Building blocks for polymer synthesis by enzymatic catalysis

Stefan Semlitsch
Front cover: Graphic interpretation of the isolation of molecules from renewable resources, the enzyme-catalyzed synthesis of oligomers and the final formation of polymer networks.
Für Melanie, Clemens und Miriam
Abstract

The search for alternatives to oil-based monomers has sparked interest for scientists to focus on the use of renewable resources for energy production, for the synthesis of polymeric materials and in other areas. With the use of renewable resources, scientists face new challenges to first isolate interesting molecules and then to process them.

Enzymes are nature’s own powerful catalysts and display a variety of activities. They regulate important functions in life. They can also be used for chemical synthesis due to their efficiency, selectivity and mild reaction conditions. The selectivity of the enzyme allows specific reactions enabling the design of building blocks for polymers.

In the work presented here, a lipase (Candida antarctica lipase B (CalB)) was used to produce building blocks for polymers. An efficient route was developed to selectively process epoxy-functional fatty acids into resins with a variety of functional groups (maleimide, oxetane, thiol, methacrylate). These oligoester structures, based on epoxy fatty acids from birch bark and vegetable oils, could be selectively cured to form thermosets with tailored properties.

The specificity of an esterase with acyl transfer activity from Mycobacterium smegmatis (MsAcT) was altered by rational design. The produced variants increased the substrate scope and were then used to synthesize amides in water, where the wild type showed no conversion. A synthetic procedure was developed to form mixed dicarboxylic esters by selectively reacting only one side of divinyl adipate in order to introduce additional functional groups.

Keywords: Enzyme, Enzyme Engineering, Biocatalysis, Lipase, CalB, MsAcT, Substrate specificity, Selectivity, Polymer Chemistry, Polymer Synthesis
Sammanfattning

Sökandet efter alternativ till råolja har gjort att forskare fokuserar på förnyelsebara råvaror för till exempel energiproduktion och syntes av polymera material. Utmaningarna med dessa råvaror ligger både i att utvinna och isolera beståndsdelar som kan användas som startmaterial, och sedan i att bearbeta dem till produkter.

Enzymer är naturens egna kraftfulla katalysatorer och finns i alla biologiska system där de reglerar en mängd livsviktiga processer. Enzymerna är specifika och katalyserar olika typer av reaktioner. Eftersom att enzymerna återfinns i biologiska system är dessa ofta effektiva katalysatorer även under milda betingelser och användning av selektiva enzymer vid framställning av till exempel byggstenar för polymerer kan ge både mindre miljöbelastning och möjliggör design av makromolekyler.

I denna avhandling används ett lipas (Candida antarctica lipase B (CalB)) för att producera byggstenar för polymerer. Som råvara används epoxy-funtionella fettsyror både från vegetabiliska oljor och från näver. Effektiva syntesvägar till oligomerer, där de funktionella grupperna i dessa fettsyror kombineras med andra funktionella grupper (maleimid, oxetan, tiol, metakrylat) har utvecklats. Beroende på kombinationen av funktionella grupper i oligoestrarna kunde de härdas selektivt till olika härdplaster med definierade egenskaper.

Vidare, redovisas i denna avhandling användningen av ett esteras med acyltranferasaktivitet från Mycobacterium smegmatis (MsAcT). Olika varianter av enzymet producerades via rationell design för att generera mutanter med varierande specificitet. Varianterna användes sedan till syntes av amidr i vatten, där vildtyps-enzymet inte uppvisat någon aktivitet. En variant av MsAcT användes även till att utveckla en syntesväg för blandade dikarboxylsyraestrar där endast ena sidan av divinyladipat reageras för att introducera ytterligare funktionella grupper.

Nyckelord: Enzym, Enzymutveckling, Biokatalys, Lipas, CalB, MsAcT, Substratspecificitet, Selektivitet, Polymerkemi, Polymersyntes
List of appended papers

Paper I

Paper II

Paper III
Nameer, ‡S., Semlitsch, ‡S., Martinelle, M. & Johansson M. One-pot enzyme-catalyzed synthesis of dual-functional polyester macromers towards surface active hydrophobic films. Manuscript

Paper IV
Hendil-Forssell, †P., Semlitsch, †S. and Martinelle, M. Engineering the esterase/acyltransferase from Mycobacterium smegmatis: extended substrate scope for amide synthesis in water. Manuscript

Paper V
Semlitsch, S. and Martinelle, M. Mixed vinyl adipate esters through selective synthesis using a designed esterase/acyltransferase. Manuscript

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Contributions to appended papers

Paper I
Major contribution to planning, execution and analysis of experiments. Performed a majority of the writing.

Paper II
Major contribution to planning, execution and analysis of experiments for the synthesis of the telechelic molecules. Minor contribution to the writing.

Paper III
Major contribution to planning and part of execution and analysis of experiments. Minor contribution to the writing.

Paper IV
Major contribution to planning, execution and analysis of experiments. Contributed to the writing.

Paper V
Planned, executed and analyzed all the experiments. Wrote the manuscript.

Papers not included in this thesis

Paper VI
List of abbreviations

A/Ala Alanine
AE Acyl enzyme
BVE 1,4-Butanediol vinyl ether
BVEVA (4-(vinyloxy)butyl) vinyl adipate
CalA Candida antarctica lipase A
CalB Candida antarctica lipase B
D/Asp Aspartic acid
DMA Dimethyl adipate
DVA Divinyl adipate
E.C. Enzyme Commission
EFA 9,10-Epoxy-18-hydroxyoctadecanoic acid
EGDMA Ethylene glycol dimethacrylate
EMLO Epoxidized methyl linoleate
E/Glu Glutamic acid
E’ Loss modulus (tensile)
E” Storage modulus (tensile)
F/Phe Phenylalanine
G’ Loss modulus (shear)
G” Storage modulus (shear)
H/His Histidine
HEMA Hydroxyethyl methacrylate
L/Leu Leucin
MUA 11-Mercaptoundecanoic acid
MsAcT Mycobacterium smegmatis esterase with acyltransferase activity
N/Asn Asparagine
OxVA (3-ethyloxetan-3-yl)methyl vinyl adipate
PDB Protein Data Bank
Q/Gln Glutamine
S/Ser Serine
T/Thr Threonine
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>$T_g$</td>
<td>Glass transition temperature</td>
</tr>
<tr>
<td>TI</td>
<td>Tetrahedral intermediate</td>
</tr>
<tr>
<td>TMP</td>
<td>Trimethylolpropane</td>
</tr>
<tr>
<td>TMPO</td>
<td>Trimethylolpropane oxetane</td>
</tr>
<tr>
<td>wt</td>
<td>Wild type</td>
</tr>
</tbody>
</table>
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1. Introduction

In nature, all living organisms depend on a functioning metabolism. Organic matter is broken down and important components of the cells such as nucleic acids and proteins have to be produced. Important to these metabolic pathways are enzymes which enable and control chemical transformations. They catalyze reactions by lowering the energy barrier and lead to rate enhancements of up to $10^{19}$ times compared to the uncatalyzed reaction.\(^1\) Another key feature of enzymes is the selectivity towards the substrates used and the type of reaction they catalyze. With these features enzymes are able to control reactions and functions in living organisms.

Enzymes are proteins and consist of one or more linear chains of the common 20 amino acids (primary structure). Monomers of proteins usually contain between 100 and 700 amino acids which allows a large sequence variety.\(^2\) Depending on the nature of the amino acid side chains and their order in the primary structure, they build up the three dimensional structure stabilized by hydrogen bonds, disulfide bridges, charge-charge interactions, van der Waals interactions, London dispersion forces and hydrophobic effects. The actual catalysis takes place in the so-called active site of the enzyme, where the substrate binds in a certain conformation. This specific binding plays an important role in both lowering of the energy barrier for the reaction and the selectivity of the enzyme towards the substrates.

