 2). All the candidates belong to subfamilies 2C7BA and 3DNMA of the hormone-sensitive lipase family and thus, are GGG(A)X-hydrolases.

Table 2: Three sequences were chosen as candidates:

Name	Accession code	Organism	Identity with Est8	Identity with PestE
Est4	B1ZHM1	*Methylobacterium populi*	49%	28%
Est5	A1AUW3	*Pelobacter propionicus* strain DSM 2379	48%	29%
Est6	A1W6M8	*Acidivorax* sp. JS42	48%	39%

A further analysis with homology models from the chosen candidates and the crystallized structure of PestE (PDB code: 2wir) reveals a high similarity in the tertiary structure. Figure 21 shows the structure alignment of PestE and the homology model of Est4. Three candidate genes were cloned, expressed and their activity towards tertiary alcohols investigated.

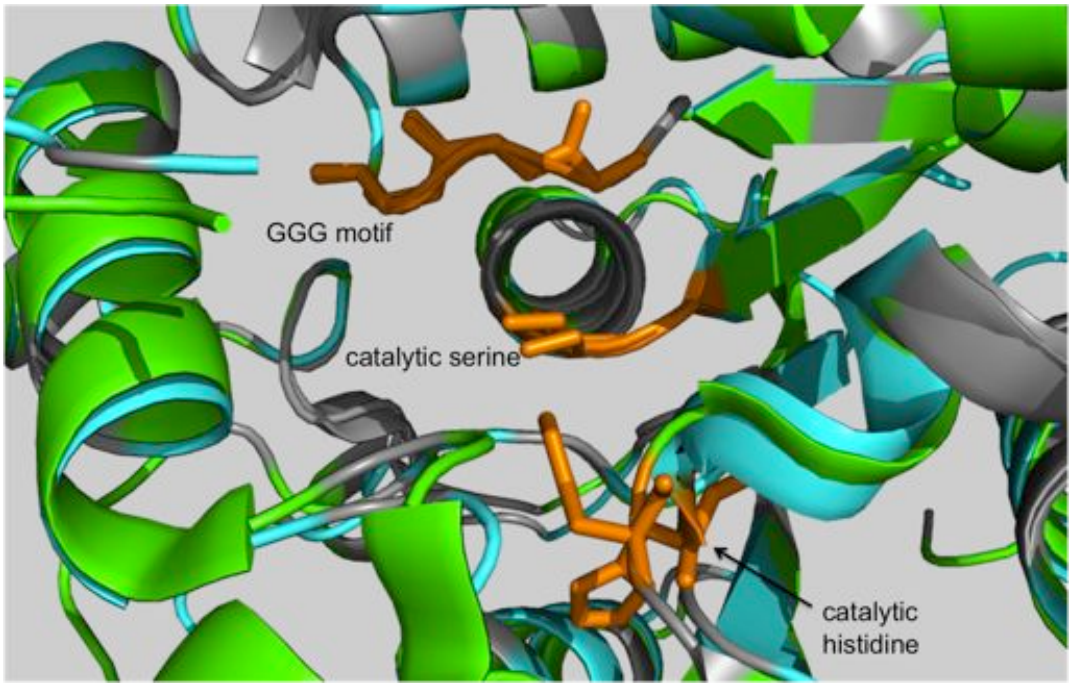


Figure 21: Structure alignment of PestE (green, PDB code: 2wir) and the homology model of Est4 (cyan) found by Phyre (http://www.sbg.bio.ic.ac.uk/~phyre/). The homology model of Est 4 was built based on the template of a protein with the PDB code c2c7bA. Regions in the structures which share identical similarity in sequence are highlighted in grey. The catalytic serines, catalytic histidines and GGG motifs of PestE and Est4 are shown in orange. The 3D model was created using PyMol molecular visualization system (http://www.pymol.org/).

In a preliminary functional screening of 72 Gram-positive Actinomycetes for the conversion of chiral tertiary alcohols, evidence was found for hydrolytic activity and enantioselectivity in the case of 14 Actinomycetes.[90, 91] The results of this initial functional screening approach include *Nocardia farcinica* of which the genome has been sequenced. Sequence information from *Nocardia farcinica* reveals the presence of three esterases containing the GGG(A)X motif which is known to confer activity of these enzymes towards tertiary alcohol esters. From three identified esterases, two esterases, Est2 and Est3, could not be cloned and expressed. The other esterase, Est1, has been successfully cloned and expressed in the working group of Dr. Gideon Grogan, University of York, UK. The enzyme was later characterized and investigated for activity towards several tertiary alcohols in our group (**Article I**).

Despite intensive optimizations, Est6 could not be expressed in the soluble fraction. Est4 and Est5 were successfully expressed and showed activity against *p*NPA. A favourable characteristic targeted with these two new enzymes is thermostability. However, in the thermostability test, the enzymes lost 60-75% of their activity after pre-incubating at 50°C for 30 min and no activity was observed after 30 min of pre-incubating at 60°C (**Article I**).

The three expressed enzymes (Est1, Est4, Est5) were further investigated for the activity towards tertiary alcohol acetates. The results are presented in Table 3.

Table 3: Enantioselectivity of Est1, Est4, and Est5 towards tertiary alcohol acetates. The data of PestE, Est8, and BS2-G105A variant from **Article III** and **IV** are shown for comparison.

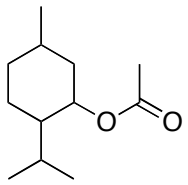
Enzyme	Substrate			
	1a	**1b**	**1c**	**1r**
Est1	6	n.c	4	n.c
Est4	24	2	3	n.c
Est5	n.c	n.c	n.c	n.c
PestE	1	2	3	10
Est8	22	9	15	22
BS2-G105A	20	68	99	n.c

n.c: no conversion

All the esterases showed activity towards *p*NPA. However, Est5 showed no activity towards any tested tertiary alcohol acetates. Est1 showed activity towards **1a** and **1c** but not towards **1b**. Nonetheless, the overall performance of Est1 towards tertiary alcohols is disappointing.

None of the enzymes could resolve the bulky tertiary alcohol **1r** compared to a moderate enantioselectivity of Est8 (**Article IV**). The highest obtained E value was from Est4 in the kinetic resolution of **1a** (E=24) while PestE showed very low enantioselectivity towards the same compound. Interestingly, the performance of Est4 towards the structurally similar compounds **1a-c** was strikingly different (Table 3). The same behaviour was observed with Est8 and BS2-G105A variant in the kinetic resolution of **1a**-**c** (**Article IV**).

With a less sterically demanding secondary alcohol ester, menthyl acetate, all the three enzymes showed activity and Est1 could resolve the compound with excellent enantioselectivity (E>100) (Figure 22).



Est1: E>100
Est4: E=5
Est5: E=8

Figure 22: Enantioselectivity of Est1, Est4, Est5 towards menthyl acetate

New biocatalysts for the synthesis of optically pure tertiary alcohols have been discovered using two different approaches, genome database mining and functional screening. Two enzymes, Est1 and Est4, showed activity towards tertiary alcohol acetates, whilst one pyridine-derived tertiary alcohol could be resolved with moderate enantioselectivity. Although the thermostability of the enzymes did not meet

expectations; with moderate enantioselectivity towards **1a**, Est4 is a good candidate for further study with protein engineering to increase the performance of the enzyme towards sterically demanding substrates.

Based on functional screening and genome database mining, new biocatalysts that show activity towards tertiary alcohols have been discovered. Est4, which belongs to the same hormone-sensitive lipase family in ABHDB with Est8 and PestE, can not resolve the sterically demanding substrate **1r**. The results from the activity study show that it is still difficult to predict the activity of new biocatalysts in spite of high sequence similarity with the template enzyme.

In the next section, a different approach of enzyme activity discovery based on an alignment-inspired method of rational protein design will be presented.

