Engineering of the Acyltranferase MsAcT from *Mycobacterium smegmatis*

Master Thesis

Etta Jost, 01430528

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Department of Chemistry, Organic and Bioorganic Chemistry, University of Graz,
Heinrichstraße 28, A-8010 Graz, Austria

Supervisors:

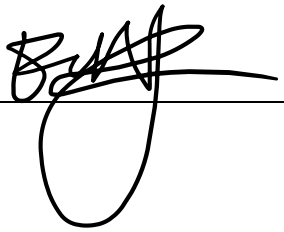
Dr.rer.nat. Christoph Winkler

Univ.-Prof. Wolfgang Kroutil

I. Declaration of Academic Integrity

The work described in this thesis was carried out at the Department of Chemistry of the University of Graz from March 01 2019 to May 15 2020.

With my signature I declare that I have authored this thesis independently and that I have not used other than the declared sources / resources. The passages which were taken from other works either literally or by content, I have marked stating the original source. Unless otherwise marked, the illustrations, pictorial representations and schemes are my own.

(City, Date) Graz, 06.07.2020 (Signature) 

II. Acknowledgements

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1 Introduction

Over the last few decades, biocatalysis has become a topic of great interest in synthetic chemistry and many applications of biocatalytic processes can be found in the chemical industry and research [1–8]. This development happened due to various benefits that biocatalysts provide. The main advantages being the increasing possibilities that biotechnology and biochemistry have to offer, due to the vast development in this field. These advances led to affordable sequencing and synthesis of DNA which favored the discovery and biochemical characterization of numerous novel and synthetically relevant enzymes. The remarkably mild conditions biocatalysts operate at and the outstanding chemo-, regio- and stereo-selectivity they possess also contribute to their popularity. Due to all these factors it has become more accessible and beneficial to implement enzymes as catalysts in chemical synthesis and especially hydrolases and oxido-reductases are already being utilized in a broad range of applications [9].

An increasing focus of the chemical industry is sustainability. Starting in the 1980s, concerns emerged regarding resources consumed and the enormous amounts of waste produced by the chemical industry [10]. The concept of "Green Chemistry" [11] was developed that focuses on sustainability *via* prevention of pollution instead of waste remediation, use of less hazardous materials, application of renewable raw materials and many more aspects of environmentally friendly synthesis design. Another important aspect is to implement catalytic amounts of reagents instead of stoichiometric, making catalysis a topic of great interest [11]. Hence, biocatalysis gained in popularity due to its many benefits in the context of Green Chemistry. Biocatalysts are produced from renewable resources, are biodegradable, perform reactions under mild conditions and generate less waste. This leads not only to more environmentally friendly procedures, but also to more cost effectiveness [12].

In order to be applicable in this extent several biochemical methods needed development. Especially the recombinant DNA technology made enzyme production more efficient, accessible, and inexpensive. Hence, the desired enzymes can be overexpressed in other host organisms, such as the fast-growing *E. coli*. Therefore, larger quantities of biocatalysts are readily accessible. The immense development of biochemical methods also led to more advanced procedures to optimize enzymes, as biocatalysts can be limited in certain factors such as substrate promiscuity, selectivity, stability and may suffer from substrate- or product-inhibition [13]. One method to eliminate those drawbacks and to increase, for example, the enzymes stereo-selectivity can be the generation of superior enzyme variants. (Site-)directed evolution provides an accessible method for enzyme engineering but needs to

be further developed. Due to the high complexity of enzymes it is hard to rationally predict beneficial mutations [14].

Hydrolytic reactions are broadly applied biotransformations in the field of biocatalysis and are catalyzed by proteases, esterases and lipases (EC 3). These enzymes represent the most widely applied biocatalysts, as they do not require a co-substrate and a large number of readily available and well characterized enzymes are active on a broad substrate scope. The enzyme-class performs the hydrolysis of esters and amides and the reversible formation of the respective compounds (Figure 1).

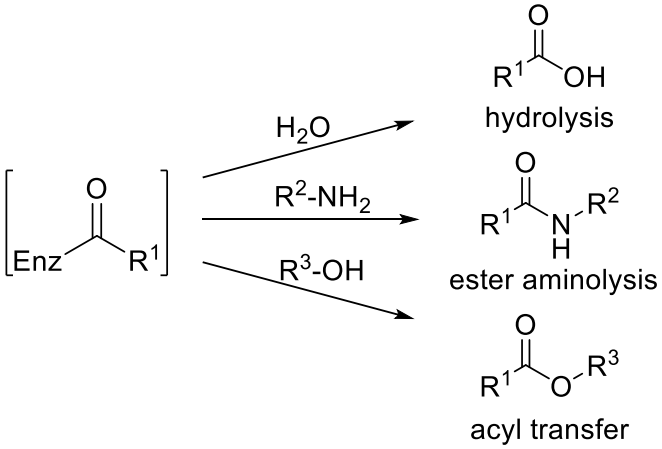


Figure 1: Scheme of possible reactions with hydrolytic enzymes (EC 3) using different nucleophiles (Nu) in order to obtain various products.

Biocatalysts performing acyl transfer work best in organic solvents at low water concentrations, since the presence of water would shift the reaction equilibrium towards hydrolysis reactions [15]. However, regarding the principles of Green Chemistry and the process compatibility with other biocatalysts, it would be desirable to perform this reaction in water. Interestingly, the Acyl Transferase from *Mycobacterium smegmatis* (MsAcT) was found to catalyze esterification faster than hydrolysis in aqueous media, due to its unique structural properties. This exceptional ability makes the enzyme a biocatalyst of great interest and opens routes to new synthetic approaches [16].

2 Literature Overview

2.1 Biocatalysis in the Context of Green Chemistry

In the last century the importance of synthetic chemistry vastly grew due to the growing demand for various fine chemicals, pharmaceuticals and other products of the chemical industry [17]. Not only the overall number of products increased, but also the accumulated waste, since a multitude of chemicals were used for these processes. Hence, a general concern emerged regarding the impact of those industrial procedures on the environment [10]. This change of perspective led to actions, as for example the United Nations founded the World Commission for Environment and Development. In their report "Our common Future" from 1987, common concerns and challenges regarding environmental impact were emphasized and a focus was brought to sustainability in development of industrial processes [18].

Subsequently the term "Green Chemistry" was defined by Anastas and colleagues [11] in the early 1990s in order to further define this aspect of chemical process development. This concept was represented by the 12 principles of Green Chemistry which focus on waste prevention, avoidance of toxic or harmful chemicals, safer product design, the use of innocuous solvents, energy efficiency, the use of renewable raw materials and the use of catalytic rather than stochiometric reagents. This led to a more defined term of sustainability in chemical processes and triggered the development of feasible solutions and improvements for industrial procedures [11].

Regarding waste prevention, a major cause for residuals in synthetic processes is the use of compounds in stochiometric amounts. Especially inorganic reagents accumulate in multistep syntheses or downstream processes in the production of fine chemicals and pharmaceuticals [10]. Consequently, such processes should be substituted with catalytic alternatives, since these synthetic routes provide higher atom economy[1] [11, 12]. All four categories of catalysts - heterogeneous catalysis, homogeneous catalysis, organo-catalysis and biocatalysis - can provide green and sustainable solutions for organic synthesis [10].

In regard of "Green Chemistry" especially biocatalysis stands out and grew in attraction for industrial processes over the last few decades [19]. The increased application of enzymes in synthetic chemistry is the result of numerous advantages those catalysts bring with them. They operate at very mild reaction conditions: ambient temperatures, atmospheric pressure and

[1] Atom economy is a tool to evaluate the amount of waste generated during a specific synthesis process for the chosen substance. By dividing the molecular weight of the product by the sum of the molecular weights of all compounds produced in the stochiometric equation of the reaction the atom economy is calculated [61].

neutral pH and are often performed in water. There is usually no need for additional protection and deprotection steps during synthesis, due to their outstanding chemo-, regio- and stereo-selectivity in comparison to other catalysts. The production of biocatalysts and their disposal meet the requirements of sustainability as well since enzymes are obtained from renewable resources and are biodegradable. These advantages show that biocatalysts meet many of the requirements for Green Chemistry and sustainability. Additionally, they provide cost-effective alternatives to other commonly used catalysts like transition metals, due to milder reaction conditions of biotransformations and the circumventing of the costly removal of these metals [12].

2.1.1 Enzyme Engineering

Enzymes have become increasingly more popular in synthetic chemistry due to the many advantages they offer. However, this type of catalyst has some significant limitations as well. Drawbacks like insufficient stability under operating conditions, product inhibition, narrow substrate scope, low space-time yields, insufficient stereo-selectivity and sometimes poor regioselectivity lead to a restricted application of enzymes as well as a general disregard of many organic chemists to use biocatalysis over other synthetic methods [13].

A promising field in research to overcome these problems in biocatalysis is protein engineering. Its goal is to alter the enzymes performance in different parameters, such as an increase of activity as well as selectivity, alteration of substrate specificity and enhancement of stability for the designated biocatalytic processes [20].

The first approaches in enzyme engineering were done in the 1980s when rational design by site-directed mutagenesis was introduced. This was possible due to a vast progress in molecular biology but also due to the growing structural knowledge of enzymes. Methods like oligonucleotide synthesis, DNA amplification by polymerase chain reaction and improved DNA analysis were introduced. With these tools it was possible to exchange a specific amino acid in the protein with another one of the 19 canonical amino acids based on the structural knowledge of the enzyme [21, 22]. This rational design approach unlocked a broader substrate range of enzymes and the synthesis of unusual synthetic intermediates. Hence, this provided an opportunity to optimize the biocatalysts for unnatural substrates and therefore made biocatalysis in general more appealing for synthetic applications [23]. Even though, the method provided a promising, new possibility in the field of enzyme engineering, it is limited due to insufficient understanding of the coherence between the sequence, structure and the function of proteins in order to fully predict beneficial mutations [24].

Another approach of enzyme engineering is based on nature's own mechanisms of protein optimization and started to gain more attraction in the 1990s. In directed evolution a broad variety of mutations is introduced into existing enzymes and these variants are screened for the desired traits. The biocatalysts with the best fit serve then as the starting point for the next round of mutations. Therefore, many cycles of individual random mutations with a certain goal in mind can lead to great catalysts [25]. Directed evolution also attracted more attention in that time due to the fact, that new techniques like DNA shuffling were introduced. This method includes *in vitro* recombination of fragmented oligonucleotides derived from the single-point mutants in every round of mutagenesis in order to enhance and combine beneficial mutations [26, 27]. Consequently, DNA shuffling partially circumvented the low probability of finding a beneficial mutant when performing directed evolution and became a key method in this field until nowadays. Despite the enhancements and success of directed evolution, this method also has its limitations. A big problem results from the chosen library size. Even for the biggest chosen libraries it is not realistically feasible to cover all possible variations. However, the high number of mutants comes with difficulties during enzyme expression and especially screening. The determination of the mutation frequency is not trivial either, since too few introduced mutations lower the chance of finding beneficial ones, but increasing it too much can lead to overall loss of enzyme activity [24].

Instead of developing new approaches to increase gene libraries and enhanced screening methods, the focus shifted rather on the design of smaller mutant libraries with higher qualities. This semi-rational approach [28] bases the design of the library on knowledge about the protein like the sequence, structure, function and it includes computational methods for predictions about targeted sites as well. Attention is put more on certain amino acids in the sequence which can dramatically reduce the size of a library [29]. In order to identify residues of interest an understanding of the molecular basis of the protein's properties or more specifically the structure-function relationship is required. This is crucial for all different types of rational design techniques, since a very particular property of the enzyme needs to be identified which will be later on targeted for modification [30]. For example, steric factors often control substrate specificity as well as stereo-selectivity. Hence, a good understanding of these features is required in order to enhance the substrate scope or the selectivity of a biocatalyst [31]. Structural visualization is one method to identify hot spot amino acids for mutagenesis. With this design approach the shape-conformity of the active site with the transition state of the substrate is investigated. This is crucial to predict the activity of the enzyme on the specific substrate and to check if a specific compound is accepted by the biocatalyst at all [32]. Knowledge about the

preferred binding mode of a substrate in an enzyme is of great interest for rational design as well. The specific positioning results from a certain geometry of the active site and enables the preference towards binding of a distinct substrate-enantiomer or formation of one configuration of a chiral product. In addition to that, unwanted binding-modes should be blocked in the active site to prohibit the formation of undesired products. Another factor that determines the selectivity of an enzyme is the nature of the entrance tunnel. The structure as well as the chemical properties of a tunnel affect the passage of a substrate [33].

In rational design, information about the active site is used to enhance desired properties such as the blocking of unfavored binding modes by introducing bulkier amino acids. Hence, modifications that favor a specific binding mode, provide less space for unfavored substrate positioning, or adjust the enzymatic entrance tunnel which can readily enhance selectivity and activity of the enzyme. However, many mutations cannot be predicted due to the high complexity of biocatalysts. Certain protein dynamics or effects on protein folding can be problematic when pre-estimating new mutations of an enzyme. Detailed information about the protein, more knowledge about the catalysis mechanism itself, inhibition processes, as well as structural insight about reaction intermediates can minimize these difficulties. However, it is hard to obtain enough enzyme characteristics *via* conventional methods like NMR or XRD because these techniques are highly time consuming and complex. Since quantum mechanical simulations can predict enzymatic characteristics such as the optimal binding modes, transition states, enzyme inhibition and mechanistic aspects, computational methods become more popular. These methods allow enzyme engineering not only for highly enantioselective catalysts and to improve pre-existing catalytic activities, but also complete redesign of the active site in order to introduce new enzymatic functions [30]. These advanced computational methods allow for large *in silico* mutant libraries which can further be evaluated. The best hits found will then be analyzed in the laboratory manually. This method increases the overall velocity of the enzyme engineering process [34].

Due to all these benefits that computational methods provide for enzyme engineering, the field becomes even more attractive. Even though *in silico* mutant libraries are a good starting point, many observed effects occurring during biocatalysis cannot be fully explained yet nor are the compositions of gene sequences completely understood. Hence, more research in this field is definitely required [24].

2.2 Hydrolytic Reactions

The class of the simplest, best investigated and most popular reaction type in biocatalysis are hydrolytic biotransformations. The respective enzyme class containing e.g. lipases, proteases and esterases (EC3) performs hydrolysis of esters and amides and in reverse the formation of the respective compounds. The lack of requirement for sensitive and expensive co-factors, as well as the multitude of readily available enzymes with a broad substrate range make these enzymes attractive for synthetic chemistry [15]. Due to the many advantages this enzyme-class offers, it has been predominantly the subject of research in the early days of biocatalysis. Consequently, hydrolytic reactions are very well investigated. Numerous enzymes in this field are characterized and a large number of affordable commercial enzymes are accessible as well as widely applied in industry [19].

2.2.1 Mechanism of Hydrolytic Enzymes

The mechanism of hydrolytic enzymes is comparable to chemical hydrolysis under basic conditions. The carbonyl group of the substrate ester or amide is attacked by a nucleophilic residue from the enzymes active site and therefore activates the acyl group. The moiety performing the nucleophilic attack can be either a hydroxy group of a serine, a carboxylate of an aspartic acid, or a thiol of a cysteine. The most common functionality in most microbial lipases is serine, hence the serine hydrolase mechanism (Figure 2) will be further discussed in detail [15].

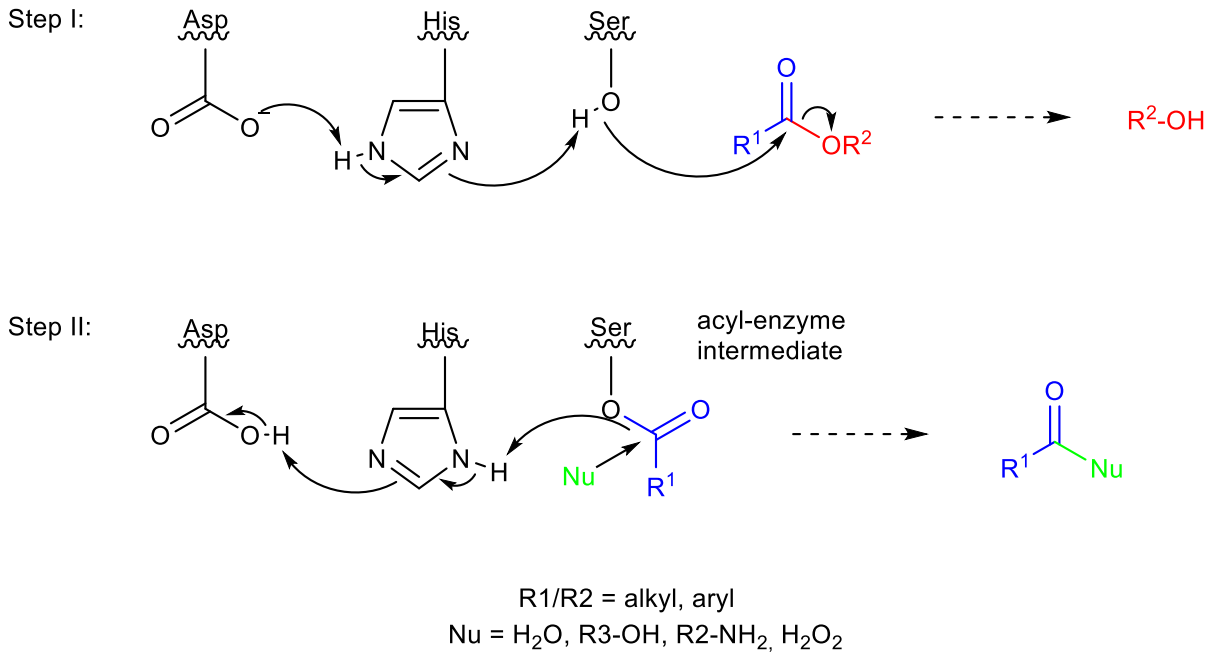


Figure 2: General serine hydrolase mechanism of hydrolytic enzymes with an ester as substrate [15].

Within this type of enzymes, the active amino acids consist of a catalytic triad: serine, asparagine, and histidine. The additional two amino acids activate the catalytic serine which lowers its pK_a and thereby enables it to perform the nucleophilic attack on the substrates carbonyl group [35–38]. This leads to the formation of a covalently bound acyl-enzyme-intermediate by releasing the leaving group (in case the substrate is an ester, the leaving group is an alcohol). Further on a nucleophile can attack the acyl-enzyme-intermediate. Depending on the nucleophile (Figure 3) different products can be formed [15].