Biocatalysis is using natural catalysts, such as enzymes, for chemical transformations of organic compounds. It works on the border of biotechnology and organic chemistry. Enzymes, found in nature, are studied, modified and applied in synthetic reactions. The advantages of enzymes in synthesis over traditional chemical catalysis are substrate selectivity, a low amount of byproducts, often mild reaction conditions and low energy requirements and the potential to reduce the number of
synthetic reaction steps. But there are disadvantages as well: The development of the biocatalysts can take a lot of time and effort and the stability is often very limited in respect to solvents, temperatures and pH. Native enzymes are rarely suitable for industrial processes, but advancements in enzyme engineering, immobilization methods and the use of enzymes in non-aqueous media have led to increased applicability. Quite a few enzymes have become interesting for different industries including; fine and bulk chemicals, pharmaceuticals, food, cosmetics, textiles, pulp and paper and polymers as reviewed on several occasions.11-16

1.1 Enzyme specificity
In order to have a working metabolism, enzymes have to control the transformations they are performing. Enzymes have to be specific towards the reaction they catalyze and the substrate they use.

The reaction specificity describes the type of reaction that is catalyzed by the enzyme. Metabolic enzymes are specific towards one type of reaction which enables control in the organism (for example hydrolysis, transfer reactions or oxidation/reduction reactions).

The substrate specificity describes how good an enzyme is at converting one substrate into product. It is determined by the specificity constant \((k_{cat}/K_M)\) which is an apparent second-order rate constant.

1.2 Enzyme selectivity
The selectivity of the enzyme makes the enzyme distinguish between different substrates according to their functionality and the positioning of the functional groups. It is determined by the ratio of the specificity constants of two substrates A and B \(((k_{cat}/K_M)_A/(k_{cat}/K_M)_B)\).

Usually selectivity is divided into three main types: chemoselectivity, regioselectivity and stereoselectivity. Chemoselectivity describes the ability to discriminate between substrates with the different functional groups. For example, *Candida antarctica* lipase B (CaLb) is almost \(10^5\) times more selective for alcohols than for thiols (Figure 1A).17 Regioselectivity describes the ability to distinguish between similar chemical groups on the same molecule which are in different positions on the molecule. CaLb has been shown to prefer the primary hydroxy-groups
over the secondary ones in carbohydrates (Figure 1B).\textsuperscript{18-19}

Enantioselectivity, which is part of stereoselectivity, describes the
discrimination between stereoisomers of a chiral molecule. As an
example, \textit{CalB} is very selective towards the R-enantiomer of 2-octanol
due to the conformation in the active site (Figure 1C).\textsuperscript{20}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Schematic overview of the types of substrate selectivity of enzymes. (A) shows the chemoselectivity of \textit{CalB} towards 1-hexanol over 1-hexanethiol in the transacylation with ethyl octanoate.\textsuperscript{17} (B) displays the regioselectivity of \textit{CalB} towards the C-6 hydroxy-group of glucose.\textsuperscript{18} (C) shows the enantioselectivity of \textit{CalB} towards the R-enantiomer of 2-octanol in a transacylation reaction.\textsuperscript{20}}
\end{figure}
2. Carboxylic ester hydrolases

The enzymes used in this thesis, *Candida antarctica* lipase B (CalB) and *Mycobacterium smegmatis* esterase with acyltransferase activity (MsAcT), belong to the enzyme sub-subclass of carboxylic ester hydrolases (E.C. 3.1.1). The enzymes in this class are then further divided according to their natural substrates into esterases, lipases, cutinases and others. Many of these enzymes have drawn attention to both industry and research. Lipases, for example, are used in the detergent industry, pulp and paper, food industry, cosmetics, pharmaceutical industry, and other areas. There is also research activity in many areas such as polymer synthesis and biodiesel production. MsAcT found industrial application in biobleaching under the tradename PrimaGreen® EcoWhite.

2.1 Reaction mechanism

The reactions of triacylglycerol lipases, such as CalB, as well as the MsAcT follow a ping-pong bi bi mechanism. The catalytic triad constitutes of serine, histidine and aspartate/glutamate. The whole hydrolysis reaction consists of two half-reactions: acylation and deacylation (Scheme 1). In the acylation part, the first substrate, acyl donor, enters the active site to form an enzyme-substrate complex. The catalytic histidine acts as a base, removing a proton from the catalytic serine hydroxy group to activate it. This activated serine attacks the carbonyl carbon on the substrate forming a tetrahedral intermediate (TI 1). Under this process, the carbonyl oxygen gets negatively charged which is stabilized by hydrogen bonds with the amino acids in the oxyanion hole, while the positive charge of the histidine is stabilized by aspartate/glutamate. The first half-reaction ends with the collapse of TI 1, which results in the formation of the acyl enzyme (AE) and a first, leaving product, an alcohol. This is due to the catalytic histidine which acts as an acid and donates a proton to the
The enzyme mechanism of serine hydrolases is exemplified by a hydrolysis reaction. The catalytic serine nucleophilically attacks the first ester and the acyl enzyme and the first product, an alcohol, are formed. The second substrate, water, attacks the acyl enzyme to form and release a carboxylic acid. By using an alcohol, amine or thiol as second substrate, the second product will be an ester, amide or thioester, respectively.

leaving group of the first substrate. The second half-reaction starts with the binding of the second substrate, acyl acceptor, to the AE. The acyl acceptor is activated by the histidine and attacks the carbonyl carbon of the AE. This results in the TI 2, which has charge stabilization by the residues of the oxyanion hole and the catalytic aspartate/glutamate. Finally a proton is transferred from the second substrate via the histidine to the serine oxygen, which enables the release of the second product from the enzyme, which is regenerated for the next catalytic cycle.

Depending on the substrates that are used, it is possible to get different products: by using a carboxylic ester as the first substrate and water as the second substrate, hydrolysis will occur. By using another carboxylic ester, amine or thiol as a second substrate, transacylation reactions take place resulting in a new ester, amide or thioester and a leaving alcohol. Depending on the reaction conditions and the availability of several substrates in the system, the selectivity of the enzyme for the different substrates will play a big role in the ratio of the final products.
2.2 *Candida antarctica* lipase B (CalB)

*Candida antarctica* (also known as *Pseudozyma antarctica*) lipase B (CalB) (E.C. 3.1.1.3) was discovered and isolated from a lake sediment in Antarctica.\(^\text{36}\) It has an α/β-fold like other lipases, 317 amino acids and a molecular weight of 33 kDa (Figure 2). CalB shows a hydrophobic surface around the entrance of the active site to facilitate the access for natural substrates, triacylglycerides, during hydrolysis. In contrast to many lipases it has no lid on top of the active site\(^\text{37-38}\) and shows no interfacial activation.\(^\text{39}\) The catalytic triad of CalB consists of Ser105, His224 and Asp187; the hydrogen bonds in the oxyanion hole are donated by Thr40 and Gln106.\(^\text{37}\)

The enzyme has attracted interest of both industry and academia and is used for the resolution of secondary alcohols and amines at an industrial scale by for example BASF.\(^\text{28}\) CalB has also been used to catalyze polyesterification reactions\(^\text{30-32}\) and non-conventional reactions like aldol and Michael-type additions.\(^\text{40-42}\) There have also been successful engineering approaches to change substrate and reaction specificity.\(^\text{43-49}\)

![Figure 2](image.png)

**Figure 2.** The structure of CalB (PDB code 1LBT). The catalytic triad is shown in red in the center.
2.3 *Mycobacterium smegmatis* esterase with acyl transferase activity (*MsAcT*)