2.3. Alignment-inspired method for the rational protein design of EstA from *Paenibacillus barcinonensis*

EstA is an esterase isolated from *Paenibacillus barcinonensis.*[92] A sequence alignment of EstA with the GGG(A)X motif esterase BS2 from *Bacillus subtilis* revealed high similarity (49%). In the previous studies, BS2-G105A variant from *Bacillus subtilis* resolved several tertiary alcohols with high enantioselectivity.[11, 68] Therefore, EstA was identified as a potential biocatalyst for the kinetic resolution of tertiary alcohols. However, the activity of the wild-type enzyme towards **1g** was very low (**Article II**). A further study with structural alignment of EstA and BS2 showed a high similarity between in these two enzymes, especially in the active site (Figure 23). As mentioned before, ABHDB (3DM) covers two large groups of esterases and lipases that have been shown to be active towards tertiary alcohol esters. While PestE, Est8 and Est4 (**Article I**) are enzymes from the hormone-sensitive lipase-like family, EstA and BS2 both belong to the second family, which share a high similarity to acetylcholine esterases and mammalian liver esterases.

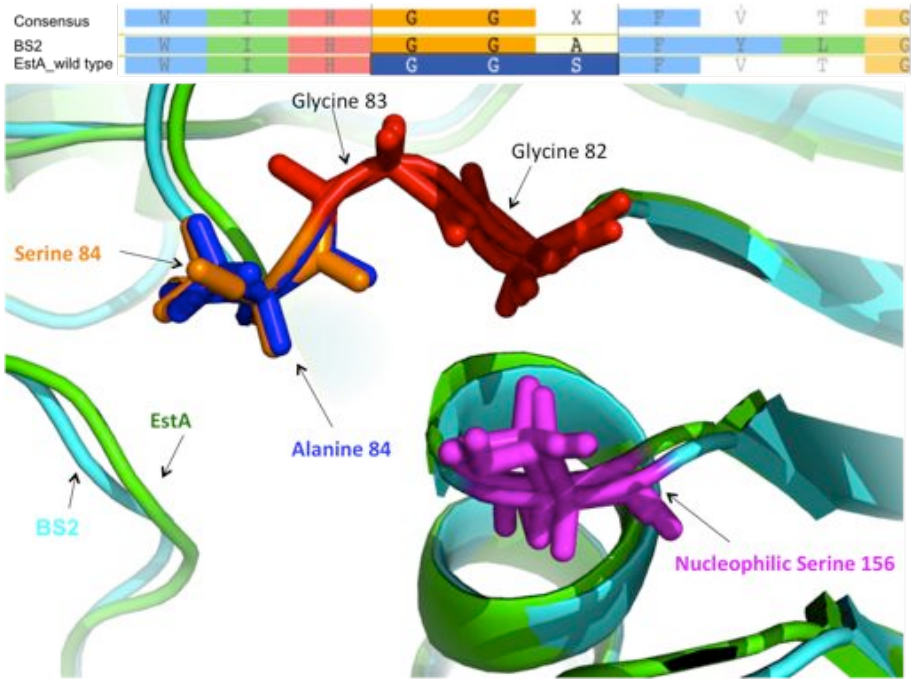


Figure 23: Sequence and structure alignment of active site regions of BS2 (PDB code: 1qe3, **cyan**) and homology model of EstA (**green**) reveals a high similarity in structure between two enzymes. In the oxyanion hole region, the amino acid residue at position 84 is alanine in case of BS2 (**orange**) and serine in case of EstA (**blue**).

At positions 82-84 in the consensus fold, the three glycines of the GGG(A)X-motif are known to be highly conserved. Therefore, it was unexpected to find the serine residue of EstA at position 84. In a further analysis of amino acid abundance using ABHDB at these three positions of the family to which BS2 and EstA belong, serine at position 84 has only 10% abundance in comparison with 64% for glycine and 21% for alanine (Figure 24). The appearance of the serine residue at position 84 could account for the low performance of EstA towards tertiary alcohol acetates.

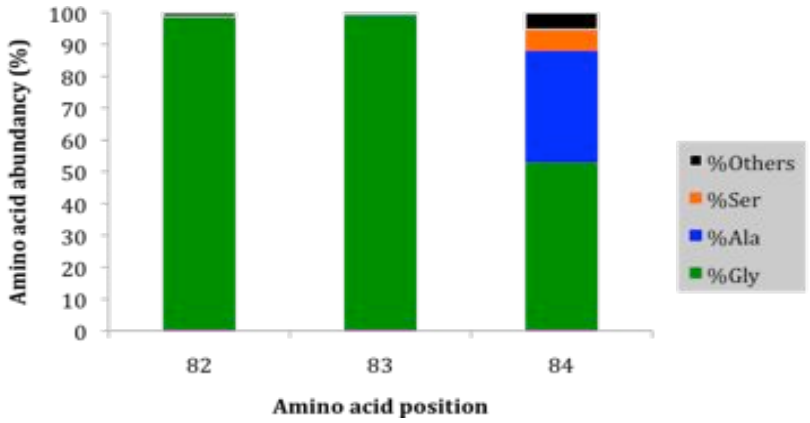


Figure 24: Amino acid abundance in 1343 sequences of the same family with similarity to acetylcholine esterases for GGG(A)X motif (position 82-84).

From this assumption, different variants of EstA were constructed to investigate the role of these amino acid residues in this important region in the kinetic resolution of tertiary alcohols. The variant EstA-GGG was created based on the highest conserved

residue glycine according to the ABHDB analysis (Figure 24). Based on the corresponding mutant BS2-G105A, which showed an excellent enantioselectivity towards many tertiary alcohol acetates,[68, 93] the analogous mutant at position 82, EstA-AGA, was also constructed. Other variants, GGA, GAG, and GGA, were created to obtain a better understanding of the relationship between structure and function of these residues.

Table 4: Activity and enantioselectivity of EstA variants towards **1g** at 37°C (**Article II**). The enantioselectivity values of BS2 wild-type (BS2-GGA) and BS2-G105A (BS2-AGA)[11] are shown for comparison.

Enzyme	Specific activity (U/mg)[a]	Conversion (%)[a]	Enantioselectivity[b]
EstA-GGS[c]	34.75	3	5
EstA-GGG	13.22	80	5
EstA-GGA	13.59	50	8.5
EstA-GAG	2.15	n.c	Not determined
EstA-AGG	2.99	100	7
EstA-AGA	2.86	10	55
BS2-GGA[c]			42
BS2-AGA			>100

[a] purified enzymes in the hydrolysis of *p*PNA, data from Table 2, **Article II**; [b] data from Table 3, **Article II**; [c] wild-type enzyme; n.c: no conversion.

Variants of EstA created by Arnau Bassegoda, University of Barcelona, Spain were later investigated in our group. The activity and enantioselectivity of these variants towards **1g** are given in Table 4.

In spite of lower specific activities towards *p*NPA, all variants except for the EstA-GAG variant showed higher activity towards **1g** in comparison to the wild-type enzyme. Two variants, EstA-GGG and EstA-GGA, displayed a 26-fold and 16-fold higher activity towards **1g**, respectively, when compared with EstA wild-type. This result supports our hypothesis for the poor performance of EstA wild-type due to the serine residue at position 84.

The variant EstA-GAG did not convert **1g** despite its activity towards *p*NPA. The same results were observed in the investigation of the GGG(A)X motif of pig liver esterase (PLE).[94] All variants containing alanine at the central position of the motif displayed very low activity towards tertiary alcohol acetates. The amino acid abundance analysis from ABHDB, which is displayed in Figure 24, also shows the major appearance of glycine in the middle position of the motif (>99%). The result from EstA-GAG underlines the importance of the second glycine residue in the conversion of tertiary alcohols.

Two variants, EstA-AGG and EstA-AGA, were chosen for a further investigation of tertiary alcohol acetates. These variants were chosen because of their high activity and enantioselectivity towards **1g**, respectively. EstA-AGG showed low enantioselectivity towards **1h-1j**. On the contrary, EstA-AGA resolved **1h** and **1i** with excellent enantioselectivity, but no activity was observed with **1j** despite the small difference between **1i** and **1j** in the position of the fluorine substituent on the phenyl ring (Table 5). The same behaviour was observed in the kinetic resolution of the structurally similar compounds **1a**-**1c** with the BS2-AGA variant (BS2-G105A) (**Article IV**).

Table 5: Enantioselectivity of EstA variants towards further tertiary alcohols (**Article II**). The data from BS2-AGA (BS2-G105A) [68, 93] are shown for comparison.