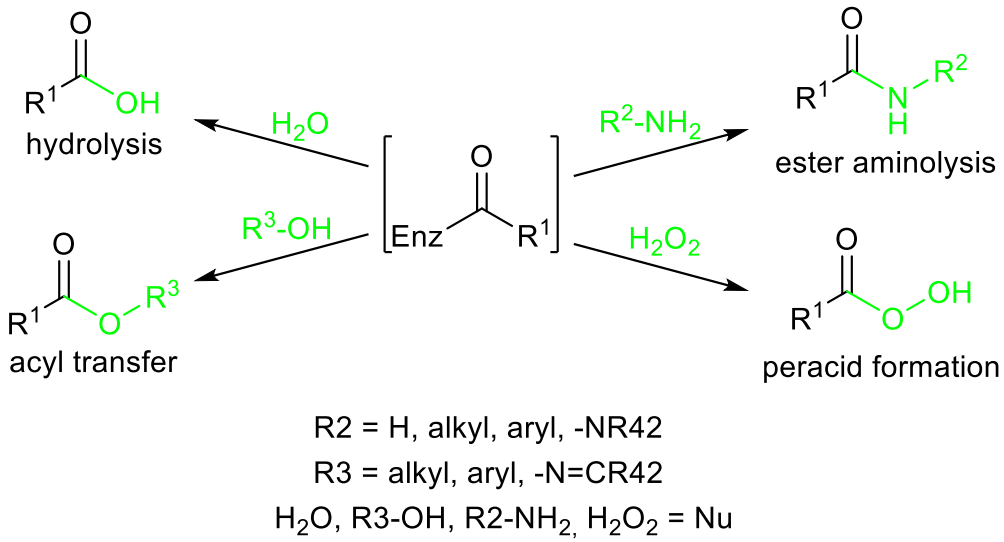


Figure 3: Possible reactions with hydrolytic enzymes (EC 3) using different nucleophiles (Nu) in order to obtain various products [15].

This type of enzyme is mainly used to perform hydrolysis and acyl-transfer reactions. Hydrolysis or perhydrolysis of esters or amides can be accomplished with water or hydrogen peroxide as a nucleophile in order to release a carboxylic acid or a peracid and an alcohol or amine. Acyl-transfer reactions can be performed to obtain a variety of esters or amides with an acyl donor and an alcohol or amine acting as a nucleophile [15]. Acyl-transfer reactions are usually performed in organic solvent, since in aqueous environment the high concentrations of the present water shifts the equilibrium towards hydrolysis of the formed ester or the acyl donor [39].

2.2.2 Chiral Differentiation and Kinetic Aspects of Hydrolytic Reactions

Two important parameters that affect the enzymes reactivity heavily are steric and electronic interactions with the substrate. As stereoisomers have different 3D structures, the enzyme, being chiral itself, may show different interaction with the two enantiomers and therefore different activity for the two molecules. The differentiation of a biocatalyst towards stereoisomers may be quantified by kinetics, i.e. the reaction rate of the enzyme with the two

substrate enantiomers. According to that it is crucial to have knowledge about the kinetics in addition to the steric and electronic properties of the enzyme of choice and as well the chirality of a substrate, in order to fully understand the chiral recognition and optimize the reaction appropriately [15].

Regarding hydrolytic reactions of racemic substrates such as esters or amides, differentiation between the two enantiomers happens [40]. Chiral discrimination of enzymes is not only occurring if the substrate has a central chirality but is also recognizing axial and planar chirality. The chiral recognition of a substrate with an enzyme originates from the chirality of the enzyme itself as well as the 3D shape of its active site. Hence, one enantiomer of the substrate fits the active site of the enzyme better than the other. This leads to a higher conversion rate of the preferred enantiomer of the substrate. This type of reaction is called kinetic resolution, as the two enantiomers may be resolved by the different kinetics the enzyme shows for them and is one of the most commonly used enzymatic reaction types among hydrolytic enzymes [15]. The reason why this reaction type is so frequently applied is, that racemates are far more abundant than prochiral or *meso*-compounds. These two groups of chiral compounds differ from each other because prochiral and *meso*-compounds have only two variable functional groups (R_1, R_2) which makes them symmetric and racemic substrates possess three (R_1, R_2, R_3), hence being asymmetric (Figure 4) [41].

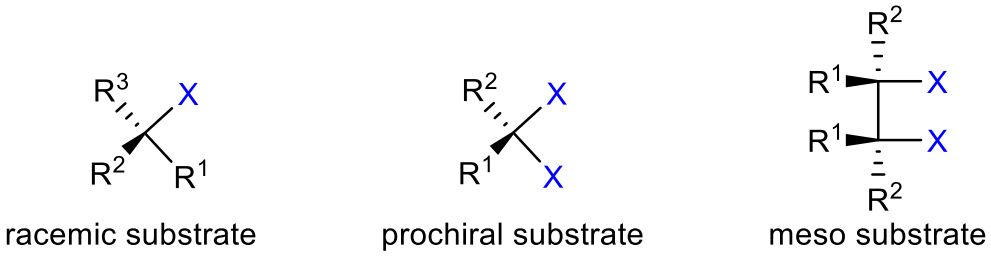


Figure 4: General structures of a racemic substrate, a prochiral substrate and a meso substrate. The blue X marks the residue, that is targeted in the synthesis [15].

However, in contrast to kinetic resolutions, desymmetrization reactions can theoretically yield 100% of product. In contrary to that, the maximum theoretical yield that can be obtained in a successful kinetic resolution is 50%. In those ideal cases (Figure 2Figure 5) the favored enantiomer is transformed extremely fast and the corresponding mirror image is not converted at all. This leads to 50% of yield for an optical pure substrate as well as product due to the high difference in reaction rates [15].

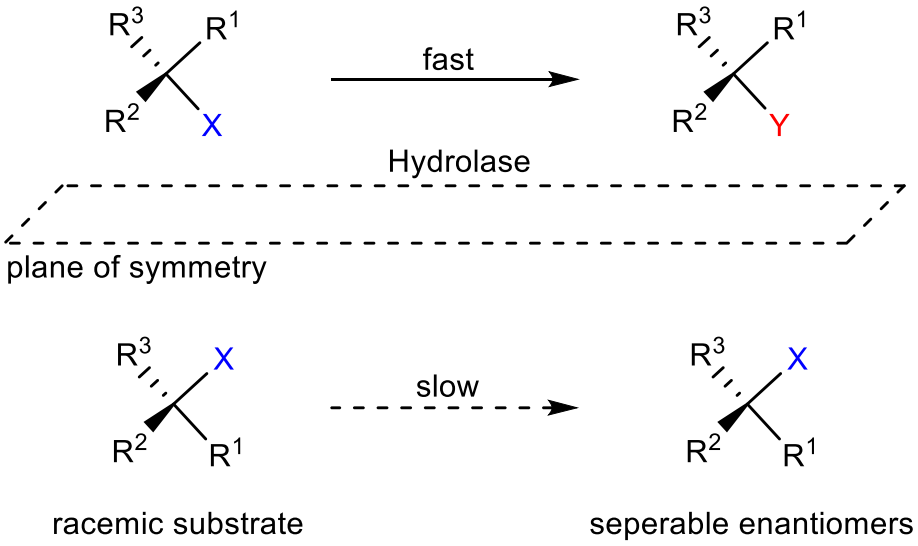


Figure 5: General scheme for kinetic resolutions of a racemic substrate using a hydrolase with high enantio-preference for only one enantiomer [15].

Nevertheless, looking at these reactions in practice shows that enantio-selectivity is usually not ideal and the difference in reaction rates of the two enantiomers cannot be indefinitely high. The kinetics of the reaction can be used to quantify the selectivity of the enzyme as well as characterize the course of the reaction itself. Experimental data usually shows that the reaction does not stop at 50% conversion, yet the velocity of the reaction is decreased significantly around this point. This leads to the conclusion that the reaction speed depends on the degree of the conversion of each enantiomer. Hence, the enantiomeric excess of the substrate (e.e.$_S$) and the product (e.e.$_P$) are functions of the overall conversion [15]. In order to quantify and describe this dependency properly a new parameter was introduced based on the work of C.J. Sih [39], K.B. Sharpless [42] and K. Fajans [43]. The "Enantiomeric Ratio" or E-value (Equation 1) is a dimensionless constant and describes the selectivity of a specific enzyme on a defined substrate performing kinetic resolution. Thereby E does not change during the time of the reaction. However, it is relative to the ratio of the second-order rate constants (ν_A, ν_B) of both substrate enantiomers (A, B). The constant is connected to the k_{cat} and K_M values of the enantiomers following the Michaelis Menten kinetics as well [44, 45].

Equation 1: Enantiomeric Ratio (E-value) derived from the rate constants of the Michaelis Menten kinetics.

$$Enantiomeric\ Ratio:\ \ E = \frac{\nu_A}{\nu_B} = \frac{\left(\frac{k_{cat}}{K_M}\right)_A}{\left(\frac{k_{cat}}{K_M}\right)_B}$$

2.2.2.1 *Irreversible Hydrolytic Reactions*

When regarding the kinetics of a hydrolytic reaction irreversible and reversible reactions must be treated separately. This type of reaction can be considered irreversible if hydrolysis is performed in water, since water is present in such high concentrations. The same principle applies to acyl transfer reactions in organic solvent. A requirement for irreversible reactions is that inhibiting factors must be negligible, hence the two enantiomers can compete without constraints for the active site of the biocatalyst. If this is the case Michaelis-Menten kinetics (Figure 6) can describe the reaction [15].

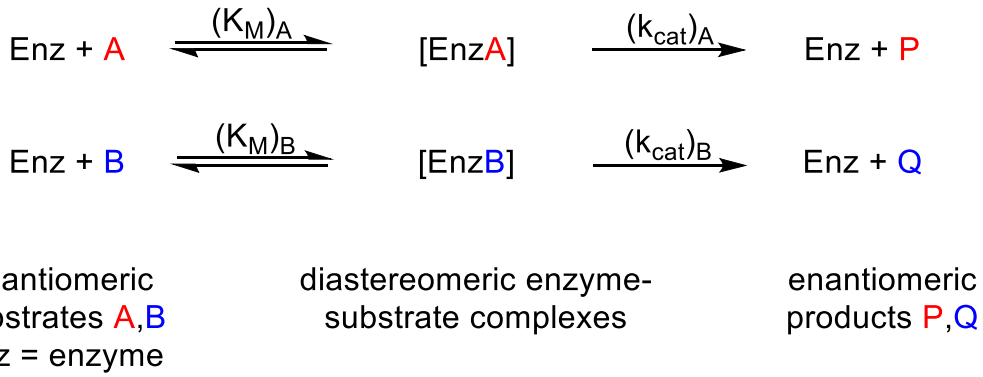


Figure 6: Michaelis-Menten kinetics of an enzymatic kinetic resolution of an irreversible hydrolytic reaction [15].

However, determining the kinetic constants of a reaction can be an elaborate task, whereas measuring the conversion (c) and the enantiomeric excess of the substrate (e.e.$_S$) as well as the product at a certain point of the reaction is far more feasible. The E-value can be calculated with c, e.e.$_S$ and e.e.$_P$ (Equation 2) since these values are mathematically linked to the ratio of the reaction rates. This constant can be determined at any given point of the reaction and is independent from conversion. Nevertheless, in cases of very low or high conversions, measuring errors can lead to unreliable results for the Enantiomeric Ratio. Therefore, the E-value is usually determined only using the enantiomeric excess of the substrate and the product.

Equation 2: Calculation of the E-value with the conversion of the reaction, the e.e.P and the e.e.S.

$$E = \frac{\ln[1 - c(1 + e.e._P)]}{\ln[1 - c(1 - e.e._P)]} \qquad E = \frac{\ln[(1 - c)(1 - e.e._S)]}{\ln[(1 - c)(1 + e.e._S)]}$$

$$E = \frac{ln\frac{[e.e._P\,(1 - e.e._S)]}{(e.e._P + e.e._S)}}{ln\frac{[e.e._P\,(1 + e.e._S)]}{(e.e._P + e.e._S)}}$$

$$E = Enantiomeric\ Ratio$$

$$c = conversion$$

$$e.e. = enantiomeric\ excess\ of\ the\ substrate(S)\ or\ the\ product\ (P)$$

Since the e.e. is a function of the conversion for kinetic resolutions and the Enantiomeric Ratio is relative to those values, the correlation between them can be visually depicted with a graph [15].

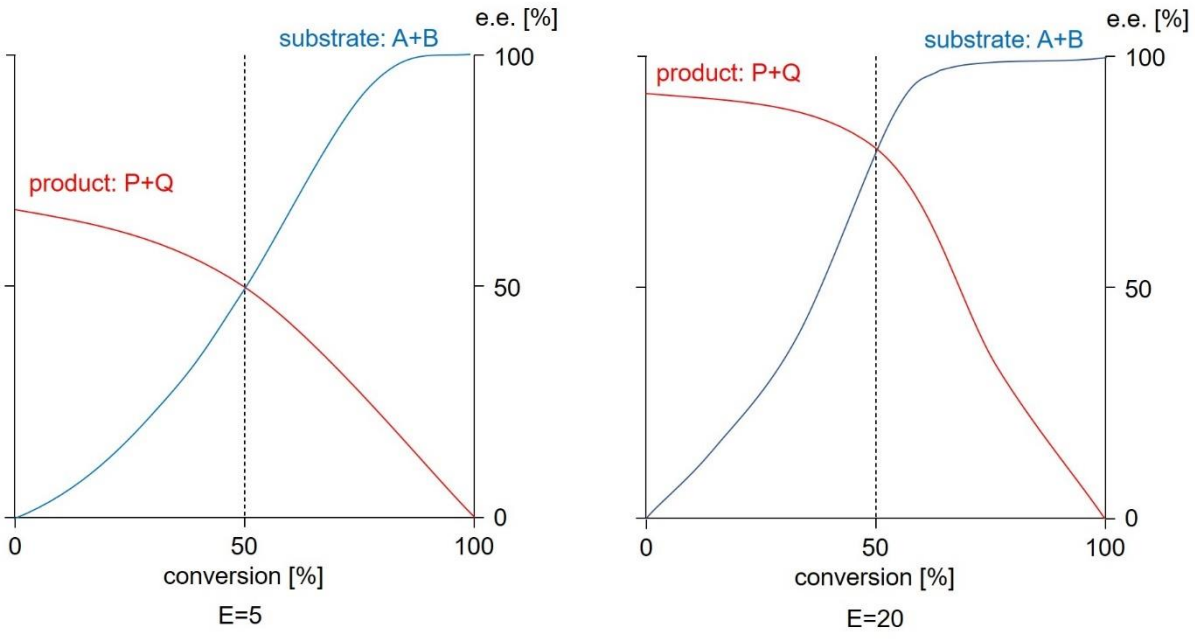


Figure 7: Comparison of kinetic resolutions with different Enantiomeric Ratios (left: E=5, right: E=20) in order to show the dependency of the optical purity on the conversion and the enantio-selectivity overall [15].

These functions can characterize the selectivity of an enzyme and the course of the reaction itself. For example, comparing two enzymatic resolutions with E=5 and E=20 (Figure 7) in a graph, the difference in enantio-selectivity is clearly depictable, due to the higher reachable optical purity with higher Enantiomeric Ratio. Another important characteristic of those graphs is, that the highest achievable optical purity for the product is obtained before 50% conversion

since the substrate enantiomers can freely compete for the active site. After the preferred enantiomer is transformed, the enzyme will also accept the "poor-fitting" enantiomer, as it is present in a relative high concentration. This leads to a drop in optical purity of the product after the enzyme has converted 50% of the substrate.

Contrary to that, the e.e. of the substrate increases steadily over the course of the reaction. The highest optical purity for the substrate can be reached after exceeding the reaction over around 60% conversion. Hence, in general, good $e.e._S$ can be obtained, but with a decreased yield. In order to achieve the ideal result of optical pure substrate and product with a yield of 50% conversion, the enantio-selectivity of the enzyme must be very high. E-values below 15 are usually not sufficient for practical purposes and above they are moderate to good. Enantiomeric Ratios beyond 200 cannot be precisely determined, since inaccuracies during measurement of the optical purity at this level can lead to drastic differences in the result [15].

2.2.2.2 Reversible Hydrolytic Reactions

In contrast to irreversible hydrolytic reactions more factors need to be encountered in the case of reversible reactions [46, 47]. In this type of biotransformation, the nucleophile which attacks the acyl-enzyme intermediate is not present in excess. Hence, the equilibrium constant K cannot be neglected in contrary to irreversible reactions. For reversible reactions the optical purity is a function of the conversion which is dependent on the enantio-selectivity and additionally it also correlates to K (Equation 3). This relation can again be expressed in form of an equation and can be visualized as a graph (Figure 8, Figure 9) [15].

Equation 3: Calculation of the E-value with the conversion of the reaction, the $e.e._P$, the $e.e._S$ and the equilibrium constant K.

$$E = \frac{\ln[1-(1+K)c(1+e.e._P)]}{\ln[1-(1+K)c(1-e.e._P)]} \qquad E = \frac{\ln[1-(1+K)(c+e.e._S\{1-c\})]}{\ln[1-(1+K)(c-e.e._S\{1-c\})]}$$

$$E = Enantiomeric\ Ratio$$
$$c = conversion$$
$$e.e. = enantiomeric\ excess\ of\ the\ substrate(S) or\ the\ product\ (P)$$
$$K = equilibration\ constant$$

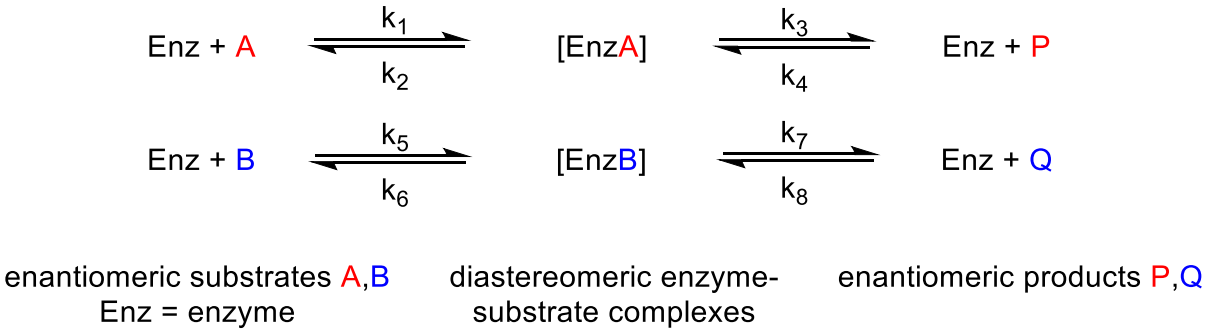


Figure 8: General reaction scheme of an enzymatic kinetic resolution for a reversible hydrolytic reaction [15].

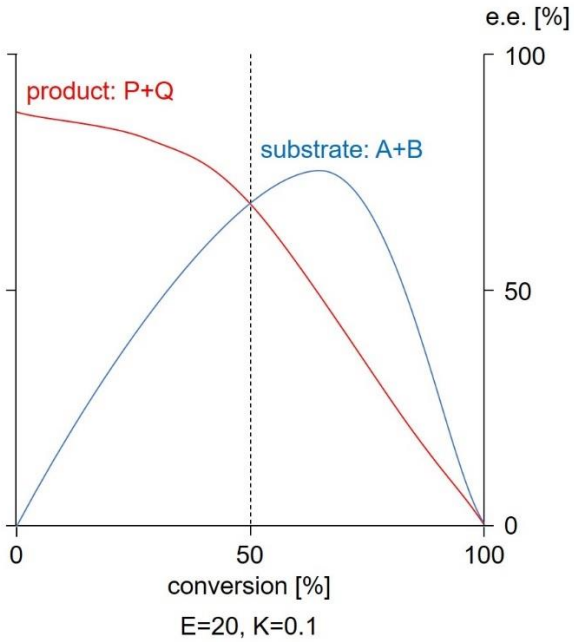


Figure 9: Function of the optical purity over the conversion for a reversible hydrolytic reaction wie E=20 and K=0.1 [15].