*Mycobacterium smegmatis* esterase with acyl transferase (*MsAcT*) (E.C. 3.1.1.3 according to the PDB database) belongs to the SGNH-hydrolase family, which is named after the conserved amino acids (serine, glycine, asparagine and histidine) important for catalysis. This enzyme family includes many types of activities, for example thioesterase, esterase, lipase, rhamnogalacturonan acetyesterase and lysophospholipase. *MsAcT* forms an octamer in vitro (Figure 3A) which is unusual for members of the SGNH-hydrolase family. This octamer can be imagined as a tetramer of dimers forming a blocklike structure with a large channel from the “front” to the “back” (Figure 3A) and crevices on the “sides” between the dimers. This quaternary structure is thought to be the key to the special properties of the enzyme. Three monomers restrict the access to the active site by forming a hydrophobic channel (Figure 3C). The structure consists of five parallel β-strands, which are flanked by seven α-helices. There are 216 amino acids per monomer with a molecular weight of 23 kDa. The catalytic triad in the *MsAcT* consists of Ser11, His195 and Asp192; the oxyanion hole from Ala55, Asn95 and Ser11 (backbone NH) (Figure 3B). Acyl transfer reactions in water using esterases are rarely reported. Above all the superfamily of *Candida antarctica* lipase A (*CalA*) has some enzymes able to catalyze these reactions. Engineering of *CalB* also led to an improved acyl transfer activity in water. Even though the 3D-structure of *MsAcT* is known for ten years now, the academic output has been limited to a few reactions: the synthesis of neopentyl glycol diacetate, the synthesis of peracetic acid for surface decontamination, oxidations of aldehyde to acid or ketones to lactones and the synthesis of amides using a cascade of enzymes.
Figure 3. (A) Octameric structure of MsAcT (PDB code 2Q0S) showing each subunit in a different color. The catalytic triad of the yellow monomer is marked in red. (B) Tertiary structure of one monomer of MsAcT showing the catalytic triad in red. (C) Visualization of the restriction of the access to the active site (substrate colored in pink) by three monomers of MsAcT forming a hydrophobic channel.
3. Building blocks for polymers

Polymeric materials are everywhere. Over 320 million tons plastics were produced in 2015. Its use ranges from packaging over construction to more specialized industrial and consumer products. Polymeric materials comprise of a wide range of different varieties ranging from thermoplastics to thermosets. Polymeric materials show many advantages over conventional materials such as the processability, chemical resistance or variety in moduli, but there are also drawbacks and challenges. The Ellen MacArthur Foundation defined three main challenges for the plastic industry in 2016: (1) Create an effective after-use plastics economy, (2) drastically reduce leakage of plastics into natural systems and (3) decouple plastics from fossil feedstocks.

3.1 Monomers from renewable resources

The use of monomers from renewable resources is a recurring theme both in the plastic industry as well as in research with an increasing interest in green chemistry, where the use of renewable feedstocks is defined as one of its twelve principles. The possible future depletion of fossil resources is driving both industry and science to look for alternatives for the production of chemicals and energy. In order to get more competitive with the fossil-based industry, which is trying to use all their side streams, the concepts of biorefinery have to be utilized. This can be achieved by among others using and commercializing by-products from for example the forest industry to convert biomass to interesting starting materials. Using monomers from renewable resources and possible transformations using biocatalysis allows for new building blocks and drop-in solutions where existing products are produced in a more biobased way.

In the work leading to this thesis, different monomers from renewable resources have been used: hydroxy fatty acids from birch bark, fatty acids from vegetable oils and adipic acid which can be produced from levulinic acid.
3.1.1 Suberin
Suberin is a natural aliphatic–aromatic crosslinked polyester and is mostly found in cell walls where it plays the role of a protective barrier between the organism and the environment. In higher plants, it is one of the main components of the outer bark cell walls.\textsuperscript{79-83} As with lignin, there is no general chemical structure for suberin. Its composition heavily depends on the type of plant. The determination of the structure is problematic, but the relative abundance of the aliphatic moieties can be determined by depolymerization and isolation methods. The main components of the aliphatic parts are ω-hydroxyfatty acids, α,ω-dicarboxylic acids and mid-chain di-hydroxy or epoxy derivates.\textsuperscript{84-86} Table 1 shows the composition for the five main suberin components for \textit{Betula pendula} (birch bark).\textsuperscript{87} By using mild alkaline hydrolysis it is possible to extract the main component 9,10-epoxy-18-hydroxyoctadecanoic acid while keeping the epoxide functionalities intact.\textsuperscript{84, 86-88} 9,10-Epoxy-18-hydroxyoctadecanoic (EFA) acid has been successfully used as monomer for lipase-catalyzed polymerization reactions.\textsuperscript{89-91}

\textbf{Table 1.} Main depolymerized suberin monomers from \textit{Betula pendula} in mg compound/g of dry starting material.

<table>
<thead>
<tr>
<th>Main depolymerized suberin monomers (after alkaline hydrolysis)</th>
<th>mg compound/g of dry starting material</th>
</tr>
</thead>
<tbody>
<tr>
<td>9,10-Epoxy-18-hydroxyoctadecanoic acid</td>
<td>99</td>
</tr>
<tr>
<td>22-Hydroxydocosanoic acid</td>
<td>42</td>
</tr>
<tr>
<td>18-Hydroxyoctadec-9-enoic acid</td>
<td>39</td>
</tr>
<tr>
<td>9,10,18-Trihydroxyoctadecanoic acid</td>
<td>30</td>
</tr>
<tr>
<td>Docosanedioic acid</td>
<td>16</td>
</tr>
</tbody>
</table>

3.1.2 Vegetable oils
Vegetable oils are triglycerides; esters of glycerol with three fatty acids. The composition can vary depending on the source of the oils in terms of fatty acid chain length and level of unsaturation. The usual fatty acids have 14-22 carbons and contain 0-5 double bonds. But there are also additional functionalities available, such as epoxy rings, hydroxy-groups or triple bonds.\textsuperscript{92} Because of the possibility of large scale production and
the low cost for production and processing, vegetable oils have become interesting for the chemical and polymer industry.\textsuperscript{93-102}

The main focus of chemical modifications of the fatty acids is on the double bonds. One possible modification is the oxidation of the double bonds to epoxides which can be achieved by using peracids.\textsuperscript{101} Epoxidized vegetable oils are very well-studied precursors of polymers.\textsuperscript{93-96, 98, 100-101} The epoxide moieties can then be ring-opened under both basic and acid conditions, which enables a variety of possible structures. Epoxy resins based on vegetable oils have found use in the synthesis of different types of polymeric structures.\textsuperscript{103-105}

### 3.1.3 Short dicarboxylic acids

The U.S. Department of Energy ranked succinic acid to one of the top value added chemicals from biomass.\textsuperscript{106} Succinic acid is a building block for a range of chemicals in the pharmaceutical, agriculture, food and chemical industry, for example as a precursor to 1,4-butanediol production or the replacement of adipic acid in the production of polyurethanes.\textsuperscript{11, 16, 107} By using genetically engineered organisms\textsuperscript{108} it is possible to replace the conventionally petroleum-based succinic acid with a bio-based version. Several companies, such as Succinity (joint venture between BASF and Corion),\textsuperscript{109} BioAmber (joint venture of DNP Grenn Technology and ARD),\textsuperscript{110} and Reverdia (joint venture between DSM and Roquette)\textsuperscript{111} have started producing it biochemically from glucose with capacities of up to 30 000 tons per year.\textsuperscript{111-113}

Adipic acid is another short dicarboxylic acid with a large interest from industry. It is mainly used as a chemical intermediate in the production of nylon 6,6, but has also applications to produce polyurethanes or in lubricants and plasticizers.\textsuperscript{114} It is mainly produced from cyclohexanol via benzene,\textsuperscript{114} but there has been academic research to produce bio-based adipic acid from renewable resources.\textsuperscript{115-118} There is also a collaboration in the Netherlands which aims to produce adipic acid from levulinic acid.\textsuperscript{77} The project started to produce adipic acid in pilot scale in 2016.\textsuperscript{78}
3.2 Enzyme-catalyzed polymerization

More than 60 years have passed since enzymes were used for the first time in synthesis reactions for polymers. Since then enzymes from four out of the six enzyme classes have been identified and used in the synthesis of different types of polymers (Table 2). Due to specificity and selectivity, enzymes open up new possibilities in both synthesis and functionalization of polymers.