Enzyme	Substrate						
	1h	**1i**	**1j**	**1a**	**1b**	**1c**	**1d**
EstA-GGS	n.c	n.c	n.c	n.c	n.c	n.c	n.c
EstA-AGA	65	>100	n.c	n.c	n.c	n.c	n.c
EstA-AGG	7	11	1	n.c	n.c	n.c	n.c
BS2-AGA	>100	>100	12	20	68	99	3

n.c: no conversion

Surprisingly, none of the EstA variants could convert the pyridine tertiary alcohols **1a-d** whilst BS2-AGA (BS2-G105A) showed activity towards all of the four compounds (**Article IV**). Moreover, BS2-AGA did resolve **1b** and **1c** with excellent enantioselectivity (Table 5). Whereas the two enzymes share a high similarity in sequence and structure alignments, adjacent amino acid residues could differ. These differences can significantly affect the substrate recognition of the enzymes.

While the tertiary alcohol acetate **1r**, bearing a bulky *tert*-butyl-carbamoyl substituent was converted by Est8 and PestE, two enzymes from the hormone sensitive lipase-like family (**Article III**), no activity was observed with EstA wild-type and its variants. BS2 also showed no activity towards **1r** and its analogues (**Article III**). It is likely that that the enzymes from the acetylcholine esterase-like family, which EstA and BS2 belong to, do not convert this sterically demanding substrate.

The activity and enantioselectivity of EstA variants towards tertiary alcohol acetates were dramatically improved by mutating the residue at position 84 of EstA from serine to glycine and alanine, the two consensus amino acid residues based on the analysis of ABHDB. The consensus concept approach to identify key residues for mutagenesis to increase the thermostability of subtilisin BPN' from *Bacillus amyloliquefaciens* has been reported in 1989 by the study of Pantoliano et al.[95] The concept is based on the assumption that any consensus amino acid of an enzyme has been tested during the evolution progress and therefore would be best fitted for

the function of the enzyme. Recently, Jochens et al. have applied an alignment-inspired method to create “small, but smart” mutant libraries for the focused directed evolution of PFE, an esterase from *Pseudomonas fluorescens*, to increase the thermostability of the enzyme[88] and its enantioselectivity towards 3-phenylbutyric acid esters.[87] In both studies, the ABHDB database has been employed to analyze the potential amino acid residues for the site-saturation mutagenesis. The obtained libraries were smaller and of higher quality when compared to control libraries.[87, 88]

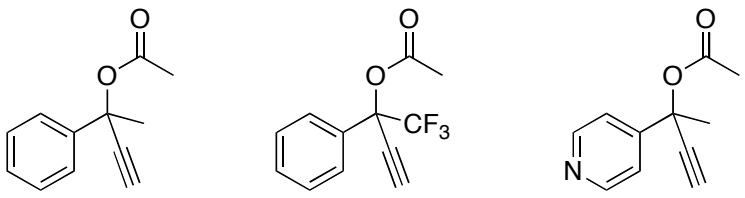
The combination of the structure-guided method based on the homology model of EstA and the database-oriented approach helped to predict the key residues in the rational protein design of EstA. The combinatorial approach can be useful when a limited amount of structural data is available.

2.4. Effects of reaction conditions on the enantioselectivity of enzymes in the kinetic resolution of tertiary alcohols.

2.4.1. Prevention of non-enzymatic hydrolysis

Non-selective chemical hydrolysis in an enzymatic resolution of an ester can lower the optical purity of the final product. In a study on the kinetic resolution of tertiary alcohol acetates, O’Hagan and Zaidi have observed the non-enzymatic hydrolysis *via* an S_N1 mechanism of compound **9** under reaction conditions (buffer pH 7.5, 37°C).[96] The chemical hydrolysis, which could be suppressed to some extend by adding dimethyl sulfoxide (DMSO) as a co-solvent, still had an effect on the enantioselectivity of the studied enzymes.[11] On the contrary, the compound **1g** did not undergo S_N1-mechanism hydrolysis on account of the electron-withdrawing nature of the $–CF_3$ group, which is known to resist against chemical autohydrolysis.[96]

The substrates **1a-c** studied in **Article IV** did not show chemical hydrolysis under reaction conditions (Figure 25). During the chemical hydrolysis *via* the S_N1 mechanism, the rate of the reaction depends strongly on the carbocation stability. In case of compounds **1a-c**, the pyridine ring could act as an electron-withdrawing group. Therefore, a carbocation in this case would be less stable and the chemical hydrolysis is partly suppressed.



9	**1g**	**1c**
unstable	stable	stable
BS2-G105A: E=55	BS2-G105A: E>100	BS2-G105A: E=99

Figure 25: Stability of **9**, **1g**, and **1c** under reaction conditions: buffer pH 7.5, 37°C and enantioselectivity of BS2 variant BS2-G105A towards each compound.[96]

2.4.2. Effect of temperature on enantioselectivity of enzymes

Holmberg et al. (1991) have observed the effect of temperature on the enantioselectivity of enzymatic reactions.[66] In most cases, the enantioselectivity increases with decreasing reaction temperature.

In the kinetic resolution of **1s** (**Article III**), the enantioselectivity of Est8 increased from E=8 to E=42 when the reaction temperature decreased from 37°C to 4°C (in combination with co-solvent optimization). In **Article IV**, the same phenomenon was observed with Est8 and the compound **1n** where the enantioselectivity increased from E=5 at 37°C to E=18 at 4°C (data not shown). However, a higher E value was not obtained with other substrates (**1k**-**1m**, **1o**) and enzymes when the same optimized reaction conditions were applied.

The temperature optimization of biocatalysis showed only a slight increase in the enantioselectivity (from E=23 at 37°C to E=26 at 4°C) (data not shown) in the kinetic resolution of **1f** using Est8 (**Article V**). Nevertheless, as presented in **Article II**, a strong improvement of enantioselectivity was achieved when the same temperature optimization strategy was implemented. Starting from an E value of 55 at 37°C, through temperature optimization, EstA mutants (EstA-AGA) could later resolve the compound **1g** with excellent enantioselectivity (E>100) at 4°C.

Despite the fact that temperature optimization does not always give the same effect to improve the enantioselectivity, the method should be considered in the kinetic resolution of tertiary alcohols, especially when the starting E value is moderate (10<E<20).

2.4.3. Effect of cosolvents on enantioselectivity of enzymes

The reaction medium is considered as one of the most important parameters that can have an impact on activity and enantioselectivity of an enzymatic reaction.[97-99] Initially, water-miscible cosolvents were added to the reaction medium to improve the solubility of organic substrates in biocatalysis.[100, 101] However, further studies showed that these cosolvents can have an influence on the enantioselectivity of biocatalysts.[97] In the study by Lam et al. (1986), the prochiral compound 3-methyl-glutarate dimethyl ester was converted by pig liver esterase to yield *(R)*-monoester in aqueous buffer (pH 7) and cosolvents. The best result obtained was with 20% methanol as a cosolvent. The enantiomeric excess of the product was 88%ee at 20°C compared to 79%ee at the same temperature if no cosolvent was added.[102] Watanabe et al. (2001) reported a dramatic enhancement of enantioselectivity in the lipase-catalysed hydrolysis of butyl 2-(4-substituted phenoxy)propionates. Starting from an E value of 4 in aqueous buffer, an excellent E value was obtained (E>100) when the concentration of the cosolvent DMSO reached 55%.[103]

In the kinetic resolution of **1r**, Est8 resolved **2r** with 56% enantiomeric excess when DMSO was added as a cosolvent in aqueous buffer (data not shown). During the optimization of the cosolvents, dimethylformaldehyde (DMF) helped to increase the

enantiomeric excess of the product to 73%ee under the same reaction conditions (**Article III**). The same effect was observed with Est8 and the compound **1n** (**Article IV**). The application of DMF instead of DMSO as the cosolvent in the kinetic resolution of **1n** with Est8 as the biocatalyst increased the E value of the enzymatic reaction from E=18 (DMSO, 4°C) to more than 100 (DMF, 4°C). Nevertheless, the same effect was not achieved in the kinetic resolution of **1f** with Est8 (data not shown) (**Article V**).