The function of the product as well as the first part of the substrate curve resembles the reaction course of irreversible reactions. Nevertheless, for higher concentrations (past 70%) major differences in the graphs can be depicted, due to the reverse reaction occurring. Since, the enantio-preference of the enzyme does not change over the course of the reaction the formed enantiopure product will be consequentially preferred for the reverse reaction as well. For example, if substrate A is preferred over B, it will be transformed into the corresponding product P quicker than product Q is formed until a certain conversion is reached. At this point the reverse reaction starts and predominantly formed product P is again preferred over Q by the enzyme. Hence, P will react faster back to A than Q to B. This leads to the conclusion, that the higher the conversion of the reaction the lower the optical purity of both the substrate and the product. Improvements of the final obtained enantiomeric purity are usually performed by shifting the reaction equilibrium, to be closer to an irreversible reaction. This can be achieved

by using the nucleophile in excess. About 20 M equivalents of the Nu in comparison of the substrate are required in order to receive equilibration constant K > 10 [15].

2.3 *Mycobacterium smegmatis* Acyl Transferase

Enzymatic acyl transfer is a widely used method in order to prepare various chiral compounds *via* kinetic resolutions [48, 49]. These reactions are commonly performed in organic solvent since biocatalysts like lipases can tolerate organic reaction medium. This is not only favorable for applications within organic synthesis (e.g. regarding substrate solubility), but also required to avoid concurrent hydrolysis. If enzymatic acyl transfer is performed in water (Figure 10), two nucleophiles (alcohol and water) compete for the enzyme-acyl-intermediate. Thus, the transesterification is reversible which can lead to decreased optical purity of both the substrate and the product and lower conversion overall. Additionally, the acyl donor can be hydrolyzed as well. This does not only lead to lower conversion, but also to a decrease in the pH due to the formation of acetic acid which can deactivate the enzyme [15, 50, 51].

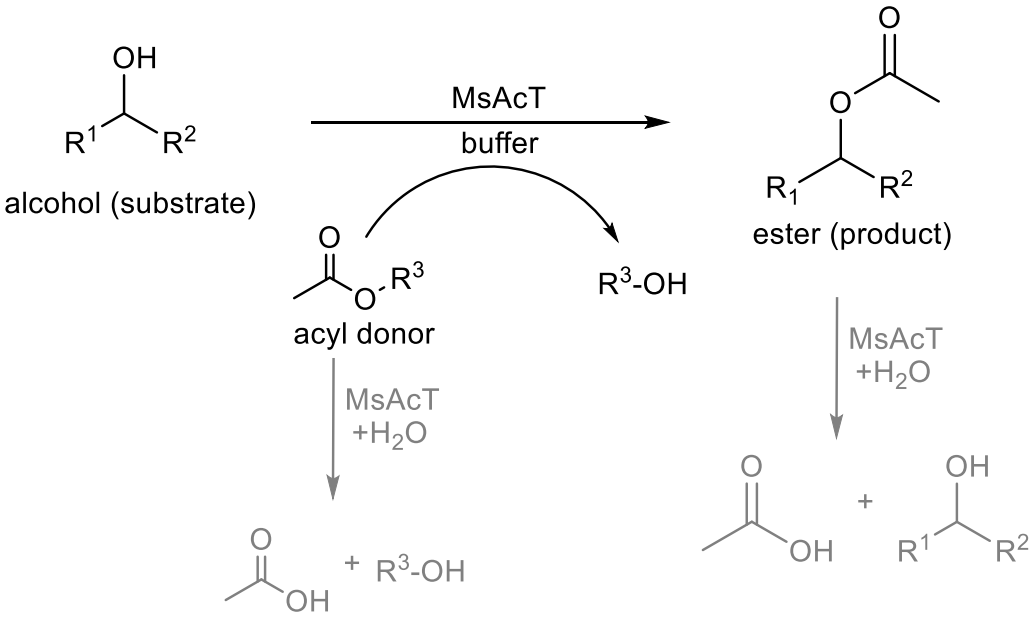


Figure 10: General reaction scheme for acyl transfer reactions in aqueous medium with reversible transesterification of an alcohol using an acyl donor and hydrolysis of the acyl donor as a side reaction.

Hence, a topic of great interest in biocatalysis remains the performance of acyl transfer reactions of alcohols in water. With a procedure as such, solvent use may be reduced and required protection and deprotection steps could be avoided. Therefore, biocatalysts that conduct these reactions in aqueous media would not only be more cost efficient but also reduce the environmental impact in regards of Green Chemistry. In 2007 an acyltransferase derived from *Mycobacterium smegmatis* was reported by Matthews *et al*. [16] to have the structural basis to perform preferentially esterification in water. The reaction selectivity derives from the fact, that

the enzyme is active as an octamer which results in a highly restricted reactive channel limiting accessibility of the active site for water. Additionally, this reactive channel has a hydrophobic nature disfavoring the access of water. These structural properties are the mechanistic basis to perform esterification over hydrolysis [16]. Since this report the MsAcT was successfully implemented in various transesterifications conducted in aqueous media (Figure 11) [52].

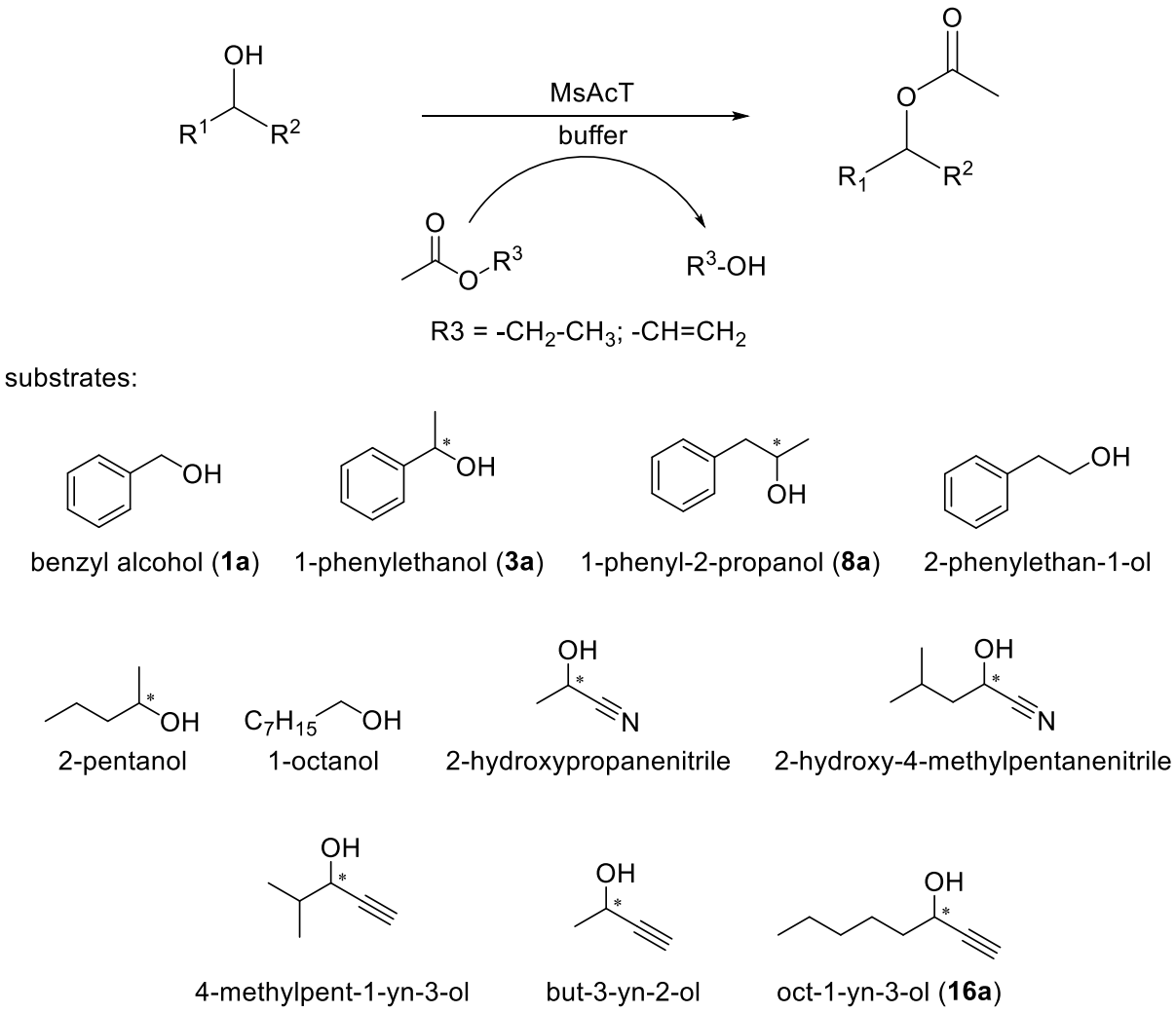


Figure 11: Overview of successfully transformed substrates by the MsAcT in aqueous medium from literature [52].

It has been shown that the biocatalyst accepts several primary and secondary alcohols. Aromatic and aliphatic alcohols, as well as cyanohydrins were transformed, and it can be used to perform kinetic resolutions. Though the selectivity of the wild type enzyme is only moderate depending on the reaction conditions [52]. Also a broad variety of acyl groups are accepted by the enzyme, ranging from the small acetyl-group to fatty acetyl groups [53]. Even though the main reaction is the acylation, the MsAcT also conducts hydrolysis of the product or the acyl donor, therefore the formed acid is one main by-product of the reaction. Consequently this enzyme is of great

interest, but further development and research is required in order to enhance substrate acceptance, enantio-selectivity and decrease hydrolysis as a side reaction [52].

2.3.1 Structure and Properties

The unique structural properties of the acyl transferase isolated from *Mycobacterium smegmatis* lead to the outstanding ability to perform preferably acyl transfer over hydrolysis in aqueous medium. The MsAcT consists of eight identical monomers with 216 amino acids per subunit, hence it is an octamer. Two of the monomers form tightly bound dimers and four of those substructures are assembled in a block like formation (Figure 12). The monomers each have five-stranded β-sheet structure sandwiched by α-helices on either side. This is characteristic for the SGNH hydrolase fold family. Though, the biggest difference between the MsAcT and other enzymes in this family, is that octamer formed in the MsAcT is extremely stable and is very unlikely to dissociate under operating conditions. Whereas other SGNH enzymes sometimes also form oligomers, they are much more likely to dissociate again and operate in a dimeric state rather than the assembled state. Hence, the tightly bound octamer of the MsAcT is a key characteristic in the selectivity of the enzyme, due to the highly restricted access into the active site of the enzyme [16].

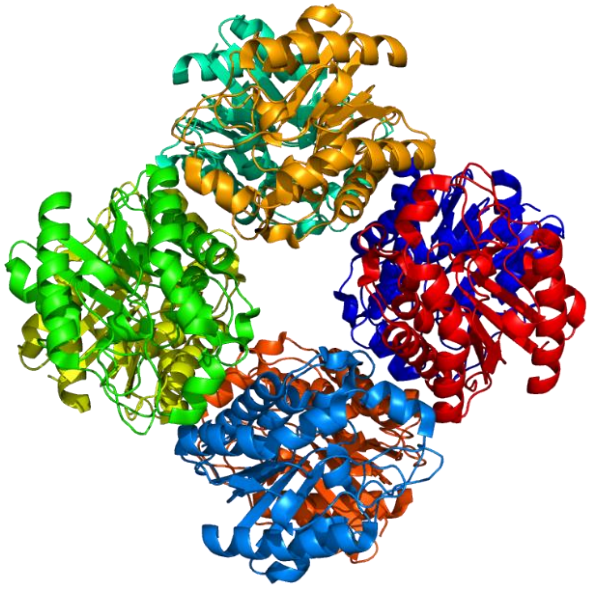


Figure 12: Block-like octamer structure of the four dimer subunits of the MSAcT (PDB code 2Q0S).

This restriction of access originates from a hydrophobic entrance channel as well. This channel leads from the surface of the protein to the active site and is formed due to the oligomerization of the monomers. The entrance tunnel constitutes of residues derived from three different monomers. Amino acids Trp16, Ala23, Pro24, Ala55, Trp149, Phe150, I153, Phe154 derive

from the first monomer and Val125 belongs to the dimer counterpart. Phe174 also is part of the channel and originates from a monomer of the neighboring dimer. The tight binding of the dimer and the added third monomer contribute all to the restricted access of the active site. Additionally, the residues are also rather hydrophobic. These factors might be the reason for the selectivity of the enzyme towards esterification over hydrolysis in water. The alkyl chains in the tunnel also indicate a direction for the substrate to the active site. Hence, the entrance channel binds or orients the substrate in a productive way. Since the MsAcT is a serine hydrolase the catalytic triad consists of a serine (Ser11), aspartic acid (Asp192) and histidine (His195) [16].

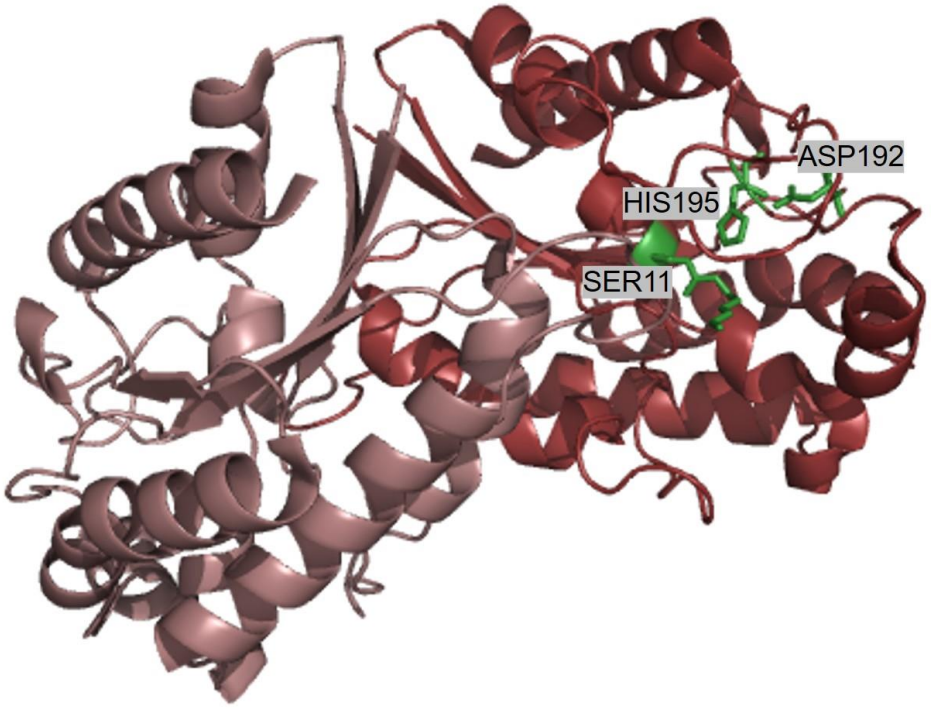


Figure 13: Structure of one dimer subunit (single monomers colored differently) of the MsAcT with the catalytic triad highlighted in green (Ser11, Asp192, His195) and bound inhibitor 4-nitrophenyl n-hexylcarbamat to the Ser11 (PDB code 2Q0S).

The functional moiety of the MsAcT is a serine, hence it follows the serine hydrolase mechanism (see section 2.2.1). This was confirmed *in silico* with DFT calculations. Consequently, the transesterification mechanism also consists of two reactions being the acylation of the enzyme with the acyl donor and the acyl-transfer on the substrate. Like other enzymes in the SGNH superfamily the MsAcT also includes an oxyanion hole which is made up of the amide groups Ser11 and Ala55 as well as the side chain of Asn94. This is required to stabilize the negative transition states with hydrogen bonds during both steps of the reaction. The binding of the substrate is also controlled by the geometry of the entrance channel. This

consist of a larger pocket (Ala55, Ser54, Lys97, Val125, Phe150, Ile153, Phe154, and Phe174) and a smaller pocket (Leu12, Thr93, and Ile194) which leads to a preferred orientation of the substrate [54].

2.3.2 Preference towards Esterification

Regarding the preference of the MsAcT towards esterification over hydrolysis one must keep in mind the kinetics of the reaction. Due to the presence of water during the acyl transfer the reaction itself is reversible (see section 2.2.2.2). Hence, hydrolysis of the acyl donor or the formed product can occur during the transesterification (Figure 10) [15].

Even though, acyl transfer is the preferred reaction by the enzyme, hydrolysis of the acyl donor and the product are the main side reactions. This cannot only lead to a drastic decrease in conversion, but also to a loss in activity of the enzyme due to the formation of carboxylic acid during the hydrolysis side reaction the pH of the reaction can drop dramatically. Since, the MsAcT operates ideally in slightly basic conditions (pH 7.5-8) activity decreases drastically the more acidic the reaction media becomes [51, 52]. Therefore, it is important to obtain a detailed understanding in the competition between esterification and hydrolysis as the main side reaction. Computational studies were performed to investigate the mechanism and specificities of the MsAcT. It was clearly found that the active site of the enzyme has a higher binding affinity towards alcohols than water, especially for substrates possessing an aromatic ring [54]. The reason for that is very likely to be the highly hydrophobic binding site and the restricted entrance tunnel to that [16].

The acyl donor itself seems to be crucial for the efficiency of the reaction as well. Donors like phenyl acetate and especially vinyl acetate, were found to increase the overall conversion vastly. In the case of vinyl acetate, the reason for that is, the irreversible tautomerization of the co-product to acetaldehyde in water that makes the reaction practically irreversible [55]. Different acyl donors were tested *in silico* to see if they can increase preference towards esterification, but they seem to have no effects on the enzyme specificity. This theoretical basis may, however, be used to predict beneficial mutations for more selective variants of MsAcT [54].

2.3.3 Enantio-selectivity

Another topic of interest in organic synthesis and industry in general is the preparation of optical pure products. Hence, in order to increase the applicability of the MsAcT the enantio-selectivity of this biocatalyst was investigated. An enantio-selectivity towards the (*S*)-2-octanol was determined. Even though the stereo-selectivity of the enzyme was not exceptional, it showed

that MsAcT might be applied for asymmetric synthesis, hence opening possibilities of stereoselective transesterification in aqueous media [51]. Further studies have been conducted implementing MsAcT successfully in kinetic resolutions of cyanohydrins and alkynols. Those two substrate groups were chosen, as they possess a similar structure, but different electronic properties. A switch from (*S*)-selectivity of MsAcT towards the alkynols to (*R*)-selectivity towards the cyanohydrins was observed indicating that the electronic properties can lead to changes in the stereo-selectivity of the biocatalyst. In general, the enantio-selectivity of MsAcT was only moderate with E values usually below 20. The selectivity could be significantly increased by applying different acyl-donors. With the use of phenyl acetate an E > 100 was obtained, but vinyl acetate and isopropenyl acetate also proved to be beneficial for increasing the stereo-selectivity [52].