3.2.1 Lipase-catalyzed polymer synthesis

Lipases are frequently used enzymes in polymer synthesis. This is partly because of the commercial availability of many lipases, such as Novozym® 435 (CalB immobilized on an acrylic resin), which is one of the most studied enzymes in polymer synthesis. There are two main types of polymerization reactions with lipases: ring-opening polymerization and polycondensation. Ring-opening polymerization uses ring-closed esters, such as lactones or lactides to open them and to perform propagation reactions of the chain (Scheme 2). Polycondensation on the other hand uses either hydroxy acids (AB-monomer) as monomers or diacids (diesters) and diols (AA/BB-monomer) to link them via ester bonds (Scheme 3).

Table 2. E.C. classes of enzymes and the macromolecules that are synthesized by enzymes of the respective enzyme classes.

<table>
<thead>
<tr>
<th>E.C. class</th>
<th>Synthesized macromolecules</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Oxidoreductases</td>
<td>Polyphenols, polyanilines, polythiophene, vinyl polymers</td>
</tr>
<tr>
<td>2. Transferases</td>
<td>Polysaccharides, cyclic oligosaccharides, polyesters*</td>
</tr>
<tr>
<td>3. Hydrolases</td>
<td>Polysaccharides, polyesters, polycarbonates, polyamides, poly(amine acid)s, polyphosphates, polythioesters</td>
</tr>
<tr>
<td>4. Lyases</td>
<td></td>
</tr>
<tr>
<td>5. Isomerases</td>
<td></td>
</tr>
<tr>
<td>6. Ligases</td>
<td>Cyanophycin*</td>
</tr>
</tbody>
</table>

* only in vivo
Scheme 2. Ring-opening polymerization. An alcohol initiates the ring-opening of a ring-closed ester which then acts as initiator for other ring-closed esters and thereby propagating the chains. The propagation stops when the hydroxy-group of the polymer chain reacts with an ester or an acid.

Scheme 3. Polycondensation reaction. 1) AA/BB-type of reaction with a diacid and a diol as monomers. 2) AB-type of reaction with a hydroxy-acid as a monomer.

The advantage of enzymes over conventional processes for polyester synthesis lies in milder reaction conditions in terms of temperature, pressure and pH. In traditional step-growth polymerizations temperatures over 200 °C are needed in presence of a strong acidic catalyst. At these temperatures side reactions are more likely to occur than at temperatures where enzymes have their ideal working temperature. The current trends and achievements in enzyme-catalyzed synthesis of polyesters have been reviewed by different working groups in recent years.
3.2.2 Lipase catalysis toward functional oligomers
By adding mono-functional monomers to the reaction mixture, the chain propagation of the polymer will be terminated. If these terminating molecules (end-cappers) contain additional groups that are not attacked by the enzyme, post-functionalization of the oligomer is possible. The presence of these end-functionalities facilitates changes in physical and chemical properties and allows for more complex structures of materials. Linear oligomers with functionalities on both sides are called telechelics. This allows the formation of both linear polymers and polymer thermosets by using linking agents to perform chain extension or the formation of polymer networks. Due to the selectivity of the enzyme, different types of functionalities can be introduced to the polymers. The regioselectivity of CalB enabled the initiation from only one of the hydroxy-groups of a glucoside. By utilizing the enantioselectivity of CalB it is possible to only use the R-enantiomer of 1-phenylethanol to initiate a ROP. Chemoselectivity allows to produce polyesters with free thiol end-groups without protection and deprotection steps. By initial control of the stoichiometry of the backbone monomers and the end-cappers, it is possible to control the degree of polymerization of the oligomer. Combining functional groups from the backbone of the oligomer with the end-functionalities, enables the formation of different networks depending on the crosslinking method that is used (Paper I, II and III).

3.3 Polymer thermosets
IUPAC defines a thermosetting polymer as a “prepolymer in a soft solid or viscous state that changes irreversibly into an infusible, insoluble polymer network by curing.” And they continue that a “cured thermosetting polymer is called a thermoset.” The polymeric material is “set” (forms an irreversible network) which means that, once cured, it cannot flow, be dissolved or processed. Making a thermoset polymer can thus be considered as a two-step process. The challenge in the first step is to polymerize the monomers without affecting the functional groups that are used in the final crosslinking step.

The mechanical properties are affected by various factors, such as the composition of the prepolymer, the number of crosslinking sites, the distance between crosslinking sites and the homogeneity of the network.
The physical properties are usually evaluated using the following parameters: glass transition temperature \((T_g)\), storage and loss modulus and \(\tan \delta\). The \(T_g\) is the temperature where mechanical properties of the material drastically change due to internal movement of the polymer chains. \(T_g\) is associated with changes in properties like stiffness, brittleness or elongation at break. Viscoelastic properties are evaluated with the loss and storage moduli, \(E'\) and \(E''\) (tensile modulus) and \(G'\) and \(G''\) (shear modulus). The homogeneity of the network can be evaluated by \(\tan \delta\), which is the ratio between the loss and storage moduli.

The main application areas for thermosets include adhesives, biomedical and dental materials and composites. The functionalities used to crosslink the oligomers in the work that lead to this thesis were epoxy-groups, thiols and alkenes which can be coupled and crosslinked through different chemistries. These functionalities represent a number of different crosslinking chemistries in order to demonstrate the versatility of lipases catalysis in making thermoset resins. Polymerizations can be triggered using various initiation routes, for example thermal and or photochemical induction. In the present work photoinduced polymerization has been utilized since it offers a good control of the reaction. The two main routes are briefly described below.

### 3.3.1 Cationic ring-opening polymerization

Cationic ring-opening polymerization is one possibility to form networks of cyclic ethers. It has a chain-growth type of mechanism, which is initiated by the initiator. The most commonly used photoinitiators are diarylidonium or triarylsulfonium salts. When excited with UV-light, the photoinitiator dissociates and generates a strong protonic acid which leads to the formation of an oxiranium ion followed by a cationic polymerization and ends in a polyether-network (Scheme 4). As a photoinitiated polymerization method, cationic polymerization has, among others, the advantages of relatively high polymerization rates, ambient temperature operation and low energy consumption. This method is mainly used in context with epoxides (Paper II and III) and vinyl ethers.
3.3.2 Radical polymerization

The radical polymerization is another photoinitiated crosslinking method. Free radicals are produced after UV irradiation of an aromatic carbonyl compound (photoinitiator). This either happens through a cleavage of C-C bonds or by abstracting a hydrogen atom. There are three main types of resins that polymerize by a photoinitiated radical mechanism: unsaturated polyester/styrene, (meth)acrylates (Paper II) and thiol-ene chemistry (Paper III).

(Meth)acrylates homopolymerize after the cleavage of the photoinitiator which leads to radical formation and the attack at the double bond of the (meth)acrylate. (Scheme 5).