The reason for the effect of reaction medium on the enantioselectivity is not yet fully understood. Fitzpatrick et al. proposed the hypothesis that in case of anhydrous solvents, the solvent could affect the conformation of the biocatalyst and therefore, influence the enantioselectivity based on changes in the molecular recognition process.[104] By measuring the initial rates of reactions in the aqueous buffer with and without the addition of DMSO, Watanabe et al. (2001) have shown that the ratio of initial rates of two enantiomers in case of DMSO addition was much higher than when only an aqueous buffer was implemented.[103] Another hypothesis was proposed by Secundo et al. (1992) in which the solvent molecules can bind with the molecules of amino acid residues in the active site and hence, intervene with the catalysis of one enantiomer more than another.[105]

In spite of the experimental data that could be used to support each particular hypothesis, the empirical rule for the prediction of the cosolvent's effect on the biocatalysis has not been generalized and defined due to a lack of consistency.[97] Nevertheless, the implementation of cosolvents in the reaction medium can be considered as one of the parameters to optimize the enantioselectivity of enzymatic reactions.

2.4.4. The influence of substrate structure on enantioselectivity

Kazlauskas et al. proposed an empirical rule based on steric interactions to predict the enantiopreference of lipases towards secondary alcohols. The rule states that a lipase in many cases will prefer the enantiomer with the configuration shown in Figure 26 for a given substrate.[106]

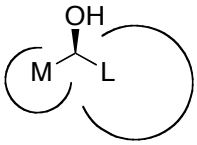


Figure 26: An empirical rule based on relative substituent size to predict the enantiopreference of lipase. L is the large sized substituent and M is the medium substituent.

For enzymatic reactions of subtilisin in aqueous buffer or in a mixture of water and water-miscible cosolvents, a rule presented in Figure 27 was suggested by Savile and Kazlauskas (2005).[107]

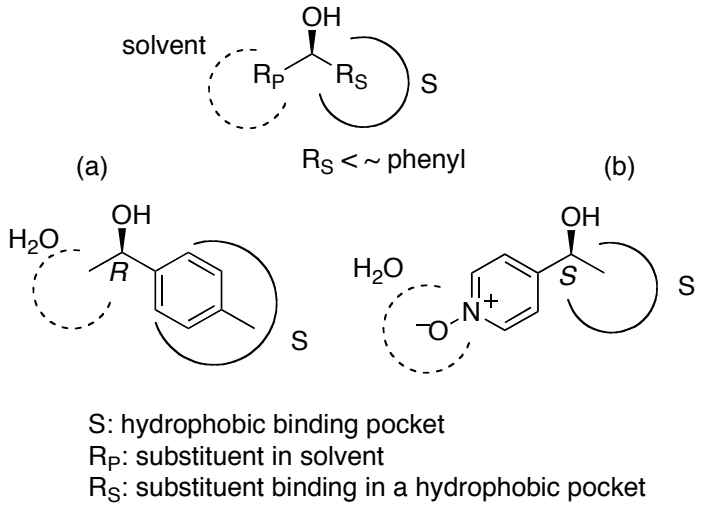


Figure 27: A rule to predict the enantiopreference of subtilisins toward secondary alcohols (adapted from Savile et al.)[107] (a) the preferred enantiomer is (*R)* because the aryl substituent is less favourable than the methyl group in the reaction medium, which contains mainly water, than the methyl group. (b) the enzyme in this case prefers the (*S*)-enantiomer because the compound contains a polar aryl substituent, which is now more favoured in aqueous environment than the methyl group.

As shown in Figure 27, the influence of substrate structure on the enantioselectivity is not only based on steric effects but also in some cases on non-steric effects, namely charge distribution, substrate solvation and polarization.[107, 108]

The striking differences in enantioselectivity of the GGG(A)X esterases toward three structurally related compounds are presented in **Article IV**. The variant BS2-105A from *Bacillus substilis* showed a contrasting enantioselectivity in the kinetic resolution of three structurally similar compounds **1a**-**1c**. While **1b** and **1c** could be resolved with excellent enantioselectivity with BS2 G105A (E>100), the enantioselectivity of the same mutant towards **1a** is only moderate (E=20). Additionally, the isoenzyme γPLE of pig liver esterase also catalyzed the biocatalysis of **1a-1c** with different enantioselectivity and enantiopreference. γPLE resolved **1a** and **1c** with excellent and moderate enantioselectivity, respectively whilst no activity towards **1b** was observed. Interestingly, two isoenzymes γPLE and PLE5, with more than 96% similarity in sequence,[109] showed an inverted enantiopreference towards **1c** as well as striking differences in enantioselectivity in the kinetic resolution of **1a-1c**.

These examples clearly showed that the influence of the substrate structure on enantioselectivity can not always be explained only by steric effects. Non-steric effects could come from the differences in charge distribution, electronic effects or polarizability.[108, 110]

Substrate solvation could contribute to the effect on enantioselectivity.[107] Wiggers et al. (2009) have reported remarkable differences of enantioselectivity in the kinetic resolution of *tert.* acetylenic alcohol esters and similar α,α-disubstituted cyanohydrin acetates (**1k**-**1o**) using BS2 and its mutants.[111] Using molecular modelling, the authors suggested that in case of cyanohydrin acetates, the nitrile groups can form hydrogen bonds with water molecules in the active site, which leads to a different

interaction of the enzyme and binding substrates. Therefore, enzymes can have different performances on these two structurally similar compounds. Figure 28 shows a theoretical suggestion of how **1c** could accommodate into the active site of BS2 esterase in comparison with **1g**.

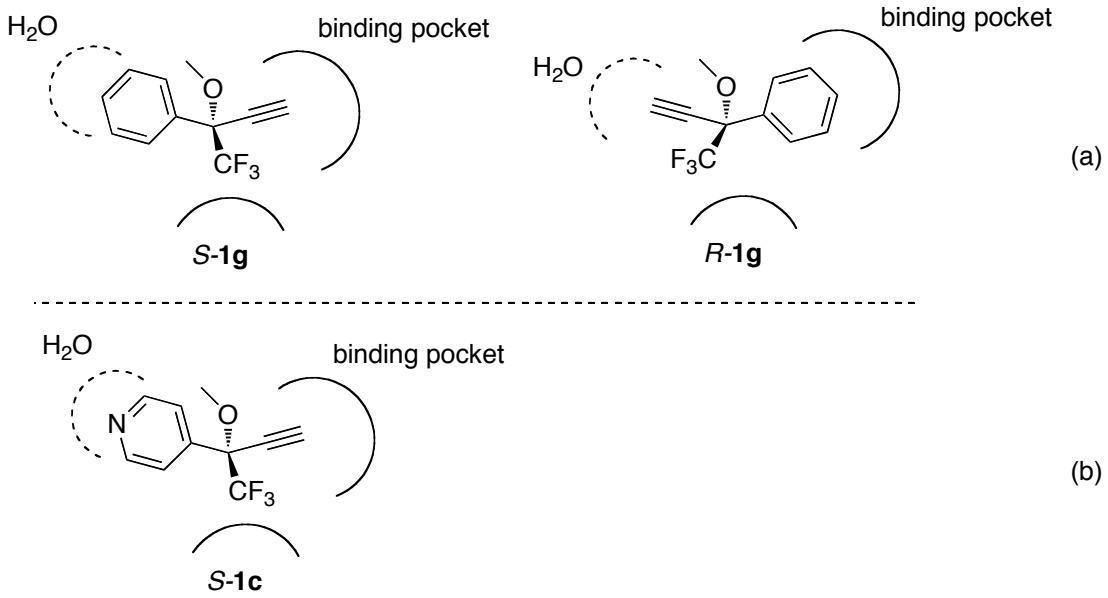


Figure 28: (a) Accommodation of *(R)*-**1g** and *(S)*-**1g** in the active site of BS2 esterase.[69] (b) A similar accommodation of *(S)*-**1c** could result in an entirely different solvation of the substrate.