However, the origin of enantio-selectivity of a biocatalyst is an important factor as well since the selection mechanism enables further knowledge about the enantio-preference and prediction for future enhancements of the enzyme. The exact source of enantio-selectivity of the MsAcT has been studied *in silico* by Kazemi *et al*. [56]. The relevant step for chiral recognition during the transesterification reaction is the second part of the hydrolytic mechanism in which the acyl-enzyme intermediate is attacked by an alcohol (nucleophile). The binding site of the MsAcT contains a small cavity (consisting of Leu12, Thr93 and Ile194) holding the acylated serine and a large cavity (consisting of Asp10, Trp16, Ser54, Ala55, Asn94, Lys97, Val125, Phe150, Ile153, Phe154 and Phe174) binding the attacking alcohol. The alcohol orients itself in a "head-in" conformation, meaning that the hydroxyl group is facing towards the acylated Ser11 residue. Due to this energetically preferred orientation the residues R and R' face either the "W-" or the "F-direction" of the binding site (Figure 14). The "W-direction" is named after the W16 residue. The "F-direction" is formed by Phe150, Phe154 as well as Ile153 and is named after the phenylalanine residues. Hence, different substrates point their residues either in one or the other direction giving rise to the enantio-preference of the MsAcT. This intrinsic knowledge of the binding site opens opportunities for enzyme engineering of the biocatalyst in order to further enhance the selectivity [56].

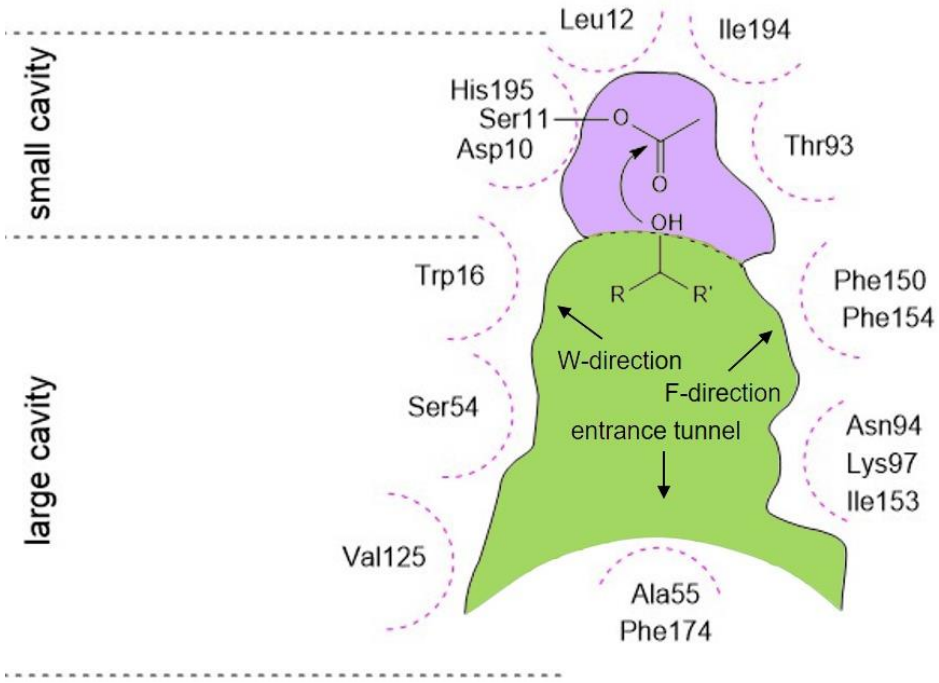


Figure 14: Schematic view of the binding site of the MsAcT with depiction of the small/ large cavity as well as the "W-direction", "F-direction" and the entrance channel for substrate orientation [56].

2.4 Aims of the project

2.4.1 Transesterification *vs*. Hydrolysis in MsAcT

The MsAcT was shown to be a biocatalyst of great interest due to its ability to perform esterification in aqueous medium. Still, the main side reaction remains hydrolysis which leads to various problems, all contributing to a poor overall productivity. Therefore, enzyme engineering provides an attractive method to further enhance the performance of the MsAcT [54]. The active site of the enzyme has been described to be quite restrictive and mostly hydrophobic which contributes to the unique selectivity of the acyltransferase [16]. This leads to the conclusion, that enhancing these properties may lead to a higher selectivity towards esterification over hydrolysis. Several mutation strategies were tested with computational simulation by M. Kazemi and F. Himo in the report of 11.06.2018 (unpublished results). For all the calculations benzyl alcohol (**1a**) was used as a substrate and EtOAc (**2**) as an acyl donor. First the active site was made more hydrophobic in order to destabilize the catalytic water required for hydrolysis. However, none of the variants were found to affect the water binding *in silico*, instead the mutants opened multiple binding positions for the alcohol and therefore suboptimal binding. In another strategy the introduction of a "gatekeeper-residue" into the active site was tested. A loop (residues 121-131, Figure 15) near the active site was identified to be a good candidate for mutagenesis, since it is flexible enough to proceed large structural

changes. A phenylalanine was introduced at its center in order to open the active site in the presence of an alcohol and close it in the absence of the substrate.

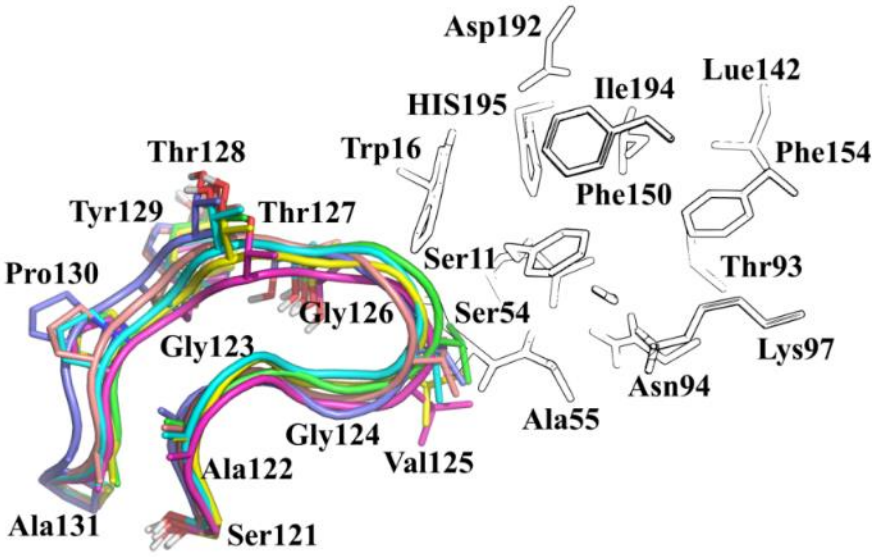


Figure 15: Loop for the introduction of a "gatekeeper-residue" consisting of residues 121-131 of the MsAcT from the computational simulations by M. Kazemi and F. Himo in the report of 11.06.2018 (unpublished results).

A set of simulations were performed, identifying the most promising mutations in the locations L (residues 121-131), S (residues 16 and 25), T (residue 150) and C (residue 54). Positions C (S54A) and T (F150N) were fixed, and L as well as S were modified leading to 14 variants of interest. Location S was modified in two ways, namely: S1 which consists of W16A and Thr25, as well as S2 consisting of W16M and T25S. For the loop itself several different variations were proceeded. (Table 1)

Table 1: Overview of all variants which were designed to increase preference of the MsAcT towards esterification over hydrolysis.

variation L1 in the loop		**variation L2 in the loop**	
variant	*sequence of location L*	*variant*	*sequence of location L*
L1aS1	SAGTAFGATYP	L2aS1	SAGPAFGTTYP
L1aS2	SAGTAFGATYP	L2aS2	SAGPAFGTTYP
L1bS1	SAGTAFGSTYP	L2bS1	SAGPGFGTTYP
L1bS2	SAGTAFGSTYP	L2bS2	SAGPGFGTTYP
L1cS1	SAGGAFGATYP	L2cS1	SAGPAFGSTYP
L1cS2	SAGGAFGATYP	L2cS2	SAGPAFGSTYP
L1dS1	SAGTGFGATYP		
L1dS2	SAGTGFGATYP		

To introduce these mutations, the genes were ordered and cloned into an appropriate vector to access the engineered enzymes. For the determination of the specificity of the MsAcT variants, a chromatographic activity assay was performed with GC measurements, using the same substrate (**1a**) and acyl donors (**2**) as in the computer simulations.

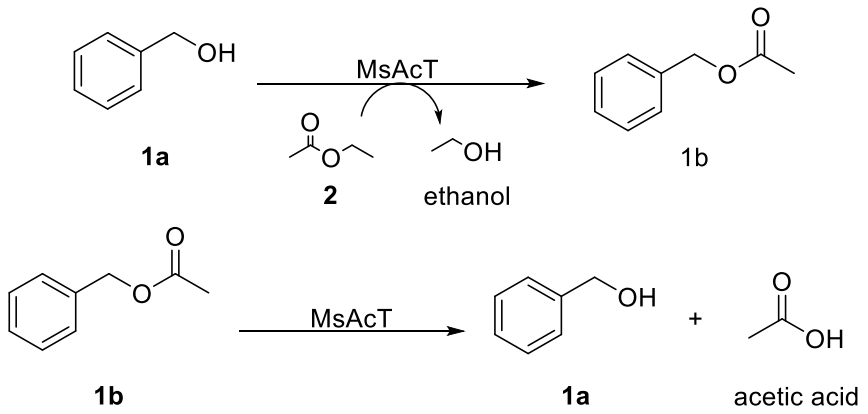


Figure 16: Transesterification and hydrolysis assay performed with MsAcT to test the in silico found variants for improved affinity towards acyl transfer over hydrolysis.

2.4.2 Stereo-selectivity of MsAcT

In order to enhance the applicability of the MsAcT, an increase of the enantio-selectivity would be desirable, since former studies showed only moderate selectivity of the biocatalyst (Table 2). Generally, lower conversions were obtained for larger, especially aromatic, substrates. For example, 1-phenylprop-2-yn-1-ol (**13a**) was not accepted at all by the WT MsAcT [52]. This indicates that the restricted active site of the MsAcT WT might not be ideally suited for larger substrates. Hence, enlarging the active site might increase the reactivity of this biocatalyst towards bulkier substrates. In a former enzyme engineering study an increase in activity towards larger diesters (divinyl adipate, octyl vinyl adipate, 4-(vinyloxy)butyl vinyl adipate) was achieved by decreasing bulk in the active site with the variants MsAcT T93A/F154A and L12A. These results suggest that a feasible option to enhance conversion for bulkier substrates is to decrease steric demand in the active site of the MsAcT [53]. Concerning enantio-selectivity data from literature, it suggests that the MsAcT WT is rather (*S*)-selective towards most substrates. A switch in enantio-preference towards the (*R*)-enantiomer was observed for cyanohydrin compounds. However, enantio-selectivity was quite low for most substrates tested with E-values ranging from 1 to 10. Hence, an improvement of enantio-selectivity would be favorable in order to obtain an applicable biocatalyst in kinetic resolutions [51, 52].

Table 2: Comparison of the results using different substrates from literature applied in kinetic resolutions with the MsAcT WT.

substrates	***conversion [%]***	***e.e.$_P$ [%]***	***E***
1-phenyl-2-ethanol (**3a**)[a]	19	14 (*S*)	1.4 (*S*)
1-phenyl-2-propanol (**8a**)[a]	7	81 (*S*)	10 (*S*)
2-octanol (**12a**)[b]	19 (estimated)	-	8 (*S*)
1-phenylprop-2-yn-1-ol (**13a**)[c]	-	-	-
oct-1-yn-3-ol (**16a**)[c]	23	-	3 (*S*)

*[a] Performed with acyl donor **2** (1 M), substrates **3a**, **8a** (100 mM), MsAcT (lyophilized CFE, 2 mg/mL) and IS dodecane (8 mM) in buffer (KPi, 200 mM, pH 7.5) with a final volume of 1 ml at 21 °C for 5 h (for substrate **3a**) or 2 h (for substrate **8a**)* [52].
*[b] Performed with acyl donor **2** (2.5 % v/v), substrates **12a** (100 mM) and MsAcT (lyophilized CFE, 2.5 µg/mL) in buffer (KPi, 50 mM, pH 8) at room temperature for 96 h* [51].
*[c] Performed with acyl donor **4** (1 M), substrates **13a**, **16a** (100 mM), MsAcT (lyophilized CFE, 2 mg/mL) and IS dodecane (50 mM)in buffer (KPi, 200 mM, pH 7.5) with a final volume of 1 ml at 21 °C for 5 h (for substrate **13a**) or 2 h (for substrate **16a**)* [52].

In this case, site directed mutagenesis presents a promising option to further improve the stereo-selectivity [51, 52, 56]. A set of computer simulations on feasible variants were performed by M. Kazemi and F. Himo in the report of 17.08.2018 (unpublished results) in order to enhance stereo-selectivity of the acyltransferase. These calculations were performed with 1-phenylethanol (**3a**) as a substrate and EtOAc (**2**) as an acyl-donor. Three locations were identified, which may lead to higher enantio-selectivity of the biocatalyst by decreasing or increasing bulk in those positions (Figure 17).

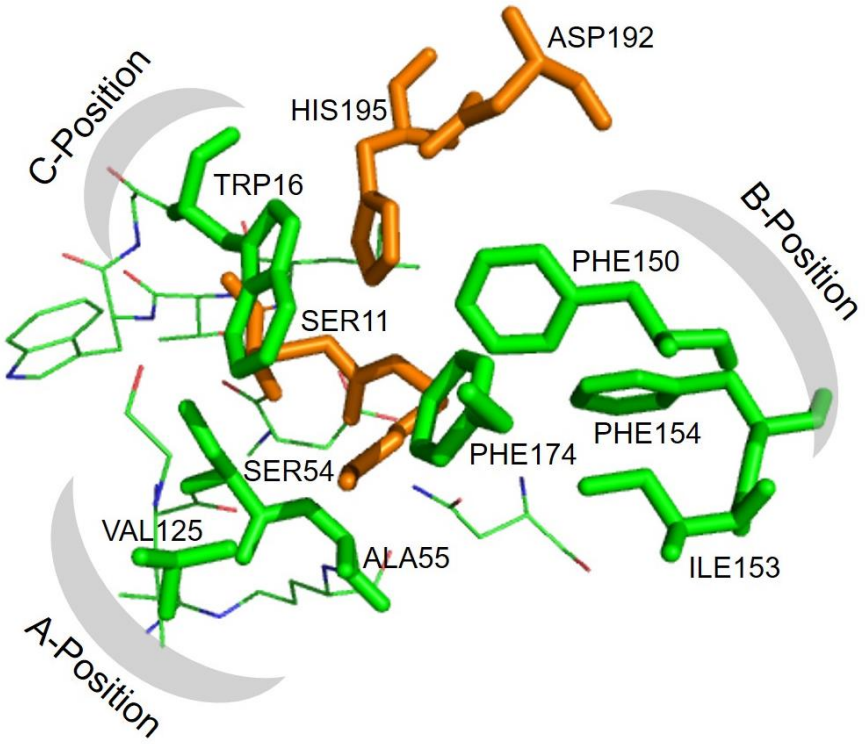


Figure 17: Positions in the active site of the MsAcT that may influence its stereo-selectivity MsAcT, identified in the computational simulations by M. Kazemi and F. Himo in the report of 11.06.2018 (unpublished results). Ser11 is bound to the inhibitor 4-nitrophenyl n-hexylcarbamat in this graph (PDB code 2Q0S).

A shift to a better binding of the (*R*)-isomer of **3a** may be accomplished by increasing bulk in positions A (correlates to the entrance channel see section **Fehler! Verweisquelle konnte nicht gefunden werden.**) and C (correlates to "W-direction" see section **Fehler! Verweisquelle konnte nicht gefunden werden.**) and d ecreasing it in position B (correlates to "F-direction" see section **Fehler! Verweisquelle konnte nicht gefunden werden.**). A shift to the (*S*)-isomer of **3a** may be accomplished by increasing bulk in position B and decreasing it in positions A and C. (Table 3)

Table 3: Overview of all the variants which were designed to increase enantio-selectivity of the MsAcT towards either the (R)- or (S)-enantiomer

higher affinity towards *R*-isomer		**higher affinity towards *S*-isomer**	
increasing bulk in position A and C	*decreasing bulk in position B*	*increasing bulk in position B*	*decreasing bulk in position A and C*
A55S	F150V	I153M	W16F
A55V	F150A	F154W	W16V
S54M	F154V	F154L	W16A
V125L	F154A	F150W	
V125F	F174V	F150L	
	F174A	F174W	
	I153A	F174L	
		I153F	

Since these variants show only a single mutation, they can be readily obtained *via* site-directed mutagenesis and therefore, this was be the method of choice. The engineered MsAcT enzymes were tested in the biotransformation which was analyzed by chromatography (GC) on a chiral phase.

3 Results and Discussion

3.1 Engineering MsAcT for Increased Transesterification- *vs*. Hydrolysis-Activity

3.1.1 Cloning, Expression and Purification of MsAcT and its Variants

Several variants were identified *via* computational simulations by M. Kazemi and F. Himo in the report of 11.06.2018 (unpublished results), which are intended to improve preference towards transesterification over hydrolysis of the MsAcT. The gene sequences of those variants with the desired mutations were ordered and cloned into pET-28a(+) vector with an N-terminal His6-tag between the restriction sites NdeI and XhoI. All variants were successfully cloned and transformation in the expression host *E. coli* BL21 (DE3) cells was performed.

The recombinant *E. coli* BL21 (DE3) cells containing MsAcT wild type and its variants were overexpressed in a small-scale cultivation with consecutive purification using His SpinTrap TALON Spin Column. Overexpression and purification were successful for the wild type and all variants except for MsAcT variant L1aS2 which seems to not have overexpressed properly.

3.1.2 Spectrophotometric Activity Assay of MSAcT and its Variants

In order to measure the differentiation of the wild type MsAcT and its variants between hydrolysis and the preferred esterification, a spectrophotometric activity assay was established. Since, neither the substrate benzyl alcohol (**1a**) nor the product benzyl acetate (**1b**) is detectable in UV/Vis, a coupled assay with a photo-detectable process was required. Coupling of the reaction with the oxidation of the (co-)product (alcohols) with a nicotinamide-dependent alcohol dehydrogenase (ADH) would allow to follow the production of the co-product by measuring formation of NAD(P)H [55]. In the case of the hydrolysis of **1b** the formed alcohol **1a** was be oxidized by a NAD(P)H dependent ADH to benzaldehyde. During the transesterification **1a** is acetylated with ethyl acetate (**2**) acting as an acyl-donor. Hence, ethanol is formed during the esterification, which can be oxidized by an NAD(P)H dependent ADH. The problem regarding this reaction is, that two different alcohols are present in this specific reaction mixture. Hence, a selective ADH is required. In both assays $NAD(P)^+$ is consumed by an ADH, leading to the formation of NAD(P)H over time which can be photometrically measured and indicates the kinetics of the MsAcT indirectly, assuming the transesterification reaction is the rate determining step. YADH1, derived from *Saccharomyces cerevisiae*, was found to be a suitable enzyme for the acyl transfer reaction, since its highly selective towards ethanol [57]. HL ADH derived from bovine liver was implemented for the hydrolysis activity assay of **1b** (Figure 18) [58].