In the thiol-ene addition the initiator starts the radical formation and causes the formation of a thyl radical. The double bond of the alkene then adds to this radical (Scheme 6).
4. Aim and motivation

Scientific discoveries about pollution and its consequences and the public awareness of these pollutions gave rise to the area of green chemistry which aims to reduce or eliminate hazardous substances.\textsuperscript{75,135} At the same time there is a demand to change from the fossil-based industry to renewable resources both for energy production, but also for the synthesis of polymers.\textsuperscript{95,136-139} Both, green chemistry and renewable (re)sources have also become more and more important in academic research. This trend becomes obvious when looking at the references with these keywords found in Scifinder (Figure 4). In the last 20 years, the total numbers of publications containing these concepts has increased basically every year, apparently reaching a plateau in the last years. New monomers from new resources might need new types of catalysts for both isolation and processing. Enzymes offer a wide variety of different activities and selectivities and nature offers a diverse (and chiral) selection of monomers.\textsuperscript{140-144}

\textbf{Figure 4.} References with “green chemistry” and “renewable (re)sources” resp. from 1960-2016 found in Scifinder\textsuperscript{145} (accessed July 2017)
The main aim of the thesis is to show the potential of enzymes in polymer synthesis in general and the versatility of lipases in particular. It is possible to perform polymerization reactions enzymatically, but also to produce new building blocks that cannot be polymerized using conventional chemistry. Not only is it possible to react specific groups with enzymes because of their selectivity, it is also possible to create multifunctional building blocks by combining different functionalities within one molecule. This is showcased through the synthesis of polymeric structures based on epoxy fatty acids from birch bark and vegetable oils, respectively, while having the mid-chain epoxy-groups unaffected. This might need tedious protection/deprotection steps with conventional chemistry, if the synthesis is possible at all. Enzymes often allow reactions at lower temperatures compared to conventional catalysts, which can decrease potential side reactions. Polymer chemistry and biocatalysis are two fields that could gain a lot by collaborations, as selective enzymatic catalysis enables the synthesis of new polymer structures. But it needs the knowledge of a polymer chemist to know what is needed for a polymer and the knowledge of a biochemist to know what enzymes are able to do. The work presented in this thesis wants to spark interest for both polymer chemists and biochemists to see possible future collaborations and an exchange of knowledge.

This thesis is divided into two parts. In Paper I-III a known and established enzyme, CalB, was used to synthesize EFA-based dual-functional resins. This means that new combinations of monomers with functional groups were obtained that are difficult to achieve using conventional chemical catalysis. The second part (Paper IV-V) deals with the possibilities of a rather uninvestigated enzyme, MsAcT. This enzyme and its variants are able to catalyze the synthesis of potential building blocks for polymer synthesis, such as mixed dicarboxylic esters.
5. Substrate selectivity in polymer synthesis

5.1 Extraction of monomers from renewable resources

The starting raw materials used in this thesis, which were suberin in outer birch bark and triglycerides in epoxidized vegetable oil show heterogeneity and a varied composition. This made it necessary to isolate and purify the wanted compounds (Scheme 7). The starting materials were chosen to be suitable candidates due to their availability and their low cost.

9,10-Epoxy-18-hydroxyoctadecanoic acid (EFA) (Scheme 7) was extracted from outer birch bark by an alkaline hydrolysis in NaOH/H$_2$O followed by a selective precipitation developed by Iversen et al. The basic conditions led to a break of the bark into smaller molecules. Slowly lowering the pH to 6 allowed the selective precipitation of EFA. The product was recrystallized in toluene to obtain EFA as a white(ish) crystalline solid. The main challenge in lab scale was the isolation from other compounds in the bark, as there are several different hydroxy-fatty acids.

For the isolation of epoxidized methyl linoleate (EMLO) (Scheme 7), epoxidized vegetable oil was used as starting material. In this case methanolysis was used to obtain the methyl esters of the fatty acids from epoxidized vegetable oil triglyceride. This led to a mixture of different fatty acid methyl esters. One advantage of methyl esters over acids is their solubility in organic solvents. Another benefit is the possibility to purify and separate the methyl esters using column chromatography with silica gel.

Scheme 7. Extracted monomers from renewable resources: 9,10-epoxy-18-hydroxy-octadecanoic acid (EFA) and epoxidized methyl linoleate (EMLO).
5.2 Synthesis of telechelics by enzymatic catalysis

As described earlier, lipases are highly selective towards the formation of ester bonds and therefore allow the incorporation of different functional groups within the same structure using “one-pot” synthetic routes.\textsuperscript{128, 150-152} Additionally, in the case of CalB, reactions can be run in bulk without solvent and moderate temperatures are possibly leading to low energy consumption. In order to investigate the substrate selectivity of the enzyme, different polymerization methods and different polymer architectures, two main systems were investigated: linear telechelic polymers from hydroxy fatty acids (\textbf{Paper I-III}) and a branched macromer from fatty acids (unpublished data, \textbf{Appendix}). The progress of the reaction was followed by \textsuperscript{1}H NMR and showed that the mid-chain epoxides were kept intact.

5.2.1 Linear telechelic polymers from hydroxy fatty acids

As a hydroxy fatty acid, EFA (1) has the possibility to do self-condensation reactions in presence of a catalyst. The presence of the mid-chain epoxy-groups allows a subsequent polymerization. In order to preserve these while reacting the hydroxy- and the acid-functionalities, a selective method is needed. Iversen and his coworkers\textsuperscript{88} showed the possibilities of CalB to perform this selective reaction of forming esters and not harming the epoxide-functionalities. Rüdiger et al. synthesized telechelic oligomers from EFA by using CalB for material formation purposes.\textsuperscript{91} In order to show the versatility of this method of using CalB to oligomerize EFA (1), several telechelic polyesters with different end-functionalities were formed. These functionalities are examples of different methods that can be used to later link these telechelic polymers to form networks. These groups include oxetanes, maleimides, methacrylates and thiols. The different reactions are summarized in Scheme 8.

In order to combine the chosen functional groups with the epoxide-groups from EFA (1), telechelics were formed using EFA (1) as the backbone and the other functional groups as end-cappers. For the maleimide-, oxetane- and thiol-end-functionalized oligomers, functional alcohols, \textit{N}-(2-hydroxyethyl)maleimide (2), trimethylolpropane oxetane (TMPO) (5) and 11-mercapto-undecanol (7), were used as end-cappers in combination with dimethyl adipate (DMA) (3) which worked as a spacer. The ratio of EFA (1) to spacer and end-capper determined the length of
the oligomer. Kinetic studies showed that these reactions performed similarly with self-condensation of EFA (1) and acylation of the end-cappers happening simultaneously. No rate-limiting path could be observed. Figure 5 shows the results of reaction B with TMPO (5) as the end-capper as an example for the synthesis kinetics of the three reactions A–C. The synthesis of the thiol-epoxy resin (8), reaction C, however had to be run in toluene at lower temperatures (60 °C) compared to reaction

**Reaction A**

**Reaction B**

**Reaction C**

**Reaction D**

Scheme 8. Single-step route to multifunctional oligoesters.
A and B, as side reactions could be detected leading to an insoluble network.

In reaction C (Scheme 8), 11-mercapto-undecanol (7) has alcohol and thiol functionalities. Both groups can react in a reaction with CalB. Due to the chemoselectivity of the enzyme, thiol groups can be used as end-groups as CalB prefers the reaction with alcohols as acyl acceptor. The chemoselectivity ratio \( \frac{k_{cat}}{K_M} \text{OH}/\left( \frac{k_{cat}}{K_M} \text{SH} \right) \) was determined to be 88,000 for CalB for hexanol and hexanethiol. Therefore the reaction produced no detectable thioesters within the eight hours reaction time, as expected.