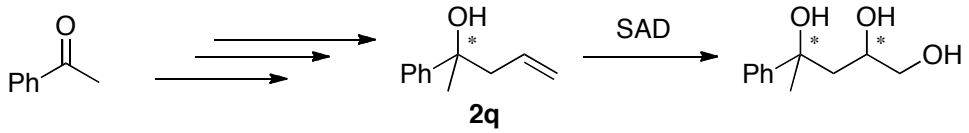
In the kinetic resolution of **1k** and **1n** with Est8, despite the almost identical size of the two compounds (different only in $–CH_3$ and $–CF_3$ substituents), **1k** could only be resolved with a low enantioselectivity in comparison with an excellent enantioselectivity toward **1n**. This again emphasizes the non-steric effects on enantioselectivity that was also observed in the study by Watanabe et al.[108]

In contrast to secondary alcohols, an empirical rule to predict the enantiopreference of tertiary alcohol esters in the kinetic resolution with esterases has not yet been established. Nevertheless, the available data indicates that the enantiorecognition of enzymes toward tertiary alcohols is also strongly influenced by non-steric effects.

2.5. Comparison of enzymatic methods with chemosynthesis pathways

Chemical synthesis of enantiopure tertiary alcohols in many cases requires heavy metal catalysts as well as harsh reaction conditions as presented in the introduction part. Biocatalytic pathways provide alternative routes to synthesize chiral tertiary alcohols under more environmentally friendly reaction conditions.

Article V presents a comparison of chemical and chemoenzymatic pathways for the synthesis of the enantiopure tertiary homoallylic alcohol **2q**, which was consequently applied for the Sharpless asymmetric dihydroxylation (SAD) to yield 1,2,4-triols (Figure 29). The chemical pathways were performed by the group of Prof. Sabine Laschat, University of Stuttgart.



2q

Figure 29: General reaction to synthesize 1,2,4-triols **11** through tertiary homoallylic alcohols with ketones as the starting compounds

Three synthetic pathways are presented in Figure 30. Following the chemical pathway I using the Evans aldol reaction with **10** as the starting compound, five synthesis steps are required (steps IIa-VIa) to achieve TBS-protected (*S*)-**2q** with 33% overall yield and excellent enantiomeric excess (>99%ee). In comparison to the chemoenzymatic pathway, (*S*)-**2q** was resolved with moderate overall yield (25%) and good enantiomeric excess (85%ee) in a three-step synthesis including two chemical reactions (step Ib and IIb) and one biocatalysis (step IIIb).

Based on the method of Walsh,[112] (*R*)-**2q** was synthesized using a separate one-step synthesis with excellent yield (99%) and enantiomeric excess (95%ee) (chemical pathway II). From the kinetic resolution of **1f** using Est8 (step IIIb), (*R*)-**1f** was isolated with 22% overall yield and good enantioselectivity (71%ee). (*R*)-**2q** can be obtained by one further step of deprotecting the acetyl group.

Despite lower enantiomeric excess and the maximum 50% yield, the synthesis of (*S*)-**1f** by the chemoenzymatic pathway required only three synthetic steps while the chemical method required six steps if **10** is the starting compound. No protection and deprotection steps (IIIa, VIIa) were required. The amount of organic solvents used in the chemoenzymatic method is considerably smaller than in the Evans aldol pathway. Additionally, a milder reaction temperature makes the biocatalytic synthetic route more efficient regarding energy consumption. The kinetic resolution of **1f** with Est8 also provided both (*S*)-**1f** and protected (*R*)-**1f** in a one-step synthesis.

With chemical synthesis, (*R*)-**2q** was obtained by a one-step synthesis with tetraallylstannane as the starting compound with excellent yield and enantiomeric excess. From step IIIb, acetyl-protected (*R*)-**2q** was already synthesized in the kinetic resolution of **2q** and Est8 in step IIb. A deprotection step (IVb) is required to yield (*R*)-**2q** with the maximum yield of 22%. In this case, the chemical pathway provides a higher yield and better optical purity of (*R*)-**2q**. However, the utilization of organotin compounds in the chemical pathway raises serious environmental concerns because of the well-studied toxicity of this type of compound.[113-116]

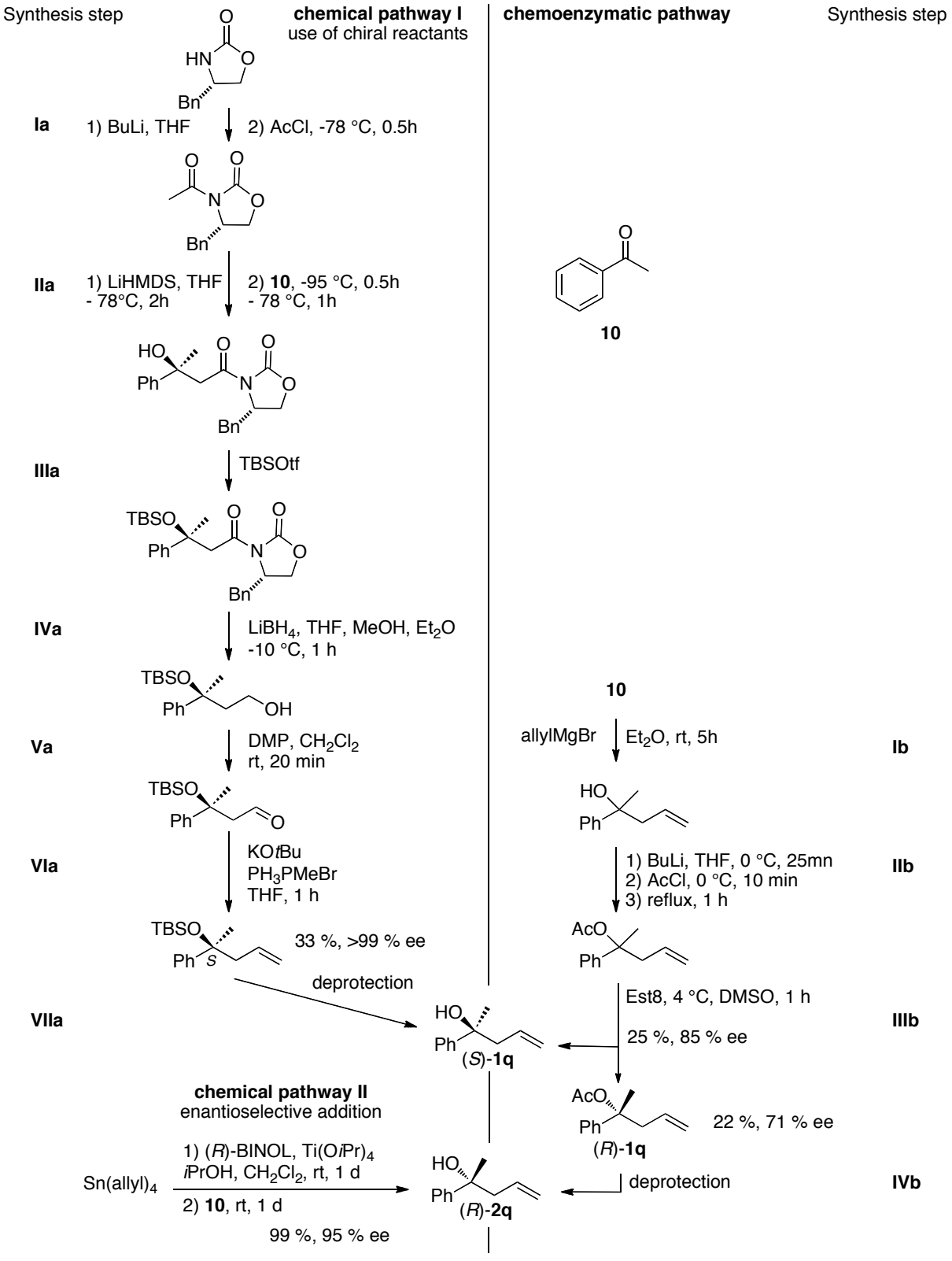


Figure 30: Three synthetic pathways for the synthesis of enantiopure allylic tertiary alcohols (Article V).