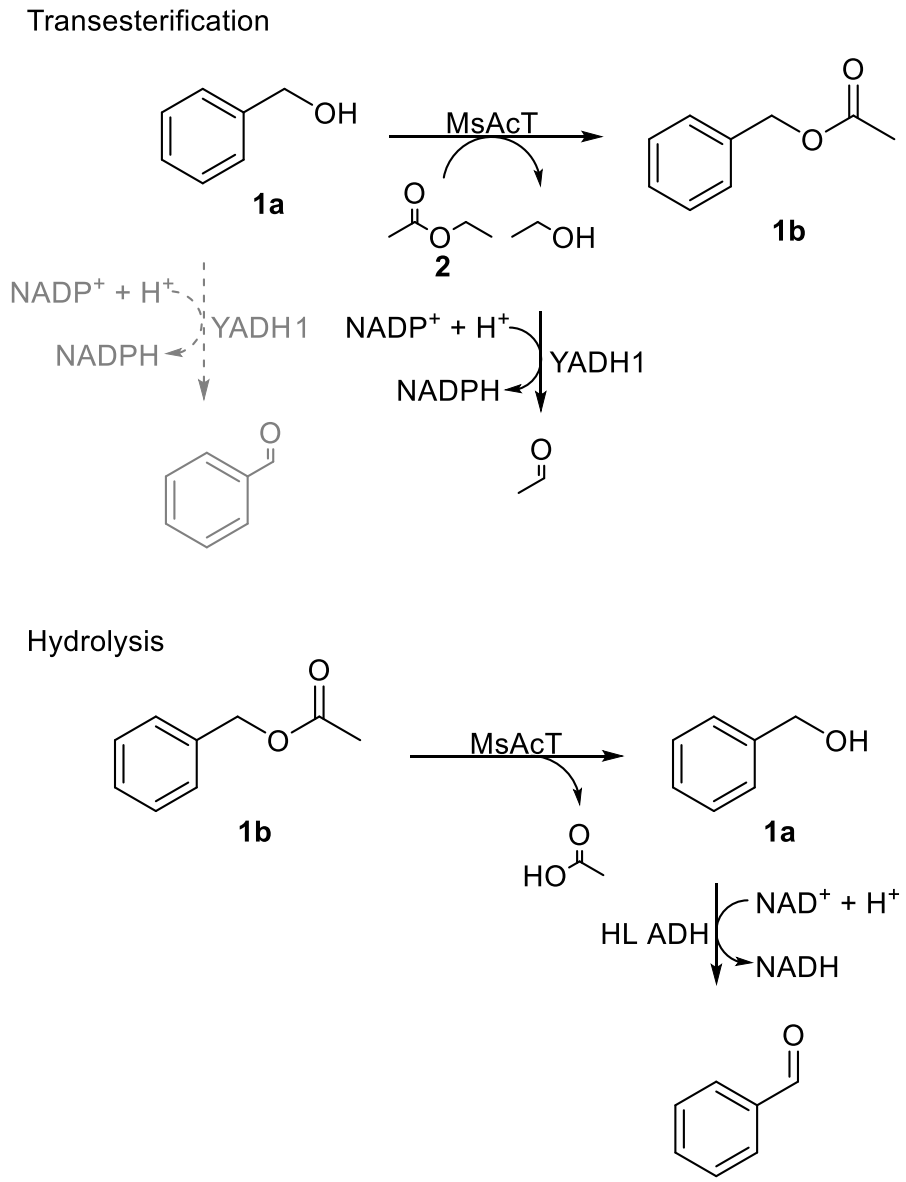


*Figure 18: Coupled spectrophotometric assay for the esterification of **1a** and for the hydrolysis of **1b** using MsAcT (WT and variants) as a biocatalyst, **2** as an acyl donor, YADH1 for oxidation of the formed ethanol and HL ADH for the oxidation of formed **1a** to produce the spectrophotometric analyte NAD(P)H.*

Even though the YADH1 performed a highly selective oxidation of ethanol over **1a**, when it was combined with MsAcT, hardly any activity was detectable anymore. The reason for that could be either other side reactions, or that the differences in the reaction rates of the acylation and the oxidation are too insignificant for the coupled assay to operate accurately. However, due to this difficulty the spectrophotometric assay could not be applied for a systematic screening of the MsAcT variants.

3.1.3 Gas-Chromatographic Activity Assay

Since, the spectrophotometric assay was not applicable for the screening of the MsAcT WT and variants, another method was implemented as follows. Biotransformations on a 1 mL scale were stopped at different time points and analyzed on GC with an internal standard (1-decanol) in

order to obtain the conversion as a measure of reactivity of the enzymes. With this the variants preference towards alcohol or water as a nucleophile can be compared with one another and the wild type itself.

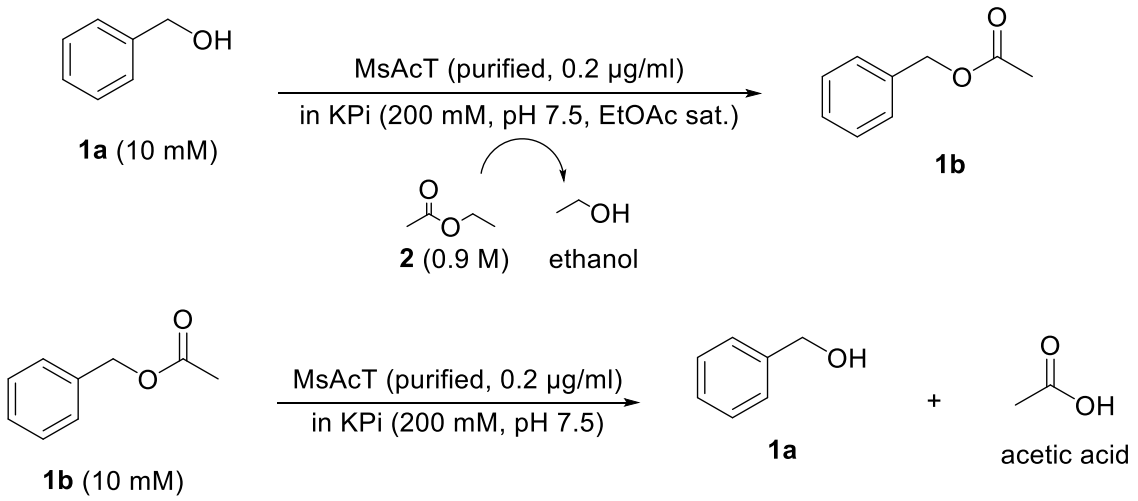


*Figure 19: Reaction scheme of acyl transfer and hydrolysis reaction performed with MsAcT for the determination of the reactivity of the MsAcT WT and its variant towards water or **1a** as nucleophile..*

3.1.3.1 Time Study of MsAcT Wild Type Enzyme

A time study with the purified WT MsAcT performing esterification was done to serve as a benchmark of the reactivities that will be measured with the variants. The time study for the acyl transfer reaction was performed with purified enzyme solution (0.2 µg/mL), **1a** (10 mM) as a substrate and **2** (0.9 M) as an acyl donor in buffer (KPi, 200 mM, pH 7.5, EtOAc sat.) at 30 °C for a time span of up to 26 h.

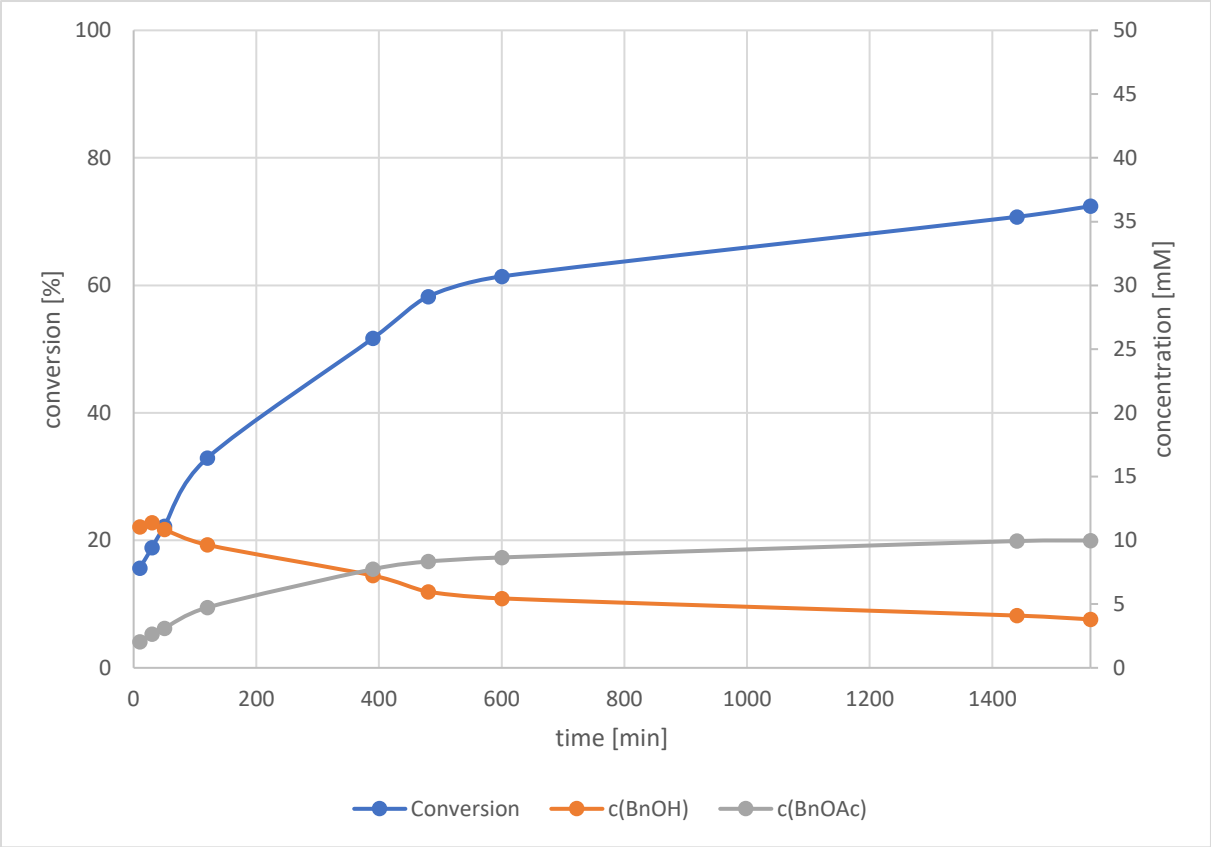


*Figure 20: Time study of the transesterification of **1a** (10mM) with the WT MsAcT (purified enzyme solution, 0.2 µg/mL) with **2** (0.9 M) as an acyl donor in buffer (KPi, 200 mM, pH 7.5, EtOAc sat.) at 30 °C for a time span of up to 26 h and measured on the GC-FID. Values shown in the graph are the average of three reactions.*

The time study (Figure 20) clearly showed that in the beginning of the reaction the esterification defines the overall reaction velocity and approximately after 24 h an equilibrium between the esterification and hydrolysis is reached and the reaction is also influenced by the transesterification and the hydrolysis. If both reactivities (transesterification and hydrolysis) would be determined, the preference of the enzyme towards acyl transfer could be calculated. In order to obtain this data, all variants were screened in the transesterification-reaction and the hydrolysis and at four representative time points (10 min, 30 min, 50 min, 24 h).

3.1.3.2 Reactivity of the MsAcT Variants in Transesterification and Hydrolysis

The acyl transfer was performed with purified enzyme solution (0.2 µg/mL), **1a** (10 mM) as a substrate and **2** (0.9 M) as an acyl donor in buffer (KPi, 200 mM, pH 7.5, EtOAc sat.) at 30 °C and the reactions were stopped at 10 min, 30 min, 50 min and 24 h. Hydrolysis was performed with purified enzyme solution (0.2 µg/mL) and **1b** (10 mM) as a substrate in buffer (KPi, 200 mM, pH 7.5) at 30 °C and reactions were stopped at 10 min, 30 min, 50 min and 24 h. Both screenings were analyzed on GC-FID.

Screening of the MsAcT variants performing transesterification led to no conversion for any of the variants or time points. Regarding the screening performing hydrolysis, conversion could only be obtained for the 24 h reaction samples of the MsAcT variants (Table 4).

*Table 4: Hydrolysis of **1b** with the MsAcT variants.*

variant	*mutations*	*conversion [%]*
L1aS1	W16A, S54A, G124T, V125A, 126F, T128A, F151N	28
L1aS2*	W16M, T25S, S54A, G124T, V125A, 126F, T128A, F151N	-
L1bS1	W16A, S54A, G124T, V125A, 126F, T128S, F151N	49
L1bS2	W16M, T25S, S54A, G124T, V125A, 126F, T128S, F151N	30
L1cS1	W16A, S54A, V125A, 126F, T128A, F151N	34
L1cS2	W16M, T25S, S54A, V125A, 126F, T128A, F151N	40
L1dS1	W16A, S54A, G124T, V125G, 126F, T128A, F151N	28
L1dS2	W16M, T25S, S54A, G124T, V125G, 126F, T128A, F151N	28
L2aS1	W16A, S54A, G124P, V125A, 126F, F151N	33
L2aS2	W16M, T25S, S54A, G124P, V125A, 126F, F151N	40
L2bS1	W16A, S54A, G124P, V125G, 126F, F151N	41
L2bS2	W16M, T25S, S54A, G124P, V125G, 126F, F151N	49
L2cS1	W16A, S54A, G124P, V125A, 126F, T128S, F151N	28
L2cS2	W16M, T25S, S54A, G124P, V125A, 126F, T128S, F151N	34
WT	-	100

*The reactions were performed with purified enzyme solution (0.2 µg/mL) and **1b** (10 mM) as a substrate in buffer (KPi, 200 mM, pH 7.5) at 30 °C for 24 h and measured on the GC-FID. *Variant L1aS2 was not overexpressed properly and enzyme concentrations could not be determined in a significant way, hence the obtained loss of activity could derive from expression and purification difficulties. Values shown in the table are the average of three reactions.*

The aim of this project was to increase the preference of MsAcT variants toward transesterification by hindering the entrance of water into the active site and thereby lowering activity of the MsAcT variants towards hydrolysis. While a decrease in the activity towards hydrolysis was obtained for all variants, no activity during transesterification of **1a** was found. This leads to the conclusion that the desired increase in preference towards esterification could not be obtained and it is very likely that the mutations led to a loss in overall activity of all the variants. To test this, two variants (L2bS1, L2cS1) were applied in acyl transfer reactions with higher substrate (**1a**, 100 mM) and enzyme concentration (6 µg/mL and 30 µg/mL). After 24 h

a maximum conversion of only 16 % could be obtained for L2bS1 (with use of 30 µg/mL). In the first screening (**1a**/**1b** 10 mM, enzyme concentration 0.2 µg/l) a conversion of 41 % for hydrolysis of the same variant was observed. In comparison with the WT MsAcT (**1a**/**1b** 10 mM, enzyme concentration 0.2 µg/l) 26% conversion for acyl transfer in comparison to 10% conversion for hydrolysis were observed after 50 min and up to 73% conversion for transesterification after 24 h. That clearly indicates that the WT MsAcT has a higher affinity towards transesterification over hydrolysis in aqueous medium and a higher activity in general than the tested variants. However, the variants are still able to perform acyl transfer, but the overall activity and preference towards acyl transfer is dramatically decreased. These results lead to the conclusion that the variants are not suitable for further application. The desired effect of an increase of preference towards acyl transfer in comparison to hydrolysis could not be observed.

3.2 Engineering of MsAcT for Higher Activity and Enantio-Selectivity

3.2.1 Site-Directed Mutagenesis and Expression

In order to enhance the enantio-selectivity of MsAcT, enzyme engineering provides an attractive opportunity to further optimize the enzyme. Especially site directed mutagenesis is appealing in this case, since specific amino acid residues of interest in the active site can be targeted directly. Therefore, this approach was chosen to obtain the desired variants of the MsAcT. Those mutants were selected based on computational simulations by M. Kazemi and F. Himo in the report of 17.08.2018 (unpublished results) and on structural visualization of the active site. Identified variants with improved enantio-selectivity were successfully obtained with site directed mutagenesis *via* PCR starting from recombinant WT MsAcT DNA in a pET-28a(+) vector. The mutants were transformed in the expression host BL21(DE3). Cell lysis was performed with the overexpressed variants for partial purification and the CFE was lyophilized. All variants were successfully obtained.

3.2.2 Screening of the Variants

Former studies showed only moderate enantio-selectivity of the MsAcT-WT [51], [52]. Hence, site-directed mutagenesis based on previous *in silico* modelling by M. Kazemi and F. Himo in the report of 17.08.2018 (unpublished results) was chosen to further improve the stereo-selectivity of the MsAcT. Three main positions in the active site were identified and by decreasing or increasing bulk in those positions by the chosen variants enantio-preference might be improved or shifted (Table 3). Therefore, an opening in “A-position” or “C-position” or an increase of bulk in “B-position” would lead to a preference of (*S*)-**3a** whereas lower steric

demand in “B-position” or increased bulk in “A-position” or “C-position” would enhance selectivity towards (*R*)-**3a**. Since in the computational study compounds **3a** and **3b** were applied as benchmark substrates and aiming for comparable data, the variants that were identified *in silico* were tested using the same model substrate **3a** for acyl transfer and corresponding ester **3b** for hydrolysis using **2** as an acyl donor.

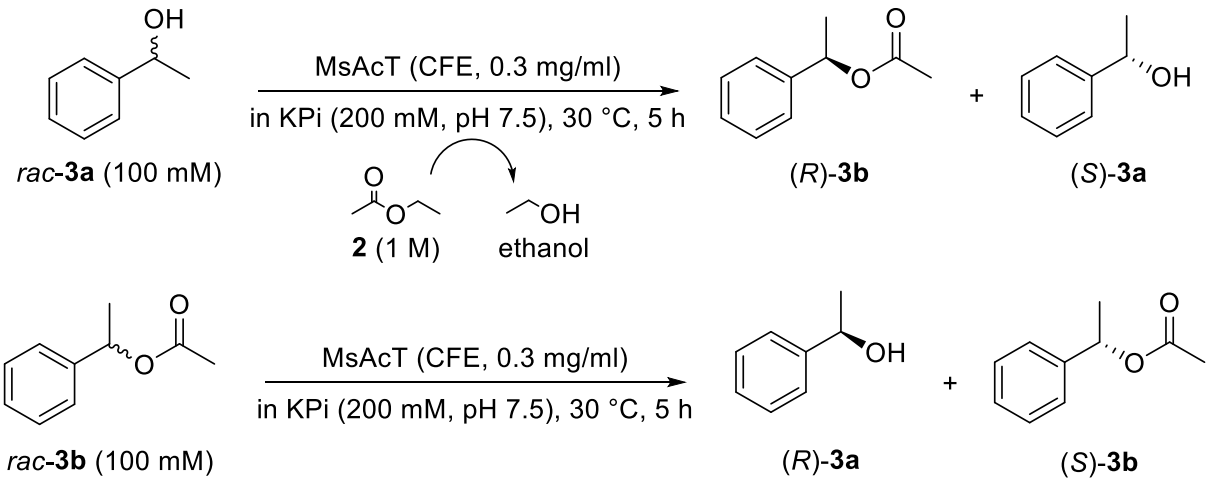


*Figure 21:Benchmark reaction for the test of the enantio-selectivity of MsAcT WT and its variants: For the acyl transfer, substrate **3a** was tested with the acyl donor **2**. For the hydrolysis substrate **3b** was applied.*

3.2.2.1 Transesterification

The transesterification screenings were performed with lyophilized cell free extract (CFE, 0.3 mg/mL), *rac*-phenylethanol **3a** (100 mM) as a substrate and ethyl acetate **2** (1 M) as acyl donor in buffer (KPi, 200 mM, pH 7.5) at 30 °C for 5 h.