For the methacrylate-end-functionalized oligomer, ethylene glycol dimethacrylate (EGDMA) (9) was used as end-capping chemical. In this reaction a rate-limiting path could be observed. Figure 6 shows the development of the reaction over time. In Figure 6A a rapid decrease of EFA-OH and a rapid increase of EFA-EFA-ester formation can be seen. This means that EFA homopolymerizes in the beginning (DP >8) and then starts to incorporate the methacrylate functionalities into the oligomers. With time the DP gradually decreases from >8 to 3.4 (see Figure 6A, EFA-EFA-ester bonds). After about ten hours reaction time,
Figure 6. Kinetic studies of reaction C with regard to the substrates EFA (1) (A) and EGDMA (9) (B) over time. (A) Distribution of the EFA (1) monomer with time, ■ (red line) represents EFA esterified to another EFA molecule, ▲ (green line) shows the consumption of the OH-group of EFA and ◊ (blue line) represents EFA with methacrylated hydroxy-group. (B) Distribution of the methacrylate group with time, ◊ (blue line) represents the consumption of EGDMA (9), ■ (red line) represents methacrylate connected to EFA via ethylene glycol moiety, ▲ (green line) represents methacrylate groups directly connected to EFA and x (violet line) represents the formation of methacrylic acid. (Paper I, reproduced by permission of The Royal Society of Chemistry).
the methacrylate conversion reached its maximum. This confirms the slow methacrylation. This happened by a methacrylation of the hydroxy-side (green line) and by using ethylene glycol by connecting the 2-hydroxyethyl methacrylate (HEMA) group to the carboxyl side (red line) (Figure 6B).

Scheme 9 shows a hypothesized pathway for the synthesis of the methacrylate-end-functionalized oligomer. It can be separated in two parts: the polymerization part (A) and the acyl transfer of the polymer chain (B). It has to be noted that acyl transfer reactions occur between all reaction species, but only a selection is shown in the scheme. The reaction starts with the homo-polymerization of EFA (1) with minor conversion of EGDMA (9) resulting in a poly-EFA (11). When EFA (1) was consumed, the acyl-transfer reactions become the dominant reactions. On the one hand fast-acyl transfer reaction with EFA (1) and poly-EFA (11), on the other hand a slower methacrylate transfer from EGDMA (9) to poly-EFA (11) to form methacrylated poly-EFA (13) and HEMA (12). HEMA was acylated by available oligomers (e.g. 11 or 13) and introduced the methacrylate moiety to the carboxyl side of poly-EFA via ethylene glycol. The formation of the methacrylated enzyme (acylation) and the following deacylation were the rate-limiting steps towards the formation of the final telechelic product (14). The formation of HEMA and the subsequent reaction with poly-EFA led to a decrease in DP. Section B of the scheme tries to explain the movement of the ethylene glycol moiety to partly end up in the middle of the oligoester (10) by different acyl transfer reactions.
Scheme 9. Development of the synthesis of poly-EFA with two methacrylate ends.
(Paper I, reproduced by permission of The Royal Society of Chemistry)
5.2.2 Branched macromers from fatty acids
Linear oligomers have different properties compared to their branched counterparts. Therefore a branched macromer was synthesized with both epoxy- and thiol-functionalities to see the differences compared to the product of reaction C (Scheme 8). Trimethylolpropane (TMP) (18) was reacted with epoxidized methyl linoleate (EMLO) (16) and 11-mercaptoundecanoic acid (MUA) (17) in the ratio 1:2:1 (Reaction F, Scheme 10).

The synthesis was performed successfully with about 96 % of the hydroxy-groups acylated, with about 90 % of the TMP (18) molecules being fully acylated. The epoxide groups were unaffected during the synthesis, as seen for reactions A-D. However, a thioester formation over time was noticed. After the synthesis the thioester content reached about 9 % of the total amount of sulfur. This is in contrast to reaction C where no thioester was detected. As lipases are more selective towards alcohol compared to thiols, no thioester was the expected result.

**Reaction E**

**Reaction F**

Scheme 10. Single-step route to a multifunctional branched macromer. (Appendix)
Figure 7. Kinetic studies of reaction E with regard to the acylation states of TMP, the methyl-group content (primary y-axis) and the percentage thioester of total sulfur content (secondary y-axis). (Appendix)

The acylation of TMP (18) occurs as expected. The amount of starting material with three free hydroxy-groups decreases and the content of TMP (18) with two free hydroxy-groups and then one free hydroxy-group increases before they both decrease again while the final product, the fully acylated TMP (18), is formed (Figure 7). After 47 h the reaction results in 90 % fully acylated and 9 % double acylated TMP (18). A higher acylation level could not be achieved at these conditions mainly because of the difficulty to acylate the third hydroxy-group and the thioester formation. CalB is more selective towards alcohols than towards thiols, but due to steric hindrance the third hydroxy-group is more difficult to acylate and therefore the thiol starts to be more competitive. Additionally the conditions change during the reaction. The acid group of MUA (17) is more polar than the methyl ester of EMLO (16). The hydrophobic thiol group is more prone to get into the active site of the enzyme in a polar environment. When the methyl ester is consumed, the formation of thioester steadily increases. In a reaction with only TMP (18) and MUA (17) (Reaction F, Scheme 10), more acid is available and the thiol will
react more easily. In this reaction a faster increase of thioesters up to twelve percent of the total amount of sulfur could be seen, which is then reduced over time to about seven percent where it stays constant, while having the same amount of acylation as in the reaction shown in reaction F (Scheme 10). In this TMP (18) and MUA (17) reaction the thioester formation follows the same trend as the curve for the double acylated TMP (Figure 8). This confirms that it is harder for the enzyme to acylate the third hydroxy-group because of steric hindrance. The thioester content is also dependent on the conversion of TMP (18). In reactions with smaller amount of fully acylated TMP and more double acylated TMP (64 % and 31 %, respectively), the amount of thioester was below 2 % of the total amount of sulfur. With lower conversion, the steric hindrance of acylating the third hydroxy-group does not affect the reaction in the same way as with higher conversion.

Figure 8. Kinetic studies of reaction F (Scheme 10) with regard to the acylation states of TMP (primary y-axis), and the percentage thioester of total sulfur content (secondary y-axis). (Appendix)
5.3 Formation of polymer thermosets

One of the main goals in this thesis has been to use and apply the synthesized telechelics to form material, thereby using the different functionalities. By varying and combining polymerization methods, materials with different properties could be obtained. The goal was to perform a selective polymerization by reacting only certain functional groups at a time while having other functional groups unaffected. This concept of orthogonality has found its way into polymer chemistry enabling the synthesis and the functionalization of complex polymer materials and surfaces.\textsuperscript{153-154}

The mid-chain epoxide groups in EFA can be crosslinked via cationic polymerization (\textbf{Paper I-III}). In \textbf{Paper II} methacrylate-functionalized EFA (10) (Reaction D) was the starting point to form materials. While the polymerization of the epoxides occurs through a cationic polymerization, the methacrylates react through a radical mechanism. Three different types of thermosets were achieved when reacting the functional groups individually or combined (Figure 9).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{networks.png}
\caption{Schematic representation of the networks achieved in \textbf{Paper II}. (Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission.)}
\end{figure}
All three combinations led to materials with different mechanical properties which were evaluated using $T_g$ and $\tan \delta$ (Figure 10). In Network A (Figure 9) only the end-groups were reacted through radical polymerization, which led to the thermoset with the lowest $T_g$ (3 °C) and the narrowest tan $\delta$ peak. This is mainly due to the low crosslink density which leads to a looser but highly homogenous network compared to the other methods. As there are more epoxide groups per chain than methacrylate groups, the crosslink density gets higher when using cationic polymerization of the epoxides and therefore also the $T_g$ (24 °C) (Network B). By combining cationic and radical polymerization, the crosslink density increases and leads to the highest $T_g$ in this study (40 °C) (Network C).