The comparison between biocatalysis and chemical catalysis for the synthesis of enantiopure compounds has rarely been studied. Zeror et al. (2010) have made a comparison between biocatalyzed reactions by *Saccharomyces cerevisiae* and

ruthenium-catalyzed reactions to synthesize chiral alcohols by ketoester reductions in water at 30°C (Figure 31).[117]

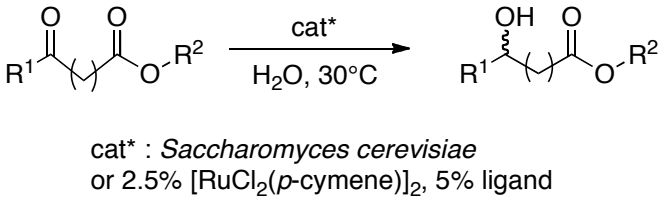


Figure 31: Enantioselective reduction of ketoesters.[117]

In most cases, the reduction of ketoesters by biocatalysts gave a better yield and enantioselectivity. The products are easily separated from the catalysts in both procedures.[117] Nevertheless, a chemocatalytic pathway requires the employment of ruthenium, a metal that is strictly regulated in pharmaceutical production.[42]

In another study of Kosjek et al. for the synthesis of chiral allylic alcohol **12**, chemocatalytic and biocatalytic methods have been applied with an α,β-unsaturated ketone **11** as the starting compound.[118]

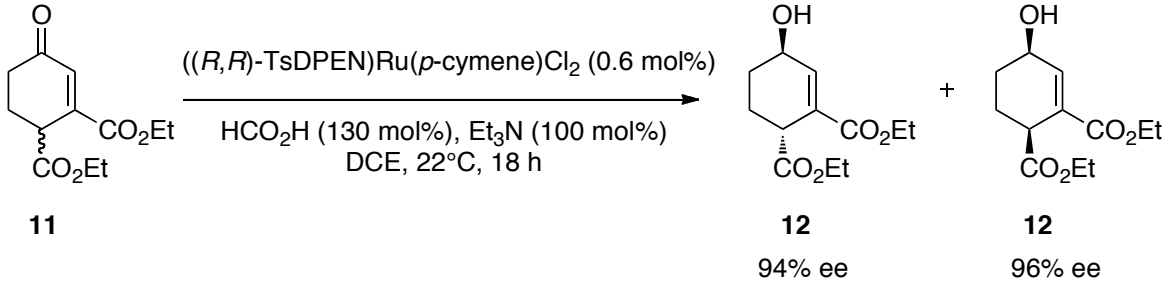


Figure 32: Ruthenium-catalyzed approach for the synthesis of **12**.[118]

Both diastereomers of the compound **12** could be resolved with high enantiomeric excess by using ruthenium-catalyzed method (Figure 32). Nevertheless, the diastereoselectivity of the reaction was moderate (47% *cis*) and further attempts to prepare **12** as a single enantiomer via epimerization were not successful.[118]

In the biocatalytic approach using isolated enzymes, after a preliminary screening, a ketoreductase (KRED108) was identified as the potential biocatalyst. The enzyme could resolve **12** with both high enantio- and diastereoselectivities (up to 95% ee *cis* and 99% cis, respectively) in a one-step reaction (Figure 33).

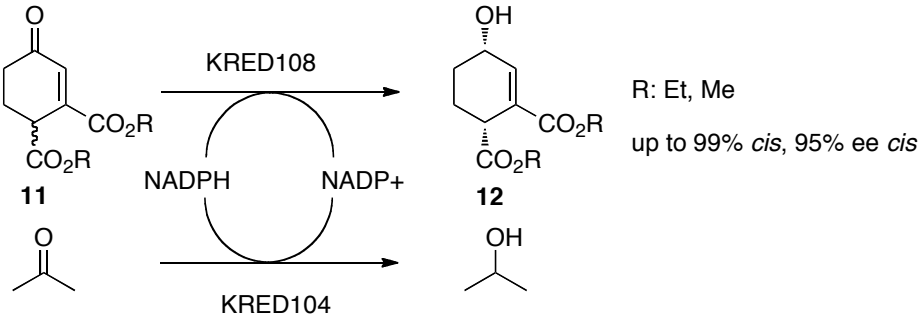


Figure 33: Biocatalytic pathway for the synthesis of **12** by ketoreductase (KRED 108, KRED 104).[118]

In this example, biocatalysts provides a more efficient pathway to synthesize chiral allylic alcohols from α,β-unsaturated ketones when compared with the chemocatalytic pathway.

An asymmetric reduction of several ketones, such as aryl, dialkyl, α,β-unsaturated and acetylenic ketones, *via* two different methods, whole cell bioconversion and ruthenium(II)- and iridium(I)-catalyzed transfer hydrogenation, has been investigated in the study of Hage et al.[119] The obtained results showed that both biocatalytic and metal-catalyzed systems could be used to reduce a wide range of prochiral ketones with complementary product configuration. While the biocatalysis approach using whole cell conversion by *Merulius remellosus* is mostly suitable for enantioselective reduction of chloro-substituted ketones, metal-catalyzed system can be used to reduce α,β-unsaturated ketones with high enantioselectivity.[119]

These examples show that in many cases, biocatalysis can provide an alternative efficient pathway for the synthesis of optically pure compounds. A combinatorial method between biocatalytic and chemocatalytic pathways should also be considered so that the advantages from both approaches can be achieved.

3. Concluding remarks

In this thesis, the application of enzymes for the synthesis of enantiomerically enriched tertiary alcohols has been considerably extended based on the established catalytic platform of GGG(A)X motif enzymes. Diverse types of tertiary alcohol could be synthesized with high optical purity by several enzymes from different sources (Figure 34). Furthermore, factors that influence the enantioselectivity of biocatalysts towards tertiary alcohols have also been studied.

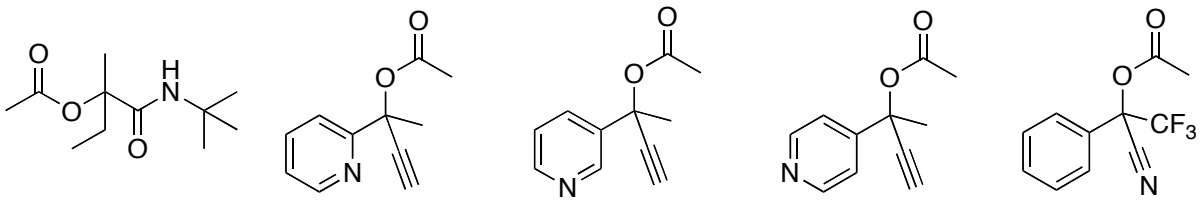
A new chemoenzymatic pathway, which includes the Passerini-multicomponent reaction and a subsequent enzymatic hydrolysis, has been developed to synthesize protected α,α-dialkyl-α-hydroxycarboxylic acids, important building blocks in organic chemistry, with good enantioselectivity. Moreover, pyridine-derived tertiary alcohols and tertiary cyanohydrins could be resolved with excellent enantioselectivity using enzymes from different sources such as a variant from the recombinant esterase BS2-G105A from *Bacillus subtilis,* an isoenzyme γPLE (PLE1) from pig liver esterase, and a metagenome-derived esterase (Est8).

Two new biocatalysts have been discovered using two different approaches: functional screening and genome database mining. The new enzymes show activity towards tertiary alcohols and will be potential candidates for further studies of protein engineering.

An alignment-inspired method has been successfully applied for the prediction of key residues in a rational protein design of EstA, an enzyme from *Paenibacillus barcinonensis*, to create new variants that are highly selective biocatalysts. The method is particular useful when only a limited amount of structural data is available.

The results from the activity study of new biocatalysts (Est4, Est5) and rational protein design of EstA have shown the limitation of enzyme activity prediction that is based solely on sequence alignment. In spite of up to 49% sequence similarity, the activity and enantioselectivity of Est4 and Est5 towards tertiary alcohols are lower than of Est8. EstA wild-type show a very low activity towards tertiary alcohols when compared with BS2 wild-type despite of 49% sequence identity. Nevertheless, key residues for rational protein design of EstA have been successfully identified with the application of ABHDB.