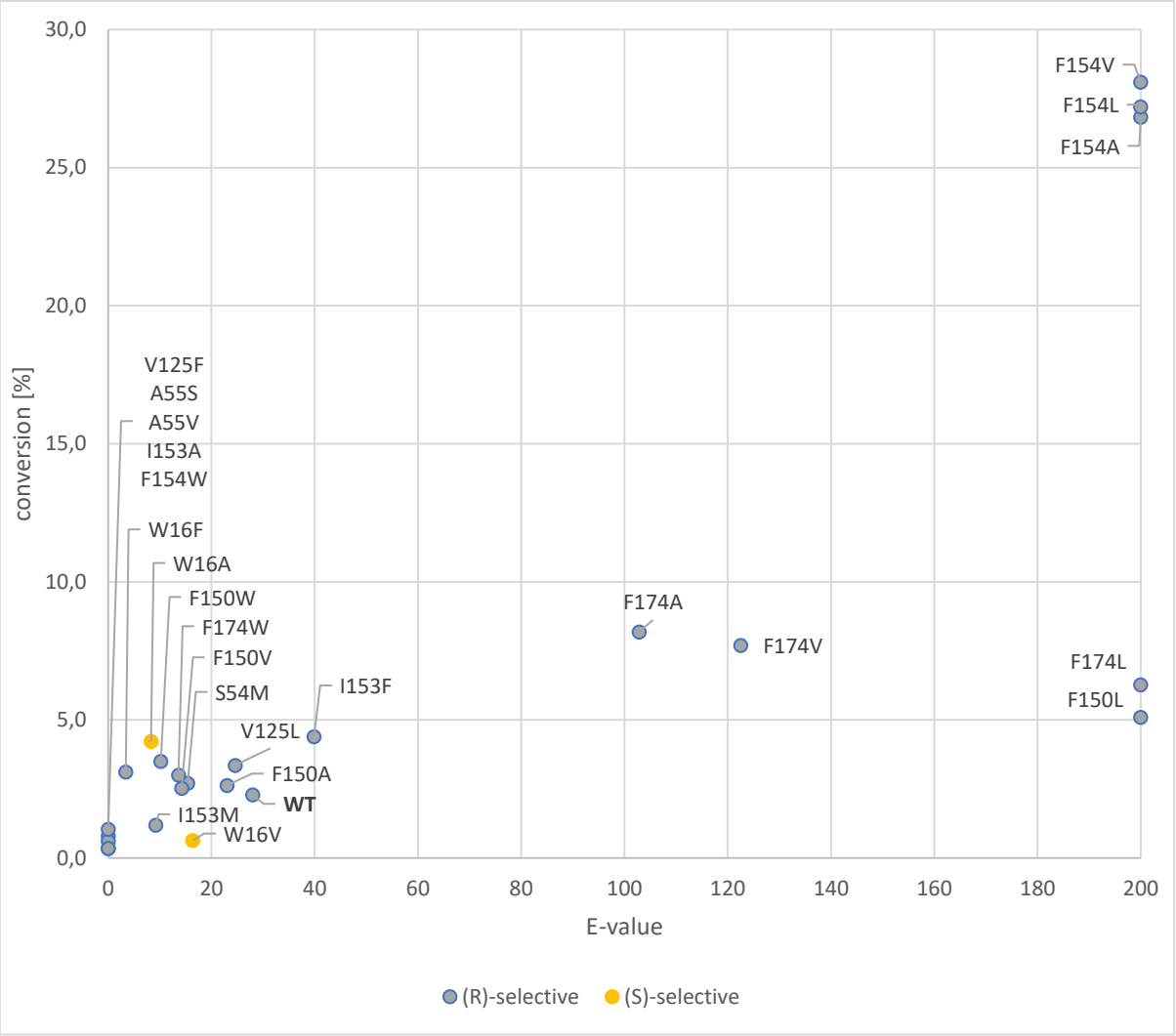


*Figure 22: Transesterification of **3a** with the MsAcT variants. The reactions were performed with lyophilized cell free extract (CFE, 0.3 mg/mL), **3a** (100 mM) as a substrate and **2** (1 M) as an acyl donor in buffer (KPi, 200 mM, pH 7.5) at 30 °C for 5 h. The E-value was calculated from e.e.P and e.e.S. Below 5% conversion the E-value was not determined (n.d.) for some variants. Values shown in the graph are the average of three reactions.*

With these screenings a few variants of interest and mutation hot spots could be determined. Good conversion and excellent selectivity were obtained for MsAcT F154L, F154V and F154A. Moderate conversion and good selectivity were observed for MsAcT F174A, F174V, F174L and F150L. All positive variants decreased the bulk in the active site. This eases the access of the substrate and thereby increases activity. Additionally a reduction in bulk in the "B-position" (Figure 14) enhanced the preference for the (*R*)-enantiomer of substrate **3a**. These results are in contrast to previous work, where it has been described that the MsAcT is selective towards the (*S*)-**3a** [52]. However, in this previous study as well as in the work herein conversions were quite low and the selectivity of the WT MsAcT is in general poor which makes the data not necessarily significant.

A clear shift to the opposite enantio-selectivity of the WT MsAcT could be determined for W16V and W16A, both opening space in the "A-/C-position" (Figure 14) of the active site.

Even though the activity and selectivity of these variants were only moderate, a switch in stereo-selectivity with only one amino acid substitution is remarkable. These variants may serve as good starting point to further enhance those enzymes with inverted stereo-selectivity. Since these residues are in the "C-position" (Figure 14) further decrease of bulk in this region may lead to a stronger shift of enantio-preference towards (*S*)-enantiomer of the substrate **3a**/**3b**.

3.2.2.2 Hydrolysis

In addition to the transesterification, all MsAcT variants were tested in the hydrolysis direction. The screenings were performed with lyophilized cell free extract (CFE, 0.3 mg/mL), **3b** (100 mM) as a substrate and **2** (1 M) as a co-solvent in buffer (KPi, 200 mM, pH 7.5) at 30 °C for 5 h.

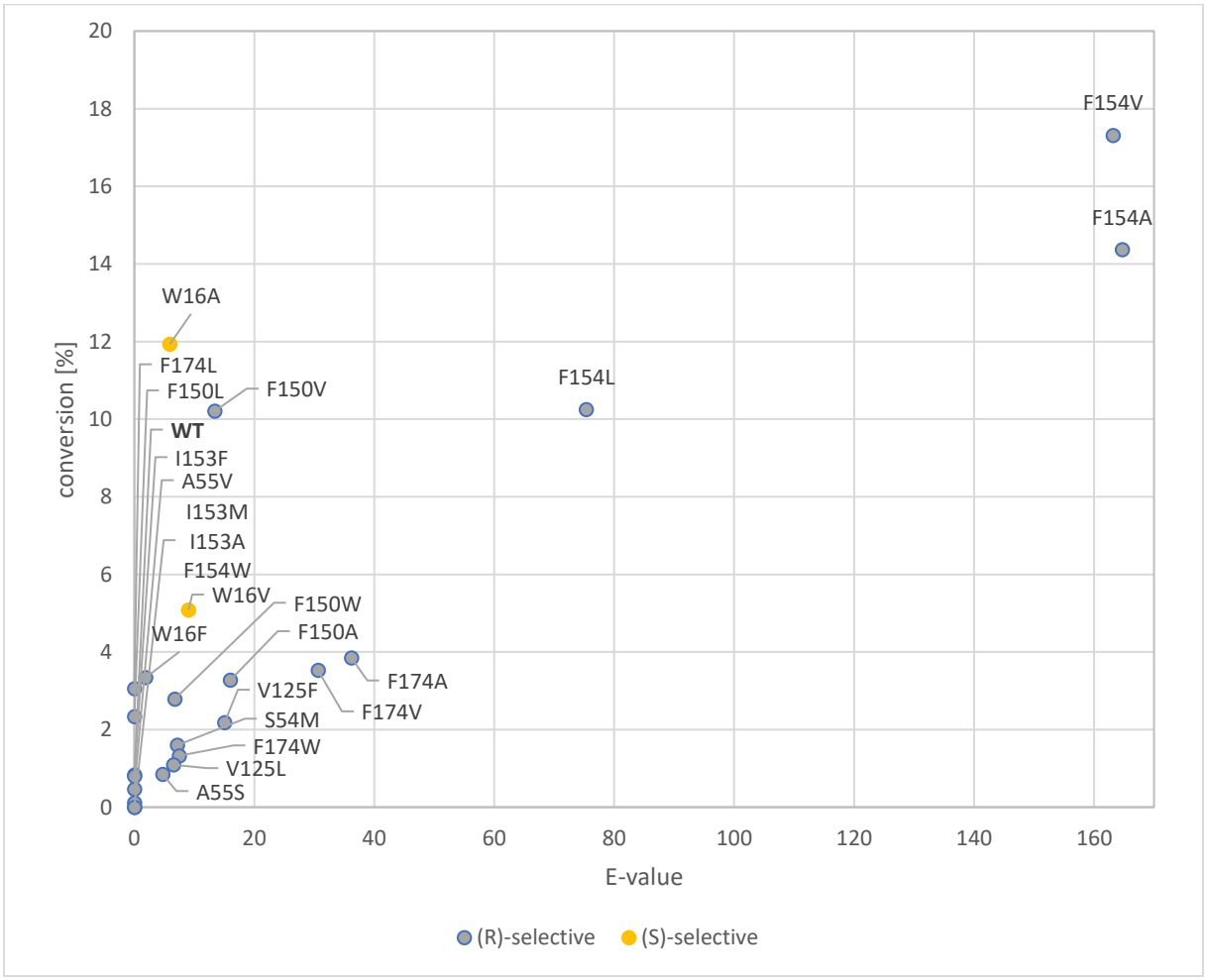


*Figure 23: Hydrolysis of **3b** with the MsAcT variants. The reactions were performed with lyophilized cell free extract (CFE, 0.3 mg/mL), **3b** (100 mM) as a substrate and **2** (1 M) as a co-solvent in buffer (KPi, 200 mM, pH 7.5) at 30 °C for 5 h. The E-value was calculated from e.e.$_P$ and e.e.$_S$. Below5% conversion the E-value was not determined (n.d.) for some variants. Values shown in the graph are the average of three reactions.*

The variants show similar results for the hydrolysis reaction, as for the acyl transfer reactions. However, overall conversion is decreased in this case. This can be expected since the MsAcT

is reported to favor transesterification over hydrolysis in aqueous medium. Enantio-selectivity is dramatically decreased as well. The reason for that could be the presence of **2**, because the hydrolyzed substrate can consecutively undergo acyl transfer reaction. The most active and selective variants were again MsAcT F154A, F154V, F154L. MsAcT W16A, F150V also showed moderate to good activity, but lower enantio-selectivity. Again, variation of position 16 triggered a switch in enantio-preference in comparison to the WT.

The overall goal of the project was to improve the enantio-selectivity of MsAcT for acyl transfer reactions. This was especially achieved with the variants F154A and F154V, but also MsAcT F154L, F174A and F174V showed far superior properties in comparison to the WT enzyme. Since, all these enzymes have also lower activity towards hydrolysis they seem to prefer acyl transfer in aqueous medium and are still applicable for this reaction. Beneficial as well as unfavorable positions for mutagenesis were determined (Figure 24). The experimental tendencies for preference towards (*R*)- or (*S*)-enantiomers of the substrates according to the "B-position" (Phe154, Phe174) or "C-position" (Trp16) seem to correlate with the *in silico* data by M. Kazemi and F. Himo in the report of 17.08.2018 (unpublished results). However, mutation of the "A-site" did not result in benefits according to activity and enantio-preference. Nevertheless, a decrease in bulk in the entrance tunnel of the enzyme appears to be favorable in case of the position Phe174. Since the mentioned variants showed good to excellent enantio-selectivity and moderate to good activity some of them will be further tested with other substrates.

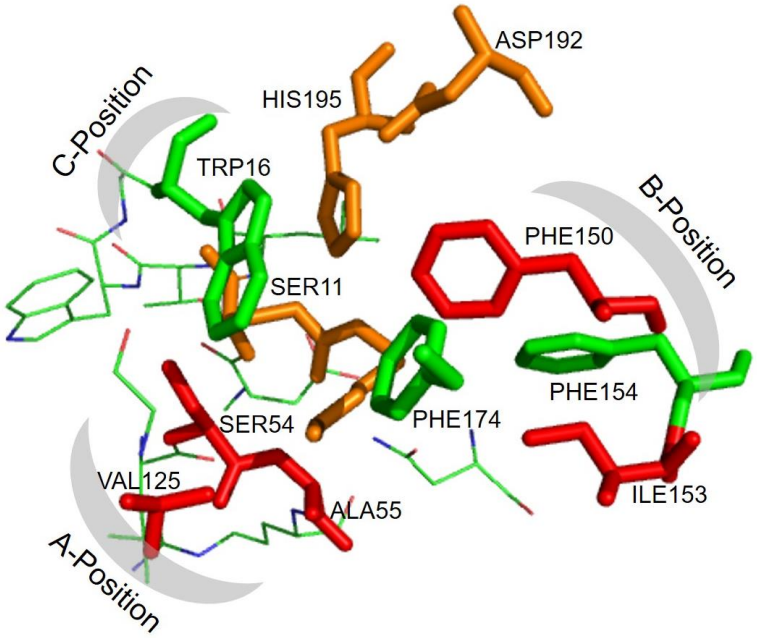


Figure 24: Active site of the MsAcT (PDB 2Q0Q) with highlighted mutation hot spots beneficial for enhanced enantio-selectivity and activity (green), unfavorable residues for mutagenesis indicated in red and the catalytic triad shown in orange. Ser11 is bound to the inhibitor 4-nitrophenyl n-hexylcarbamate in this graph.

The positions of interest (Phe154, Phe174, Trp16) were also tested *in silico* by M. Kazemi and F. Himo in the report of the 17.10.2019 (unpublished results). The valine variants of the mentioned positions were calculated and the rate-determining step TS2 was further investigated. In case of the F154V variant it was found that the change from the phenylalanine to the smaller residue (valine in the calculation) creates a new cavity which can accommodate the phenyl moiety of the substrate. This increases the preference for the (*R*)-enantiomer of **3a** since it lowers the energy of its transition state. In case of the (*S*)-configured substrate, steric repulsion occurs with the phenyl moiety of the substrate and the residues Trp16 as well as Ala55. This makes this specific configuration energetically unfavorable. For the MsAcT W16V, the exact opposite applies: The absence of the bulky tryptophan provides a cavity to accommodate the phenyl-moiety of the substrate **3a**, while in the (*R*)-configuration the phenyl groups of the "B-position" and the substrate clash. This leads to an energetical preference towards the (*S*)-enantiomer. Regarding the F174V mutant the vacant phenylalanine residue allows the Phe150 to rotate and create space in the "B-position". Hence, the (*R*)-configuration of the substrate fits the active site better than the (*S*)-enantiomer. However, the energy difference of the two transition states with this variant is not as significant as in the case of the F154V variant. This was also recognizable in the screening since, the Phe154-variants showed far higher selectivity. Thus, the results of the computational study resemble the experimental data in all aspects.

3.2.3 Reaction Engineering with MsAcT

3.2.3.1 Reaction Conditions

For further application of the MsAcT, different reaction conditions were tested in order to optimize the biotransformations. The first set of reactions was performed with varying enzyme concentrations of the WT MsAcT (lyophilized CFE, concentrations as indicated), **3a** (100 mM) as a substrate and **2** (1 M) as the acyl donor in buffer (KPi, 200 mM, pH 7.5) at 30 °C for 5 h (Figure 25).

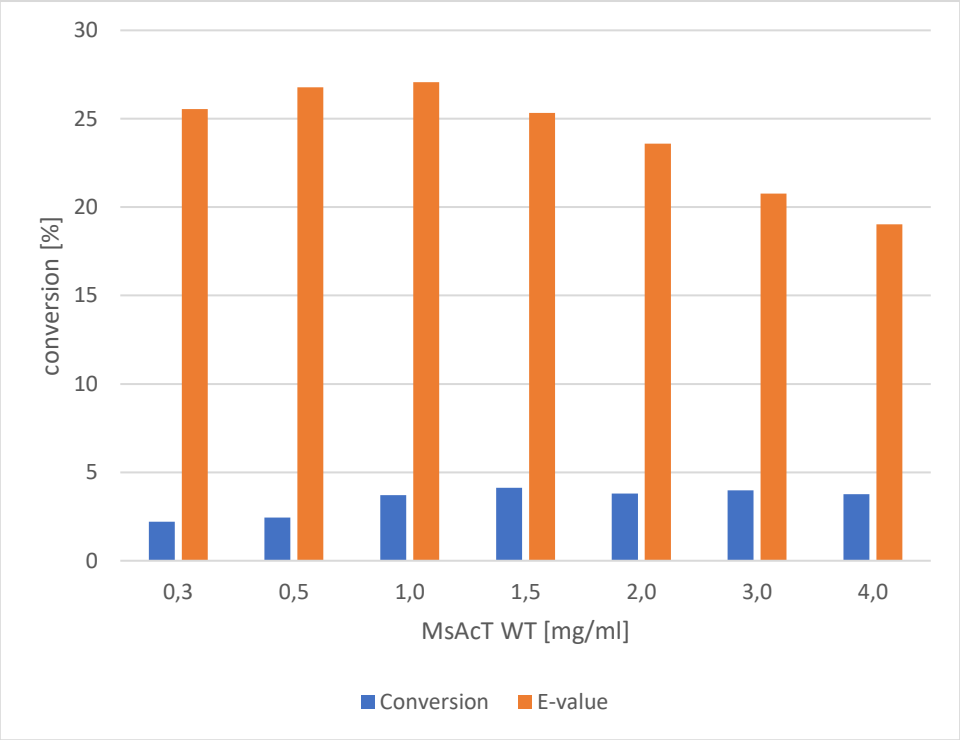


*Figure 25: Transesterification of **3a** (100 mM) with different concentrations of MsAcT WT. The reactions were performed with lyophilized cell free extract (CFE, concentration as indicated) and **2** (1 M) as an acyl donor in buffer (KPi, 200 mM, pH 7.5) at 30 °C for 5 h. Values shown in the graph are the average of three reactions.*

It is clearly demonstrated that an increase in enzyme concentration leads to a higher conversion for concentrations up to 1.5 mg/mL. However, beyond this concentration no increase in conversion was observable anymore. Regarding the selectivity of the MsAcT WT the E-value rises slightly until 1.0 mg/mL of CFE but then dramatically drops. Those two effects might result from the fact, that with higher enzyme concentrations present, the reaction runs faster and thereby also the concurrent hydrolysis of the product starts earlier, leading to a loss of the product as well as a loss of optical purity. Hence, higher concentrations of the enzyme should be avoided for highly enantioselective processes. For further biotransformations enzyme concentrations of 0.5 mg/mL were used.