The product of reaction B (6) (Paper I) (Scheme 8) has an oxetane functionality. The oxetane is a cyclic ether, as the epoxide, and has similar reactivities. Therefore both can be reacted cationically, leading to material with differences in mechanical properties. In this case mid-chain-epoxy-groups can react with oxetane end-groups. Reaction A (Paper I) has a maleimide-functionality. The maleimide enables the possibility to both couple other molecules to the network and form a network by for example using a Diels-Alder reaction.

<table>
<thead>
<tr>
<th>Polymerization</th>
<th>$T_g$ [°C]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Radical</td>
<td>3</td>
</tr>
<tr>
<td>Cationic</td>
<td>24</td>
</tr>
<tr>
<td>Radical + cationic</td>
<td>40</td>
</tr>
</tbody>
</table>

Figure 10. DMA curves of the resins formed by the three different polymerization methods. (Paper II, Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission.)
Scheme 11. Schematic overview of the synthesis of reaction C (Scheme 8) towards surface active hydrophobic films. (Paper III)

Paper III describes the combination of epoxides and thiols in a thermoset (Scheme 11). Here the epoxides are polymerized cationically while the thiols stay unaffected. This could be shown by FT-Raman and by using Ellman’s reagent (5,5’-dithiobis-(2-nitrobenzoic acid)). To further investigate whether the thiol groups on the surface of the films are active, a thiol-ene reaction with 1,6-hexanediol mono vinyl ether was performed to modify the surface.
Figure 11. Contact angle before (to the left) and after (to the right) modifying the surface with 1,6-hexanediol mono vinyl ether using thiol-ene chemistry. (Paper III)

Figure 11 shows the contact angle of a water droplet on the surface of the crosslinked film before and after the thiol-ene reaction with the vinyl ether. The measurements showed that the surface properties of the film changed after the reaction. Because of the reaction the end-groups on the surface will be changed from thiol-groups to hydroxy-groups which are more hydrophilic and lead to a lower contact angle (Figure 11, right picture).
6. Induced substrate specificity for the synthesis of building blocks for polymer synthesis

In Paper I-III multifunctional oligomers were produced which could be polymerized using the epoxy-groups leaving other functional groups available for post-functionalization. It is not necessary to synthesize oligomers in order to have a starting material for a polymerization reaction as smaller molecules can also be interesting to begin with. The MsAcT wild type (wt) enzyme shows limitations to catalyze reactions with longer acyl donors than butyrates. These limitations in the substrate scope can be used as an advantage to synthesize designed asymmetrical diesters.

6.1 Extending the acyl donor specificity in MsAcT

In Paper IV the active site of MsAcT was engineered to extend the acyl donor substrate scope. Three positions were identified to maybe have an influence on the position of the acyl donor chain. Single and double mutants were made and a hydrolysis assay showed that the best variant, T93A/F154A, could accept acyl donors up to five carbons longer than the wt enzyme with a more than 400-fold increase in specificity (Table 3).

The steric hindrance in MsAcT is visible in the simulation snapshots in Figure 12A. The acyl donor chain has to bend towards the entrance of the active site at the C3 position leading to an unfavorable conformation. The double mutant T93A/F154A opens up space on top of the acyl donor chain, making more space available to fit in a larger acyl donor (Figure 12B). It also makes space for the acyl donor to bend towards the active site entrance. The L12A mutant on the other hand (Figure 12C) opens up a pocket towards the center of the enzyme subunit, which enables a slight increase in the possible length of the acyl donor chain.
Induced substrate specificity for the synthesis of building blocks for polymer synthesis

Table 3. Substrate specificities towards different methyl esters in hydrolysis catalyzed by MsAcT wt and variants. (Paper IV)

<table>
<thead>
<tr>
<th>Methyl esters</th>
<th>n</th>
<th>wt</th>
<th>L12A</th>
<th>T93A</th>
<th>F154A</th>
<th>L12A/T93A</th>
<th>L12A/F154A</th>
<th>T93A/F154A</th>
</tr>
</thead>
<tbody>
<tr>
<td>acetate</td>
<td>0</td>
<td>3200</td>
<td>64</td>
<td>460</td>
<td>3300</td>
<td>44</td>
<td>295</td>
<td>530</td>
</tr>
<tr>
<td>propionate</td>
<td>1</td>
<td>630</td>
<td>27</td>
<td>170</td>
<td>690</td>
<td>40</td>
<td>68</td>
<td>160</td>
</tr>
<tr>
<td>butyrate</td>
<td>2</td>
<td>84</td>
<td>16</td>
<td>160</td>
<td>130</td>
<td>39</td>
<td>77</td>
<td>110</td>
</tr>
<tr>
<td>hexanoate</td>
<td>4</td>
<td>-b</td>
<td>40</td>
<td>-b</td>
<td>-b</td>
<td>11</td>
<td>80</td>
<td>64</td>
</tr>
<tr>
<td>heptanoate</td>
<td>5</td>
<td>-b</td>
<td>10</td>
<td>c</td>
<td>c</td>
<td>8.4</td>
<td>47</td>
<td>180</td>
</tr>
<tr>
<td>nonanoate</td>
<td>7</td>
<td>-b</td>
<td>-b</td>
<td>c</td>
<td>c</td>
<td>-b</td>
<td>-b</td>
<td>81</td>
</tr>
</tbody>
</table>

$k_{cat}/K_m$ [s$^{-1}$ M$^{-1}$]$^a$

The substrate specificities were determined by linear regression of the initial rates for at least 4 different concentrations. The coefficients of determination ($r^2$) varied between 0.94-1.0 for acyl donor chain length up to hexanoate. For larger acyl donors $r^2$ varied between 0.9-0.99.

$^b$ No activity detected, <0.2 s$^{-1}$ M$^{-1}$.

$^c$ Not determined.

The results in the hydrolysis assay were very promising but can only give an indication on the acyl transfer. The analytical procedures used by Wittrup Larsen et al. showed that the acyl transfer to hydrolysis ratio did not alter significantly, with F154A being an exception (Scheme 12). F154 seems to play a role in the acyl transfer to hydrolysis ratio, as a smaller amino acid as alanine increased the hydrolysis activity in all variants. This position could facilitate the access of water into the active site.
Figure 12. Simulation snapshots of the active site of MsAcT viewed from the oxyanion hole. The entrance of the active site is to the left and is marked by a black arrow in (A). The models were made with butyl hexanoate (ball&stick) after 50 ps MD simulations. The acyl donor colored in blue, acyl acceptor in yellow and mutated amino acids in red (native) and purple (alanine mutation). (A) The active site of the MsAcT wild type. The limitation of the acyl donor pocket is clearly visible and steric hindrance forces the acyl donor chain to bend outwards at C3 and adopt a more high-energetic conformation. (B) T93A/F154A, the mutation opens up a big hole in the active site that can easily fit longer acyl donor chains and allow them to continue outward towards the active site entrance. (C) L12A, the mutation opens up a pocket towards the center of the MsAcT subunit. This allows larger acyl donor chains to fit into the active site compared to the wt. (Paper IV)
6.2 Applications
Extending the substrate scope of *MsAcT* opens up new possible reactions. Two *MsAcT* variants, T93A/F154A and L12A, showed promising results as they were able to convert longer acyl donor chains compared to the wt enzyme. They were used to catalyze the synthesis of amides in water (Paper IV), where the wt enzyme gave no conversion and the selective synthesis of mixed vinyl adipate esters (Paper V).