Chemical synthesis and enzymatic resolution of a tertiary homoallylic alcohol have been performed to evaluate the efficiency of each pathway. On one hand, the chemoenzymatic pathway could resolve the compound with moderate yield and enantioselectivity. On the other hand, two enantiomers of the tertiary alcohol could be synthesized with moderate to high yield and with excellent optically purity by two chemical pathways. Nevertheless, greater synthesis steps and amounts of organic solvents as well as the employment of heavy metals have made the chemical pathway less environmentally friendly and energy efficient than an enzymatic approach.



E=42	E>100	E>100	E>100	E>100
Est8	γPLE	BS2-G105A	BS2-G105A	Est8

Figure 34: Selected examples of tertiary alcohols that can be resolved with high optical purity by GGG(A)X motif enzymes.

The presented examples on Figure 34 underline the importance of enzyme toolbox for the kinetic resolution of tertiary alcohols. Several enzymes in the toolbox can help to overcome the limitations of individual enzymes. Moreover, a wide range of tertiary alcohols can be synthesized with high optical purity by different enzymes in the toolbox.

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Summary

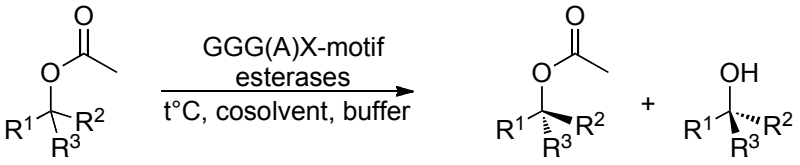
The first applied biocatalysis stemmed from ancient China, Japan, and Mesopotamia in the production of food and alcoholic drinks using isolated enzymes or whole-cell biocatalysts. Enzyme applications nowadays are found in several sectors of chemical industry such as food additives, fine chemicals, drugs, and agricultural chemicals. Many fine chemicals have been produced in multi-ton quantities by using enzymatic processes.

Enzymes play an important role in the development of a more sustainable chemicals production. In many cases, the production processes in which enzymes act as catalysts do not require high temperature, pressure or organic solvents. This helps reduce energy costs and avoid environmental impacts. Another advantage of enzymes over chemical catalysts is their high chemo-, regio- and enantioselectivity. This has made enzymes more attractive for the pharmaceutical industry, in which more than 50% of the compounds are chiral.

Nevertheless, in many cases, enzymes have a narrow substrate scope, which limits their application in industrial production. The demand for extending the substrate scope of enzymes and the discovery of new biocatalysts has led to several directions in enzyme research. One direction is to focus on the investigation of the activity and enantioselectivity of enzymes towards different types of compounds, which have potential applications. The other direction is to find new biocatalysts by modifying available enzymes by protein engineering and discovery of new enzymes through functional screening, metagenome derived sources and database mining.

Tertiary alcohols have become interesting targets for organic synthesis themselves or as building blocks for valuable pharmaceutical compounds. However, the synthesis of optically pure tertiary alcohols is still a challenge both chemical and enzymatic means. Enzymes containing the GGG(A)X motif in the active site region have been known to show activity towards these sterically demanding substrates. Several tertiary alcohols have been resolved with high enantioselectivity by using this biocatalytic synthetic route.

This thesis aims at providing a better understanding of enantiorecognition of GGG(A)X motif hydrolases in the enzymatic synthesis of enantiomerically enriched tertiary alcohols. Kinetic resolution of a wide range of tertiary alcohols using hydrolases provided insights on factors that can influence enantioselectivity of GGG(A)X motif enzymes. Additionally, a newly proposed chemoenzymatic method to synthesize protected α,α-dialkyl-α-hydroxycarboxylic acids has broadened the application of these enzymes to synthesize optically pure tertiary alcohols. Newly found biocatalysts through functional screening, database mining and rational protein design approaches provided a better enzyme platform for optically pure tertiary alcohol resolution.



Scheme 1: Kinetic resolution of enantiomerically enriched tertiary alcohols using GGG(A)X motif enzymes

A new chemoenzymatic pathway, which includes the Passerini-multicomponent reaction and a subsequent enzymatic hydrolysis, has been developed to synthesize protected α,α-dialkyl-α-hydroxycarboxylic acids, important building blocks in organic chemistry, with an E value up to 42 (Figure 1). Moreover, pyridine-derived tertiary alcohols and tertiary cyanohydrins could be resolved with excellent enantioselectivity (E>100) using enzymes from different sources such as a variant of the recombinant esterase from *Bacillus subtilis* (BS2-G105A), an isoenzyme γPLE (PLE1) from pig liver esterase, and a metagenome-derived esterase (Est8).

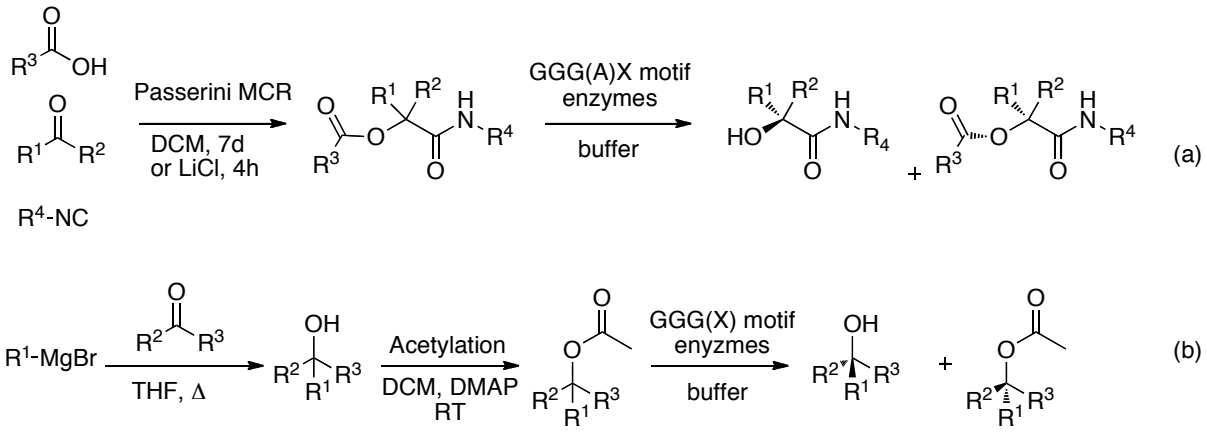


Figure 1: Chemoenzymatic pathways for the synthesis of optically pure tertiary alcohols involving chemical synthesis steps (a) through Passerini MCR, (b) through Grignard reactions and a subsequent enzymatic resolution.

An alignment-inspired method has been successfully applied for the prediction of key residues in a rational protein design of EstA, an enzyme from *Paenibacillus barcinonensis*, to create new variants that are highly selective biocatalysts. EstA shares a high sequence identity with BS2, an enzyme from *Bacillus subtilis* that shows high enantioselectivity towards several tertiary alcohols. However, the activity of EstA wild-type variant towards tertiary alcohol is very low.

Sequence and structure alignments of BS2 and EstA revealed a high similarity in the active site regions of both enzymes (Figure 2, above). Interestingly, based on a further analysis at position 84, the amino acid residue is serine in case of EstA instead of highly conserved glycine of consensus sequence or alanine of BS2. A further analysis using the new α/β-Hydrolase Fold Enzyme Family 3DM Database (ABHDB), which is a structure-based classification of 12,431 available sequences of α/β-hydrolase fold enzymes, showed that at position 84, serine only presents in

10% of total 1,343 proteins in the acetylcholine esterase family. The analysis encouraged us to create several EstA variants to study the role of these residues in this important region for the kinetic resolution of tertiary alcohols.