For further testing, a set of biotransformations with longer reaction time was performed. The acyl-transfer was carried out with MsAcT WT (lyophilized CFE, 0.5 mg/mL), **3a** (100 mM) as a substrate and **2** (1 M) as the acyl donor in buffer (KPi, 200 mM, pH 7.5) at 30 °C for 24 h (Table 5).

*Table 5:Transesterification of **3a** using MsAcT WT over longer reaction times.*

time [h]	**5**	**24**
conversion [%]	2	5
E (e.e.$_S$, e.e.$_P$)	27	26

*The reactions were performed with lyophilized cell free extract of MsAcT WT (0.5 mg/mL), **3a** (100 mM) as a substrate and **2** (1 M) as an acyl donor in buffer (KPi, 200 mM, pH 7.5) at 30 °C for 5 h or 24 h. Values shown in the table are the average of three reactions.*

Higher conversions can be expected for longer reaction times. However, the E-value decreased with prolonged reaction time. In the case of WT MsAcT, the difference in enantio-selectivity comparing results after 5 h and 24 h reaction time is not drastic. Nevertheless, it can be expected, that for variants with higher activity, the selectivity of the reaction and therefore also the optical purity of the product might decrease even more after longer reaction times. Hence, for further screenings reaction times of 5 h were implemented.

3.2.3.2 Evaluation of Different Acyl Donors

Another important factor for transesterification reactions is the choice of acyl donor. Hence, several different donors were tested on the MsAcT WT and the variants F154A as well as F174A. Reactions were carried out with lyophilized CFE (0.5 mg/mL), **3a** (80 mM) as a substrate and EtOAc (**2**), vinyl acetate (**4**), isopropenyl acetate (**5**), phenyl acetate (**6**) (1 M) as the acyl donor in buffer (KPi, 200 mM, pH 7.5) at 30 °C for 5 h.

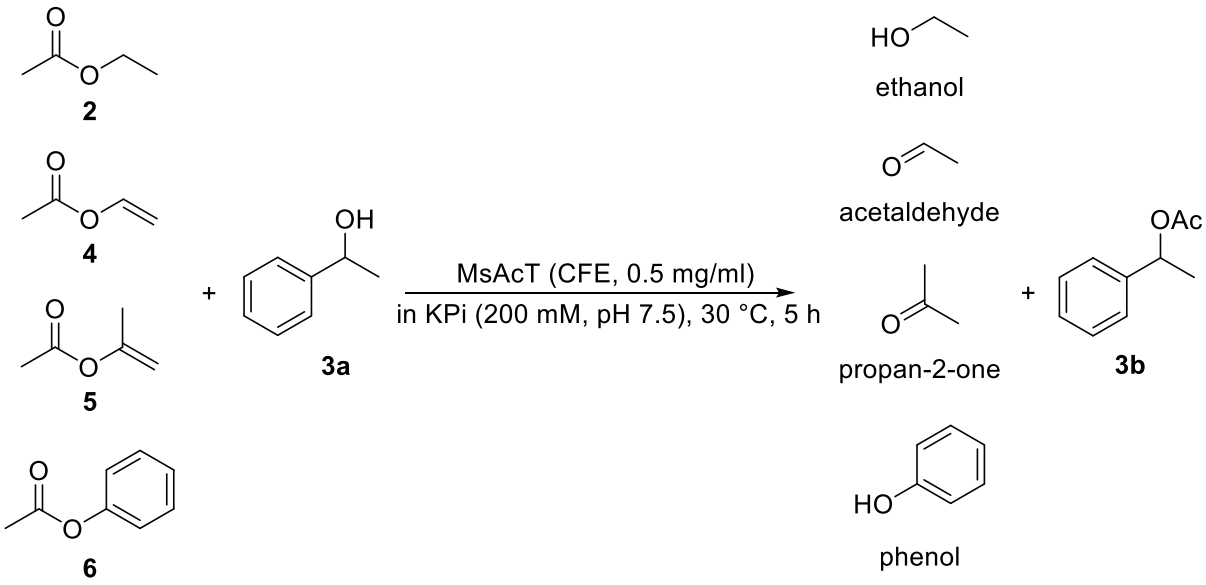


*Figure 26: Evaluation of the acyl donors **2**,**4**,**5**,**6** (1 M) for the transesterification of **3a** (80 mM) with MsAcT WT, F154A and F174A (CFE, 0.5 mg/mL)at 30 °C for 5 h.*

Table 6: Testing of different acyl donors with MsAct WT and the variants F154A, F174A.

acyl donor	***variant***	***conversion [%]***	***e.e.$_P$ [%]***	***E***
2	WT	2	93 (*R*)	27 (*R*)
	F154A	26	>99 (*R*)	>200 (2812, *R*)
	F174A	9	98 (*R*)	132 (*R*)
4	WT	3	89 (*R*)	18 (*R*)
	F154A	29	>99 (*R*)	>200 (2995, *R*)
	F174A	12	98 (*R*)	99 (*R*)
5	WT	3	91 (*R*)	22 (*R*)
	F154A	36	>99 (*R*)	>200 (1295, *R*)
	F174A	9	98 (*R*)	121 (*R*)
6	WT	3*	93 (*R*)	n.d.
	F154A	17*	>99 (*R*)	n.d.
	F174A	9*	>99 (*R*)	n.d.

*The reactions were performed with MsAcT WT, F154A and F174A (CFE, 0.5 mg/mL), **3a** (80 mM) and different acyl donors **2**,**4**,**5**,**6** (1 M) in buffer (KPi, 200 mM, pH 7.5) at 30 °C for 5 h (WT, F174A) or 2h (F154A). The E-value was calculated from e.e.$_P$ and e.e.$_S$. Values shown in the graph are the average of three reactions. Values shown in the graph are the average of three reactions. *Conversions for acyl donor **6** are approximated since peaks of phenol overlapped with substrate peaks on the GC chromatogram and E-values could not be determined (n.d.).*

Regarding the conversion of the transesterification reaction using different acyl donors (Table 6) **4** and **5** showed the highest conversion depending on the enzyme used. **2** provided significantly lower conversions as well as **6**. Due to troubles in the analytics (peaks of the acyl donor and phenol overlapped with the substrates peaks in the GC-chromatogram), conversions

for acyl donor **6** were assumed for comparison and the E-value could not be determined at all. Hence, due to low conversions and the difficulties during analysis, it was not further applied. A comparison of the E-values of transesterifications with varying acyl donors yielded no significant pattern. However, the biotransformations using **4** and MsAcT F154A led to the highest E-value and this acyl donor seems to increase conversion of the reactions in comparison to previously used **2**. Hence, it was be implemented in further acyl transfer reactions.

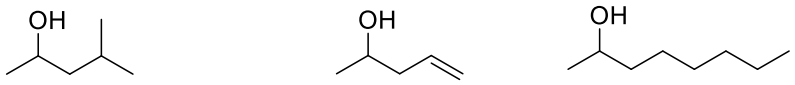
3.2.4 Substrate Screening

To further evaluate the potential of the beneficial variants F154A, F154V, F154L, F174A, F174V a broad substrate screening was performed. Substrates with different size, configuration and chemical properties were chosen and can be categorized in three main groups: aromatic substrates, aliphatic substrates and alkynols (Figure 27).

aromatic substrates:

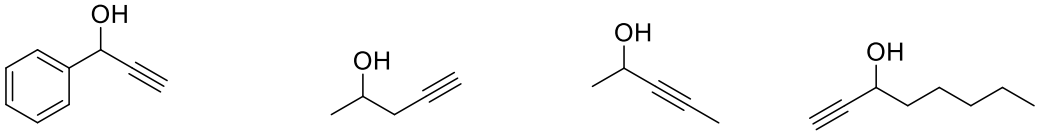
1-phenylethan-1-ol **3a** 1-phenylpropan-1-ol **7a** 1-phenylpropan-2-ol **8a** 4-phenylbutan-2-ol **9a**

alliphatic substrates:



4-methylpentan-2-ol **10a** pent-4-en-2-ol **11a** octan-2-ol **12a**

alkynoles:



1-phenylprop-2-yn-1-ol **13a** pent-4-yn-2-ol **14a** pent-3-yn-2-ol **15a** oct-1-yn-3-ol **16a**

Figure 27:Overview of the substrates screened with MsAcT WT, F154, F154V, F154L, F174A and F174V, categorized in three groups: aromatic substrates, aliphatic substrates and alkynols.

3.2.4.1 Aromatic substrates

Aromatic compounds were screened for acyl transfer with MsAcT WT, F154A, F154V, F154L, F174A and F174V (CFE 0.5 mg/mL), the substrates **3a**, **7a**, **8a**, **9a** (100 mM) and the acyl donor **4** (1 M) in buffer (KPi, 200 mM, pH 7.5). Reactions were performed at 30 °C for 5 h.

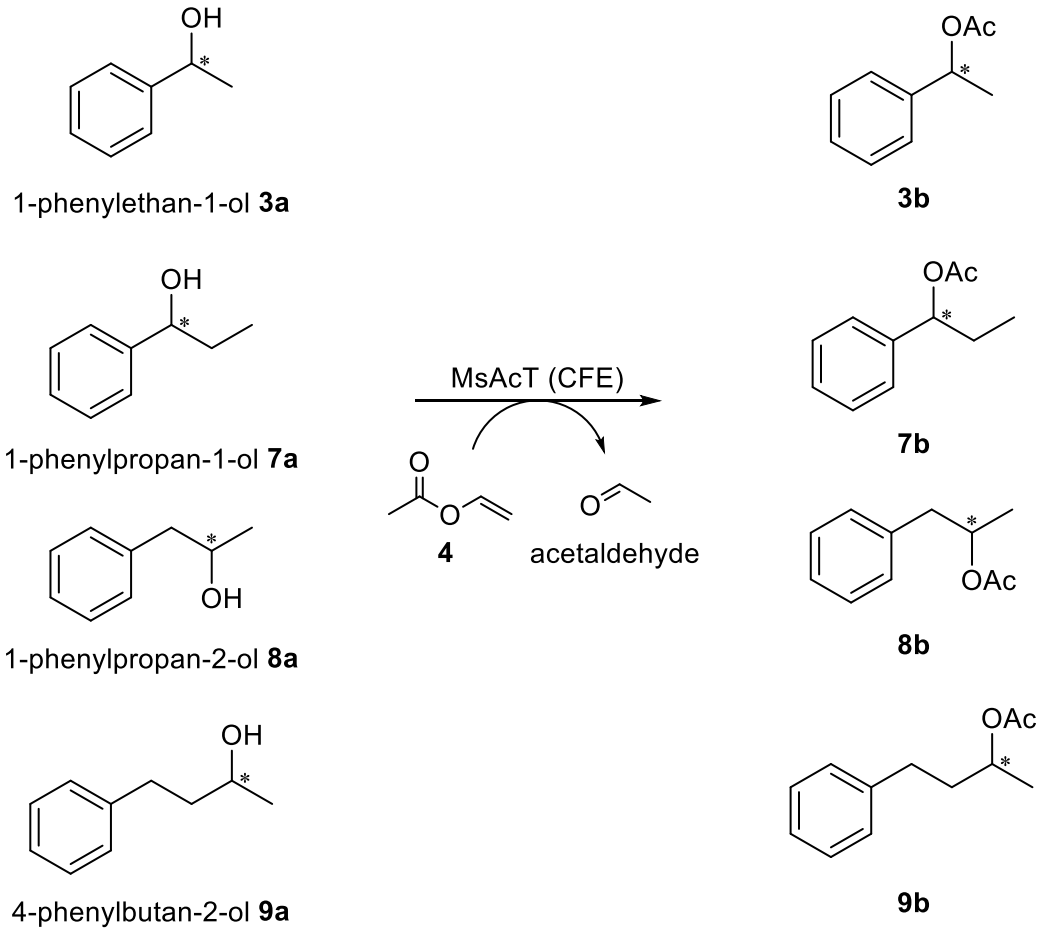


*Figure 28: Acetylation of substrates **3a**, **7a**, **8a**, **9a** (100 mM) with MsAcT WT, F154A, F154V, F154L, F174A and F174V (CFE, 0.5 mg/mL) and acyl donor **4** (1 M) at 30 °C for 5 h.*

Table 7: Acetylation of aromatic substrates.

substrate	***variant***	***conversion [%]***	***e.e.$_P$ [%]***	***E***
3a	WT	4	88 (*R*)	16 (*R*)
	F154A	41	>99 (*R*)	>200 (1309, *R*)
	F154V	49	>99 (*R*)	>200 (1090, *R*)
	F154L	45	98 (*R*)	>200 (204, *R*)
	F174A	13	98 (*R*)	93 (*R*)
	F174V	15	98 (*R*)	99 (*R*)
7a	WT	n.d.	n.d.	n.d.
	F154A	16	99 (*R*)	174 (*R*)
	F154V	28	98 (*R*)	114 (*R*)
	F154L	15	91 (*R*)	24 (*R*)
	F174A	n.d.	n.d.	n.d.
	F174V	n.d.	n.d.	n.d.

8a	WT	51	35 (*S*)	3 (*S*)
	F154A	66	53 (*R*)	22 (*R*)
	F154V	60	37 (*R*)	4 (*R*)
	F154L	49	36 (*S*)	3 (*S*)
	F174A	53	29 (*S*)	2 (*S*)
	F174V	54	26 (*S*)	2 (*S*)
9a	WT	n.d.	n.d.	n.d.
	F154A	48	95 (*R*)	120 (*R*)
	F154V	8	87 (*R*)	15 (*R*)
	F154L	n.d.	n.d.	n.d.
	F174A	n.d.	n.d.	n.d.
	F174V	n.d.	n.d.	n.d.

The reactions were performed with MsAcT WT, F154A, F154V, F154L, F174A and F174V as lyophilized cell free extract (0.5 mg/mL), ***3a****,* ***7a****,* ***8a****,* ***9a*** *(100 mM) as a substrate and* ***4*** *(1 M) as an acyl donor in buffer (KPi, 200 mM, pH 7.5) at 30 °C for 5 h. The E-value was calculated from e.e.$_P$ and e.e.$_S$. Due to the low conversions (<5%) of some variants E values and conversion could not be determined (n.d..).Values shown in the table are the average of three reactions.*

Comparing all aromatic substrates, **3a** is accepted best by the MsAcT variants and produced the highest enantio-selectivity. This is no surprise, as the variants were optimized for that substrate and as the substrate has a very defined chiral center due to the bulky phenyl moiety and the methyl group. In comparison to the WT MsAcT, an enormous increase in activity (49% conversion for MsAcT F154V, 4% conversion for WT MsAcT) and enantio-selectivity (MsAcT F154A calculated E-value: 1309 (*R*), MsAcT WT calculated E-value: 16 (*R*)) could be achieved with the variants F154A as well as F154V for substrate **3a**. However, a significant increase in both conversion and enantio-selectivity in comparison to the WT MsAcT was be obtained for every other substrate as well. The best performing variants were F154A and F154V. Especially with MsAcT F154A good to excellent E-values and conversions were obtained. This leads to the conclusion that a decrease in bulk in the "B-position" of the active site results in a definite increase in enantio-selectivity for aromatic substrates. This seems to work the best for very small residues in position Phe154, since F154L hardly led to improved results.

The only substrate that performed poorly in regards of enantio-selectivity was **8a**. Previous data from literature suggests that the WT MsAcT is selective towards (*S*)-**8a** [52]. This was confirmed here as well for the MsAcT WT, F154L, F174A, and F174V. Interestingly, for MsAcT F154A and F154V a switch to (*R*)-selectivity was observed. Nevertheless, the switch in enantio-preference could be explained due to the vast decrease of steric demand in "B-position" for variants F154A and F154V. Substrate **7a** only differs in the position of the hydroxy

moiety and led to better results. Consecutively it can be assumed that a phenyl group next to the chiral center concludes in higher enantio-selectivity than a benzyl-moiety. The reason for that might be the more defined chiral center in the case of the aromatic ring being in closer proximity to it. However, in the case of substrate **9a** the aromatic ring is even further away from the chiral center as in the case of **8a** and still higher E-values are generally obtained. Hence, the reason for the differing results of these substrates might also be different spatial orientation of the substrates in the active site due to the flexibility of the alkyl chains.

3.2.4.2 Aliphatic substrates

A set of aliphatic compounds (**10a**, **11a**, **12a**; 100 mM) was screened as substrate for acyl transfer with MsAcT WT and its variants F154A, F154V, F154L, F174A and F174V (CFE 0.5 mg/mL) and the acyl donor **4** (1 M) in buffer (KPi, 200 mM, pH 7.5). Reactions were performed at 30 °C for 5 h.