6.2.1 Amide synthesis in water
*MsAcT* T93A/F154A was used for the synthesis of N-benzylhexanamide in water (Table 4). It reached 81% conversion of the amine, while the wt did not show any activity above the background reaction. The altered substrate scope of T93A/F154A might be used for the synthesis of amides in water.
Table 4. Synthesis of \( N \)-benzylhexanamide in water: Conversion of methyl hexanoate and benzylamine for MsAcT wt, and T93A/F154A variant after 20 h using the same enzyme concentration. (Paper IV)

<table>
<thead>
<tr>
<th>Variant</th>
<th>Conversion [%]</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>benzylamine</td>
<td>methyl hexanoate</td>
<td></td>
</tr>
<tr>
<td>wt</td>
<td>&lt;2</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>T93A/F154A</td>
<td>81</td>
<td>≥99</td>
<td></td>
</tr>
<tr>
<td>Control(^a)</td>
<td>&lt;2</td>
<td>46</td>
<td></td>
</tr>
</tbody>
</table>
\(^a\) without enzyme

6.2.2 Selective synthesis of mixed vinyl adipate esters

Dicarboxylic acids and their derivatives have been used as acylating agents to obtain dimeric or hybrid derivatives of bioactive natural compounds. Especially activated esters of dicarboxylic esters have been found to be a versatile choice. Another potential application area is polymer synthesis. Dicarboxylic acid derivatives are used in polycondensation reactions in combination with diols. Vinyl esters are used as monomers in polymer research and industrial production of poly(vinyl ester)s. Vinyl esters have also been combined with glucose before polymerizing the vinyl groups to obtain material with tensioactive properties.

As seen earlier, MsAcT has limitations in the size of the acyl donors it can use. Therefore by using a symmetric, activated dicarboxylic ester, it is possible to react only one of the ester groups while keeping the other one unaffected, as the product will be too big to fit in the active site as a new acyl donor. In order to test this hypothesis, three reactions were set up to evaluate the performance of MsAcT wt, MsAcT L12A and CalB (Scheme 12). Three acyl acceptors were reacted with divinyl adipate (DVA) (22): 1-octanol (23), an aliphatic alcohol, 1,4-butanediol vinyl ether (BVE) (25), a vinyl ether at the end of an aliphatic chain and TMPO (5), a slightly bulkier substrate with an oxetane-functionality.

Table 5 summarizes the results of the reactions G, H and I. Reaction G with DVA (22) and 1-octanol (23) was promising. The MsAcT variant L12A showed high conversion producing exclusively the mixed octyl vinyl adipate (OVA) (24) according to GC analysis. CalB converted more than 97% of DVA (22) within 5 min, resulting in a bigger part dioctyl adipate,
as both vinyl groups of DVA (22) are accessible for CalB even if one end already has a long substituent.

In order to show the versatility and applicability of this system, alcohols with additional functional groups were introduced. BVE (25) was used in combination with DVA (22) (reaction H) in order to create a vinyl ester and a vinyl ether functionality within the same molecule. As BVE (25) has similar spatial properties as octanol (23), similar reactivities were expected. MsAcT L12A showed a high conversion of DVA (22) and the formation of BVEVA (26) as product after 5 hours reaction time. This result could be confirmed by \(^1\)H NMR, as the peaks for the vinyl groups of DVA (22) decreased by 50% as half of the groups were reacted with BVE (25). In the same time span, MsAcT wt was able to convert about 40% of DVA (22) with less selectivity.

TMPO (5) is a bulkier substrate than octanol (23) or BVE (25). The acyl acceptor specificity for MsAcT has not been explored yet to see the full scope of possible substrates, but a few different acyl acceptors have been used in different publications.\(^{50, 69-72}\) MsAcT L12A achieved over 90% conversion within 22 hours by almost exclusively reacting with the adipate on one side to produce the mixed OxVA (27). MsAcT wt had problems converting TMPO and only achieved 7% conversion in the same time period. This is most likely because of steric hindrance because it is more difficult for TMPO to access the active site. The products were mixed with oxetane-functionalities on one or on both sides of the adipate.
Table 5. Enzymatic syntheses of vinyladipoyl esters using MsAcT wt, MsAcT L12A and CalB. Uncatalyzed reactions were used as control. Distributions in percent are shown between the two products, acylated with the alcohol on one side (mixed) and on both sides (di).

<table>
<thead>
<tr>
<th>Catalyst</th>
<th>Alcohol</th>
<th>ratio</th>
<th>time</th>
<th>conv.(^a)</th>
<th>product distrib.(^a)</th>
<th>DVA: alc.</th>
<th>DVA [%]</th>
<th>mixed [%]</th>
<th>di [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>MsAcT L12A</td>
<td>Octanol</td>
<td>1:3</td>
<td>21</td>
<td>99</td>
<td>&gt;99</td>
<td>&lt;1</td>
<td>&gt;99</td>
<td>&lt;1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1:1</td>
<td>30</td>
<td>80</td>
<td>&gt;99</td>
<td>&lt;1</td>
<td>&gt;99</td>
<td>&lt;1</td>
<td></td>
</tr>
<tr>
<td>MsAcT wt</td>
<td></td>
<td>1:3</td>
<td>21</td>
<td>41</td>
<td>94</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1:1</td>
<td>30</td>
<td>57</td>
<td>83</td>
<td>17</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CalB</td>
<td></td>
<td>1:3</td>
<td>0.083</td>
<td>98</td>
<td>10</td>
<td>90</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1:1</td>
<td>0.017</td>
<td>68</td>
<td>59</td>
<td>41</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MsAcT L12A</td>
<td>BVE</td>
<td>1:1.5</td>
<td>5</td>
<td>99</td>
<td>&gt;99</td>
<td>&lt;1</td>
<td>&gt;99</td>
<td>&lt;1</td>
<td></td>
</tr>
<tr>
<td>MsAcT wt</td>
<td></td>
<td>1:1.5</td>
<td>5</td>
<td>40</td>
<td>92</td>
<td>8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MsAcT L12A</td>
<td>TMPO</td>
<td>1:1.5</td>
<td>22</td>
<td>92</td>
<td>&gt;99</td>
<td>&lt;1</td>
<td>&gt;99</td>
<td>&lt;1</td>
<td></td>
</tr>
<tr>
<td>MsAcT wt</td>
<td></td>
<td>1:1.5</td>
<td>22</td>
<td>7</td>
<td>85</td>
<td>15</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) determined by GC
Induced substrate specificity for the synthesis of building blocks for polymer synthesis
7. Concluding remarks

In the work presented in this thesis, the synthesis of different multifunctional building blocks for polymer materials from renewable resources has been accomplished with the use of enzymes.

In the first part, Paper I-III, two epoxy fatty acids, EFA and EMLO, were isolated from birch bark and vegetable oils, respectively. Linear telechelics based on EFA and a branched macromer based on EMLO have been synthesized by lipase catalysis. Different functional groups could be combined in the same molecule with a “one-pot”-strategy. This can be advantageous compared to the conventional protection/deprotection chemistry. This work included epoxides, maleimides, methacrylates, oxetanes and thiols, but because of the selectivity of CaLB other groups and combinations are possible to achieve different materials with different properties. The focus in this part was on the use of epoxy fatty acids as backbone for the polymeric materials, but also other structures and functional groups, such as furans, could be used.

In the second part, Paper IV and V, a rather uninvestigated enzyme, MsAcT, was engineered to extend its substrate scope for linear acyl donors to use it for the synthesis of mixed vinyl adipate esters. Three different reactions were performed to combine the vinyl group with other functionalities, but the full substrate scope of MsAcT has to be investigated to combine other functional groups to form polymers with interesting properties. The sterical limitation of MsAcT’s active site could be used to form amide building blocks for the synthesis of polyesteramides or possibly other polymer building blocks. But also medically interesting compounds could be synthesized from activated dicarboxylic esters as summarized by Bassanini et al.157

Nature offers a wide variety of molecules which possibly can be made accessible and be processed with the help of enzymes. There are also many uncharacterized enzymes which could be used for both the
production of polymer building blocks or for the future degradation of the polymers.


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