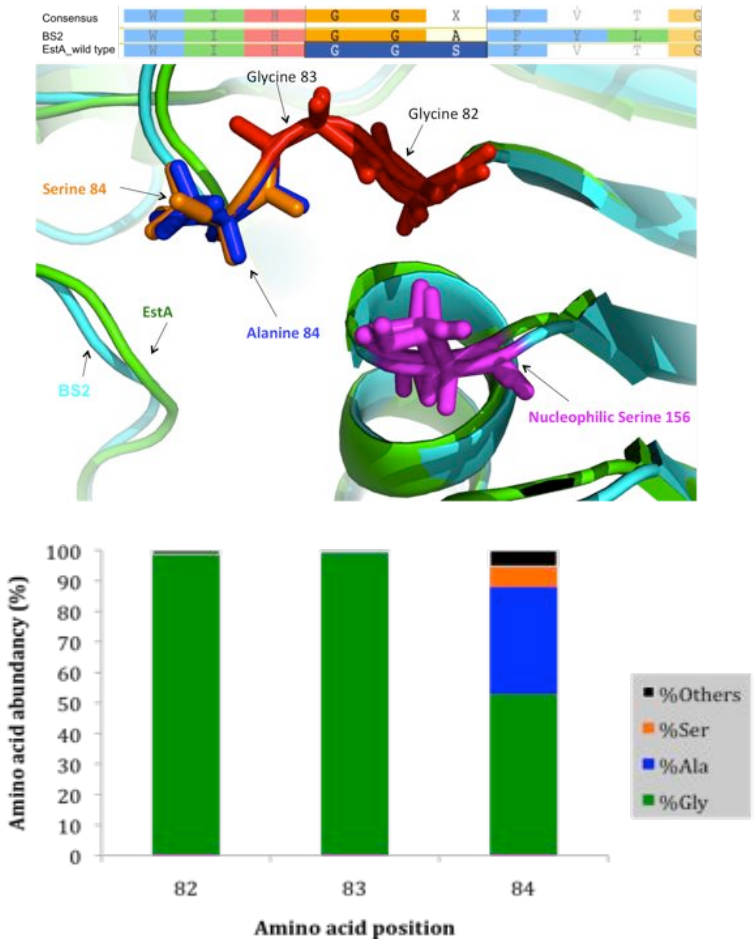


Figure 2: Above: sequence and structure alignment of active site regions of BS2 (PDB code: 1qe3, cyan) and homology model of EstA (green) reveals a high similarity in structure between two enzymes. In the oxyanion hole region, the amino acid residue at position 84 is alanine in case of BS2 (orange) and serine in case of EstA (blue). Below: amino acid abundance (analyzed in 3DM) in 1343 sequences of the same family with similarity to acetylcholine esterases for GGG(A)X motif (position III_82-84).

Two variants, EstA-GGG and EstA-GGA, displayed a 26-fold and 16-fold more activity towards **1**, respectively, when compared with EstA wild type. The highest obtained enantioselectivity is with EstA-AGA towards **1**, **2** and **3** (up to >100).

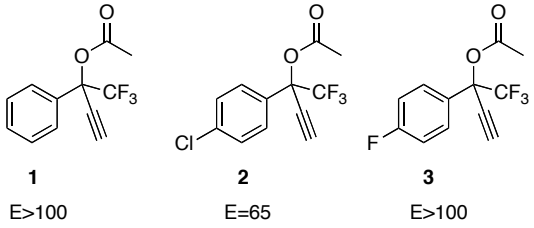


Figure 3: Enantioselectivity of EstA-AGA variant towards tertiary alcohols

The alignment-inspired method using ABHDB has been successfully applied to identify key residues for the rational protein design of EstA. Variants of EstA have shown to be useful biocatalysts for the synthesis of optically pure tertiary alcohols.

In complement with available enzymes in the catalytic platform, new biocatalysts have been discovered using sequence-structure guided genome mining. The new enzymes show activity towards tertiary alcohols and will be potential candidates for further studies of protein engineering.

The results from the activity study of new biocatalysts (Est4, Est5) and rational protein design of EstA have shown the limitation of enzyme activity prediction that is based solely on sequence alignment. In spite of up to 49% sequence similarity, the activity and enantioselectivity of Est4 and Est5 towards tertiary alcohols are lower than of Est8. EstA wild-type has very low activity towards tertiary alcohols when compared with BS2 wild-type despite of 49% sequence identity. Nevertheless, key residues for rational protein design of EstA have been successfully identified with the application of ABHDB.

Chemical synthesis and enzymatic resolution of a tertiary homoallylic alcohol have been performed to evaluate the efficiency of each pathway. On one hand, the chemoenzymatic pathway could resolve the compound with moderate yield and enantioselectivity. On the other hand, two enantiomers of the tertiary alcohol could be synthesized with moderate to high yield and with excellent optically purity by the chemical pathway. Nevertheless, more synthesis steps and amounts of organic solvents as well as the employment of heavy metals have made the chemical pathway less environmentally friendly and energy efficient than enzymatic approach.

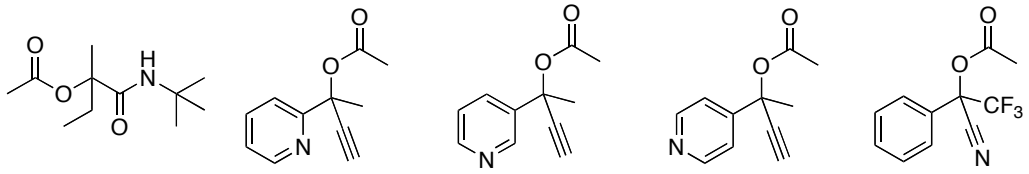


Figure 4: Selected examples of tertiary alcohols that can be resolved with high optical purity by GGG(A)X motif enzymes.

The presented examples on Figure 4 underline the importance of enzyme toolbox for the kinetic resolution of tertiary alcohols. Several enzymes in the toolbox can help to overcome the limitations of individual enzymes. Moreover, a wide range of tertiary alcohols can be synthesized with high optical purity by different enzymes in the toolbox.

Erklärung

Hiermit erkläre ich, dass diese Arbeit bisher von mir weder an der Mathematisch-Naturwissenschaftlichen Fakultät der Ernst-Moritz-Arndt-Universität Greifswald noch einer anderen wissenschaftlichen Einrichtung zum Zwecke der Promotion eingereicht wurde.

Ferner erkläre ich, dass ich diese Arbeit selbständig verfasst und keine anderen als die angegebenen Hilfsmittel benutzt habe.

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Unterschrift

Curriculum Vitae

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Education and qualification

2007-10 to 2010-12	*Greifswald University, Germany – Doctoral thesis in Biotechnology* Supervision: Prof. Uwe Bornscheuer, Greifswald University, Germany Subject: Enantioselective biocatalysis for the preparation of optically pure tertiary alcohols
2009-01 to 2010-03	*TU Delft, Netherlands* - Guest researcher in the Biocatalysis and Organic Chemistry working group of Prof. Isabel W.C.E Arends.
2006-02 to 2006-07	Participant in the Diploma Equivalent (DE) Program in Hanoi, Vietnam
2004-10 to 2007-09	*University of Natural Sciences, Ho Chi Minh City, Vietnam* Teaching assistant in the Department of Biotechnology, Faculty of Biology
2000-09 to 2004-09	University of Natural Sciences, Ho Chi Minh City, Vietnam Bachelor in Biotechnology

Awards

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Publications

2010

[1] Bassegoda, A., Nguyen, G.S., Kourist, R., Schmidt, M., Diaz, P., Bornscheuer, U.T. (2010), Rational protein design of *Paenibacillus barcinonensis* esterase EstA for kinetic resolution of tertiary alcohols, *ChemCatChem*, 2, 962-967,

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[4] Kourist, R., Nguyen, G. S., Strübing, D., Böttcher, D., Liebeton, K., Naumer C., Eck, J, Bornscheuer, U.T., (2008) Hydrolase-catalysed stereoselective preparation of protected α,α-dialkyl-α-hydroxycarboxylic acids (2008) *Tetrahedron: Asymmetry*, 19, 5, 1839-1843.

Poster presentations in conferences

Poster: “Highly enantioselective esterase-catalyzed kinetic resolution of chiral tertiary alcohol acetates”; Biotrans in Bern, Switzerland (05. – 09. July 2009)

Poster: “Hoch enantioselektive kinetische Racematspaltung von chiralen tertiären Alkoholacetaten”; DECHEMA conference in Mannheim, Germany (07. – 10. September 2009)

Poster: “Protein engineering of EstA from *Paenibacillus barcinonensis* for the kinetic resolution of tertiary alcohols based on a sequence alignment approach”; Biocat 2010 in Hamburg, Germany (29. August – 02. September 2010)

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Signature

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