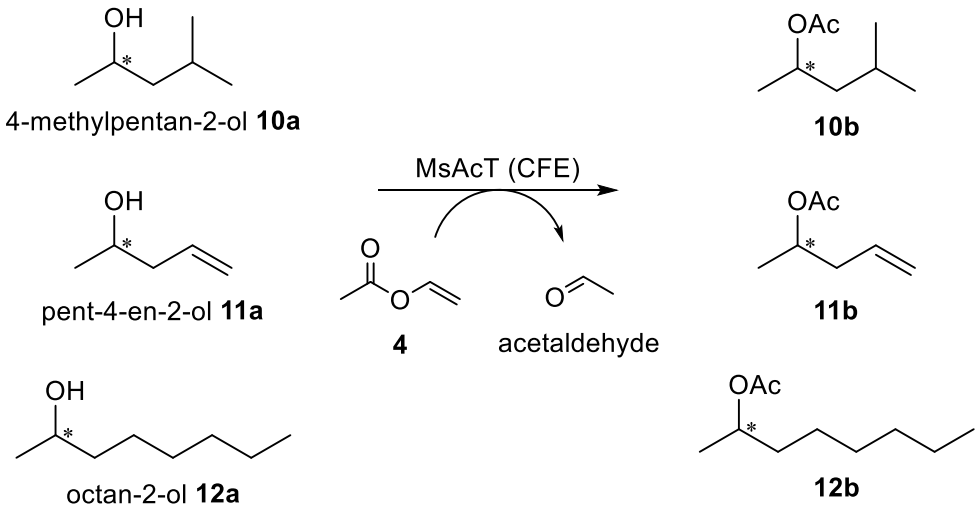


*Figure 29: Acetylation of substrates **10a**, **11a**, **12a** (100 mM) with MsAcT WT, F154A, F154V, F154L, F174A and F174V (CFE, 0.5 mg/mL) and acyl donor **4** (1 M) at 30 °C for 5 h.*

Table 8: Acetylation of aliphatic substrates.

substrate	***variant***	***conversion [%]***	***e.e.$_P$ [%]***	***E***
10a	WT	n.d.	n.d.	n.d.
	F154A	17	93 (*R*)	36 (*R*)
	F154V	51	96 (*R*)	>200 (377, *R*)
	F154L	n.d.	n.d.	n.d.
	F174A	25	88 (*R*)	20 (*R*)
	F174V	22	87 (*R*)	19 (*R*)
11a	WT	89	12 (*R*)	2 (*R*)
	F154A	44	56 (*R*)	5 (*R*)
	F154V	45	47 (*R*)	4 (*R*)
	F154L	73	21 (*R*)	3 (*R*)
	F174A	50	36 (*R*)	3 (*R*)
	F174V	59	32 (*R*)	3 (*R*)
12a	WT	28	79 (*S*)	12 (*S*)
	F154A	52	82 (*R*)	30 (*R*)
	F154V	28	46 (*R*)	3 (*R*)
	F154L	22	72 (*S*)	7 (*S*)
	F174A	8	34 (*S*)	2 (*S*)
	F174V	19	48 (*S*)	3 (*S*)

*The reactions were performed with MsAcT WT, F154A, F154V, F154L, F174A, F174V lyophilized cell free extract (0.5 mg/mL), **10a**, **11a**, **12a** (100 mM) as a substrate and **4** (1 M) as an acyl donor in buffer (KPi, 200 mM, pH 7.5) at 30 °C for 5 h. E-value was calculated with e.e.$_P$ and e.e.$_S$. for substrates **10a** and **11a**.For substrate **12a** the E value was calculated with e.e.$_P$ and the conversion (determined with calibration of the substrate). Due to the low conversions (<5%) of some variants E values and conversion could not be determined (n.d..). Values shown in the table are the average of three reactions.*

The aliphatic substrates were chosen based on size and differences in their configuration to test the overall applicability of MsAcT on this group of compounds. In general, higher conversion and lower enantio-selectivity was obtained for these compounds. Since the variants were optimized for the bulkier aromatic compound **3a** it was to be expected, that enantio-selectivity is decreased for smaller substrates. The only compound that led to good selectivity was **10a**. In this case full conversion and E-value >200 was obtained for MsAcT F154V. This was the bulkiest substrate due to the branches in the alkyl chain. Hence, these results support the hypothesis that sterically demanding compounds are better fit for enantio-selective applications of the MsAcT variants. Comparing substrate **11a** and **12a** it is also observable, that a longer alkyl chain in the substrate gives higher enantio-selectivity. However, since very few substrates were tested this finding would need to be further investigated.

MsAcT WT showed the highest enantio-selectivity towards (*S*)-**12a** with good conversion. This confirms previous literature, where it was also stated that the MsAcT prefers (*S*)-**12a** over the (*R*)-enantiomer but with low selectivity [51]. Remarkably, a switch from (*S*)-selectivity for MsAcT WT, F154L, F174A and F174V to (*R*)-selectivity for variants F154A as well as F154V was observed. As the same switch in selectivity was observed for substrate **8a** (see section 3.2.4.1) this may be a trend for the variants F154A and F154V. The reason for that is very likely the decrease in bulk in "B-position" which is favoring (*R*)-configuration of the substrate.

3.2.4.3 Alkynols

A set of alkynols (**13a**, **14a**, **15a**, **16a**; 100 mM) was screened as substrates in the acyl transfer with MsAcT WT and its variants F154A, F154V, F154L, F174A and F174V (CFE 0.5 mg/mL) using **4** (1 M) as the acyl donor, in buffer (KPi, 200 mM, pH 7.5). The reactions were performed at 30 °C for 5 h.

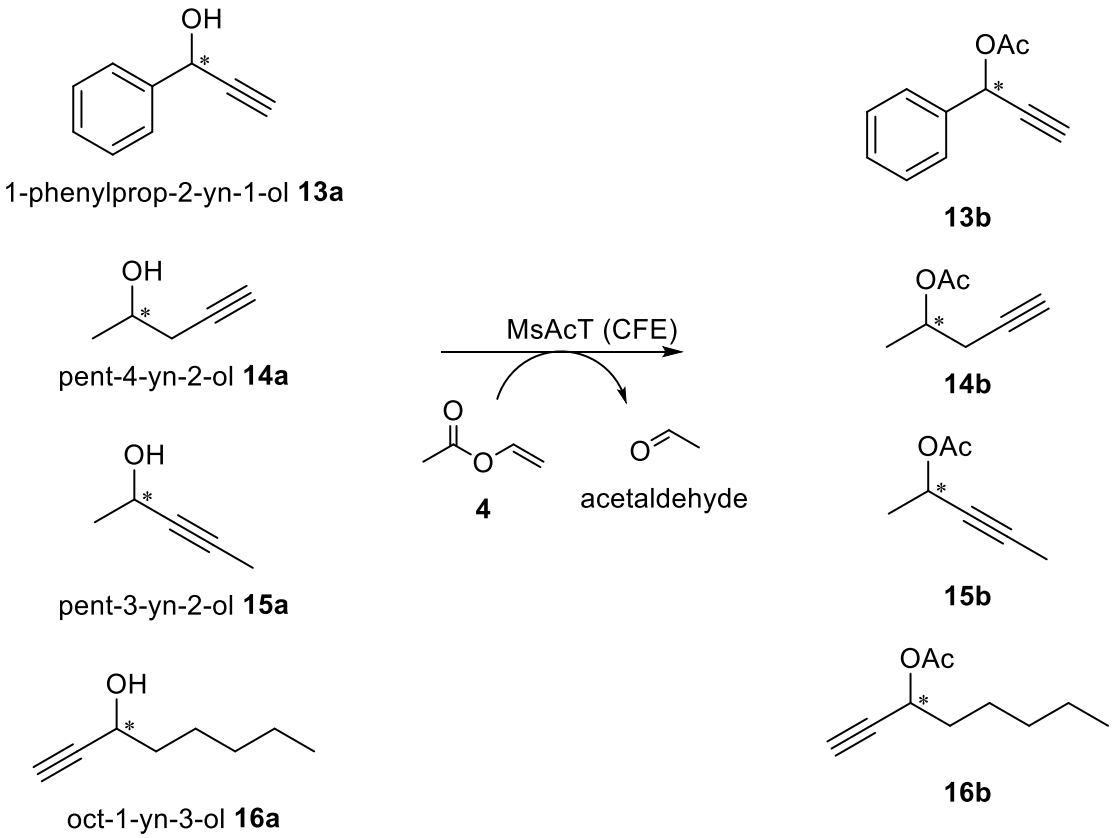


*Figure 30: Acetylation of substrates **13a**, **14a**, **15a**, **16a** (100 mM) with MsAcT WT, F154A, F154V, F154L, F174A and F174V (CFE, 0.5 mg/mL) and acyl donor **4** (1 M) at 30 °C for 5 h.*

Table 9: Acetylation of alkynols.

substrate	*variant*	*conversion [%]*	*e.e.$_P$ [%]*	*E*
13a	WT	4	93 (*R*)	33 (*R*)
	F154A	48	99 (*R*)	>200 (693, *R*)
	F154V	50	97 (*R*)	>200 (287, *R*)
	F154L	46	97 (*R*)	180 (*R*)
	F174A	7	97 (*R*)	64 (*R*)
	F174V	9	97 (*R*)	80 (*R*)
14a	WT	93	2 (*R*)	1 (*R*)
	F154A	71	34 (*R*)	5 (*R*)
	F154V	79	21 (*R*)	3 (*R*)
	F154L	90	3 (*R*)	1 (*R*)
	F174A	90	2 (*R*)	1 (*R*)
	F174V	94	3 (*R*)	1 (*R*)
15a	WT	40	91 (*S*)	39 (*S*)
	F154A	6	>99 (*R*)	>200 (213, *R*)
	F154V	89	13 (*R*)	6 (*R*)
	F154L	72	27 (*S*)	3 (*S*)
	F174A	53	57 (*S*)	7 (*S*)
	F174V	55	55 (*S*)	7 (*S*)
16a	WT	12	32 (*S*)	2 (*S*)
	F154A	49	88 (*S*)	42 (*S*)
	F154V	49	63 (*S*)	8 (*S*)
	F154L	44	2 (*S*)	1 (*S*)
	F174A	11	78 (*S*)	9 (*S*)
	F174V	12	67 (*S*)	5 (*S*)

*The reactions were performed with MsAcT WT, F154A, F154V, F154L, F174A, F174V lyophilized cell free extract (0.5 mg/mL), **13a**, **14a**, **15a**, **16a** (100 mM) as a substrate and **4** (1 M) as an acyl donor in buffer (KPi, 200 mM, pH 7.5) at 30 °C for 5 h. The E-value was calculated from e.e.$_P$ and e.e.$_S$. Due to the low conversions (<5%) of some variants E values and conversion could not be determined (n.d..). Values shown in the table are the average of three reactions.*

Comparing the overall data of the alkynol screening, the best results were obtained for substrate **13a**. In this case improved enantio-selectivity was found for all tested variants in comparison to the WT. The poor overall activity of the WT MsAcT on this substrate is partially supported by previous screenings in which **13a** was not accepted at all [52]. With the MsAcT F154A, F154V and F154L high conversions and excellent E-values were achieved. The reason for that might be, that the tested variants are optimized for aromatic substrates and enantio-selectivity

of the active site seems to be heavily influenced by steric demand. This was also shown with the overall results of the alkynol substrates with an aliphatic chain which generally led to lower E-values. Substrates **14a**, **15a** and **16a** were chosen to investigate aliphatic alkynols of different size and configuration. Additionally, substrate **14a** is the alkyne equivalent of the alkene **11a**. These compounds all performed very differently with the only similarity being generally lower enantio-selectivity. The reaction with substrate **14a** led to the highest conversion and lowest enantio-selectivity. This indicates that small compounds are not transformed in an enantio-selective way with the MsAcT WT and the investigated variants. Additionally, this compound is also more flexible than the constitution isomer **15a** since the triple C-C bond is terminal for **14a**. This might lead to more possible binding modes in the active site and therefore a diminished selectivity during the reaction. In comparison to the alkene equivalent **11a**, E-values decreased and conversions overall increased. Hence, it seems that alkynes in regards of enantio-selectivity perform slightly worse. However, the reason for these results might also be a different steric interaction with the active site due to spatial orientations. With substrate **15a** enantio-selectivity was overall improved and especially variant F154A showed excellent enantio-preference towards (*R*)-**15a** (E >200). Hence, a higher restriction in flexibility due to the rigid internal C-C triple bond seems to increase chiral recognition of the MsAcT active site. Again, decreased bulk in "B-position" in case of MsAcT F154A led to higher enantio-selectivity towards (*R*)-**15a** probably due to the sterically demanding alkynyl chain fitting the active site better this way. In addition, a switch in overall enantio-preference was seen from (*S*)-**15a** for MsAcT WT F174A, F174V, F154L to (*R*)-**15a** for variants F154A and F154V similar to the results of substrates **8a** and **12a**. For substrate **16a** only selectivity towards the (*S*)-enantiomer was observable. Previous work with WT MsAcT indicated selectivity towards (*S*)-**16a** as well [52]. This means that the rigid but smaller alkynyl residue would be preferably facing the "C-position" and the alkyl chain the "B-position". This supports the assumption, that the main factor in selection between the enantiomers for the MsAcT is the size of a residue.

3.2.5 Screening of the Double Mutant Library

In order to further improve the beneficial variants of the MsAcT, double variants with mutations at positions F150, F154 and F174 were made by switching the phenylalanine either to an alanine or valine residue (Table 10). These double variants were obtained with site directed mutagenesis *via* PCR. According to the single variants successfully obtained double variants were transformed in the expression host *E. coli* BL21(DE3) and partially isolated by performing cell lysis with subsequent lyophilization of the CFE.

Table 10: Double variants of MsAcT for further optimization of the enzymes enantio-preference for aromatic substrates.

double variants with A	double variants with V
F150A/F154A	F150V/F154V
F150A/F174A	F150V/F174V
F154A/F174A	F154V/F174V

3.2.5.1 Screening of the Double Variants with Benchmark Substrate ***3a*** for Acyl Transfer

The identified variants were tested on the model substrate **3a** for acyl transfer using **2** as an acyl donor based on the previous screening. Transesterification reactions were performed with lyophilized cell free extract (CFE, 0.3 mg/mL), **3a** (100 mM) as a substrate and **2** (1 M) as an acyl donor in buffer (KPi, 200 mM, pH 7.5) at 30 °C for 5 h. Screening conditions were chosen according to the first screening of the entire single variant library (see section 3.2.2) in order to compare the results appropriately.

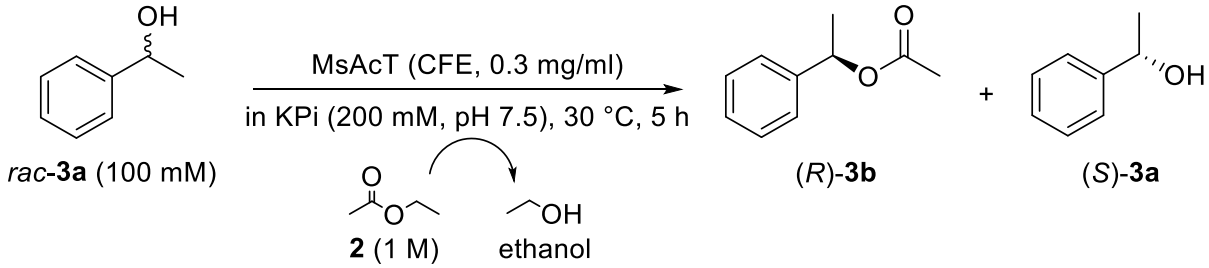


*Figure 31: Acetylation reaction of **3a** performed with the MsAcT WT and its double variants with the acyl donor **2**.*

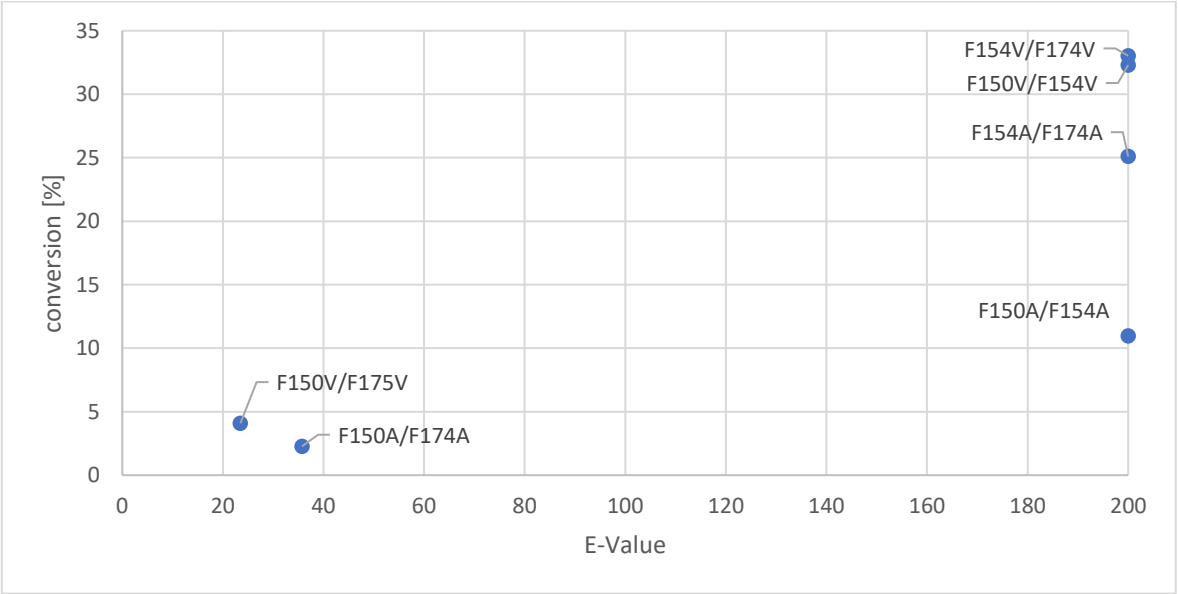


*Figure 32: Acetylation of **3a** using MsAcT WT and its double variants F154A/F174A, F150A/F154A, F150A/F174A, F154V/F174V, F150V/F154V, F150V/F174V. The reactions were performed with lyophilized cell free extract (0.3 mg/mL), **3a** (100 mM) as a substrate and **2** (1 M) as an acyl donor in buffer (KPi, 200 mM, pH 7.5) at 30 °C for 5 h. Values shown in the graph are the average of three reactions.*

Comparing the results for the screening of the double variants four of the biocatalysts led to excellent enantio-selectivity with E-values over 200. Enantio-selectivity as well as activity could even be further increased with the F154A/F174A (calculated E-value: 2775) variant in comparison to the previous best performing single variant F154A (calculated E-value: 2211) on substrate **3a**. The best combination of mutations seems to be at positions Phe174 and Phe154 for both the valine and alanine variants. This might be explained due to their positioning in the active site. Phe154 is in the "B-position" or "F-direction" of the big cavity in the active site and Phe174 is positioned in the entrance tunnel. Hence, a decrease in bulk in two different positions seem to be beneficial in regards of enantio-selectivity. Double variants with mutations at positions Phe150 and Phe154 led to a slightly lower enantio-selectivity, but still excellent E-values were achieved. The combination of mutations at position Phe150 and Phe174 lead to significantly lower conversions and enantio-selectivity. Hence, it can be assumed that a decrease in bulk at position Phe154 is required in order to dramatically increase enantio-preference of the MsAcT towards (*R*)-**3a**.

These results confirm the trends of the *in silico* data on the double variants (F154V/F174V, F150V/F154V, F150V/F174V) which were tested by M. Kazemi and F. Himo in the report of the 17.10.2019 (unpublished results). The computer simulations indicate an increase in overall enantio-selectivity with the double mutants in comparison to the single mutants especially for MsAcT F154V/F174V as well as F150V/F154V. The best performing double variant F154V/F174V in the calculations appears to have a higher enantio-preference towards (*R*)-**3a** than the single variant F154A due to the additional mutation at position Phe174. Because of the F174V mutation a cavity in the active site is formed which enables Phe150 to slightly rotate. In this position a beneficial C-H-π interaction between the Phe150 and the phenyl moiety of the substrate further stabilize the transition state of the (*R*)-**3a**.

3.2.5.2 Screening of Aromatic Substrates

In order to further test the double variants, they were all tested for acyl transfer with the previous screened aromatic substrates **3a**, **7a**, **8a** and **9a** including the aromatic alkynol **13a**. These substrates were chosen, as the MsAcT variants were optimized for aromatic substrates and in previous screenings they performed generally best regarding enantio-selectivity. Also, it is very likely, that the even more open active sites of the double variants work better on bigger substrates. Screenings for transesterification were performed with lyophilized cell free extract (CFE, 0.5 mg/mL), using **3a**, **7a**, **8a**, **9a** or **13a** (100 mM) as substrates and **4** (1 M) as an acyl donor in buffer (KPi, 200 mM, pH 7.5) at 30 °C for 5 h. Screening conditions were chosen

according to the first substrate screening with the single variants (see section 3.2.4) in order to compare the results appropriately.

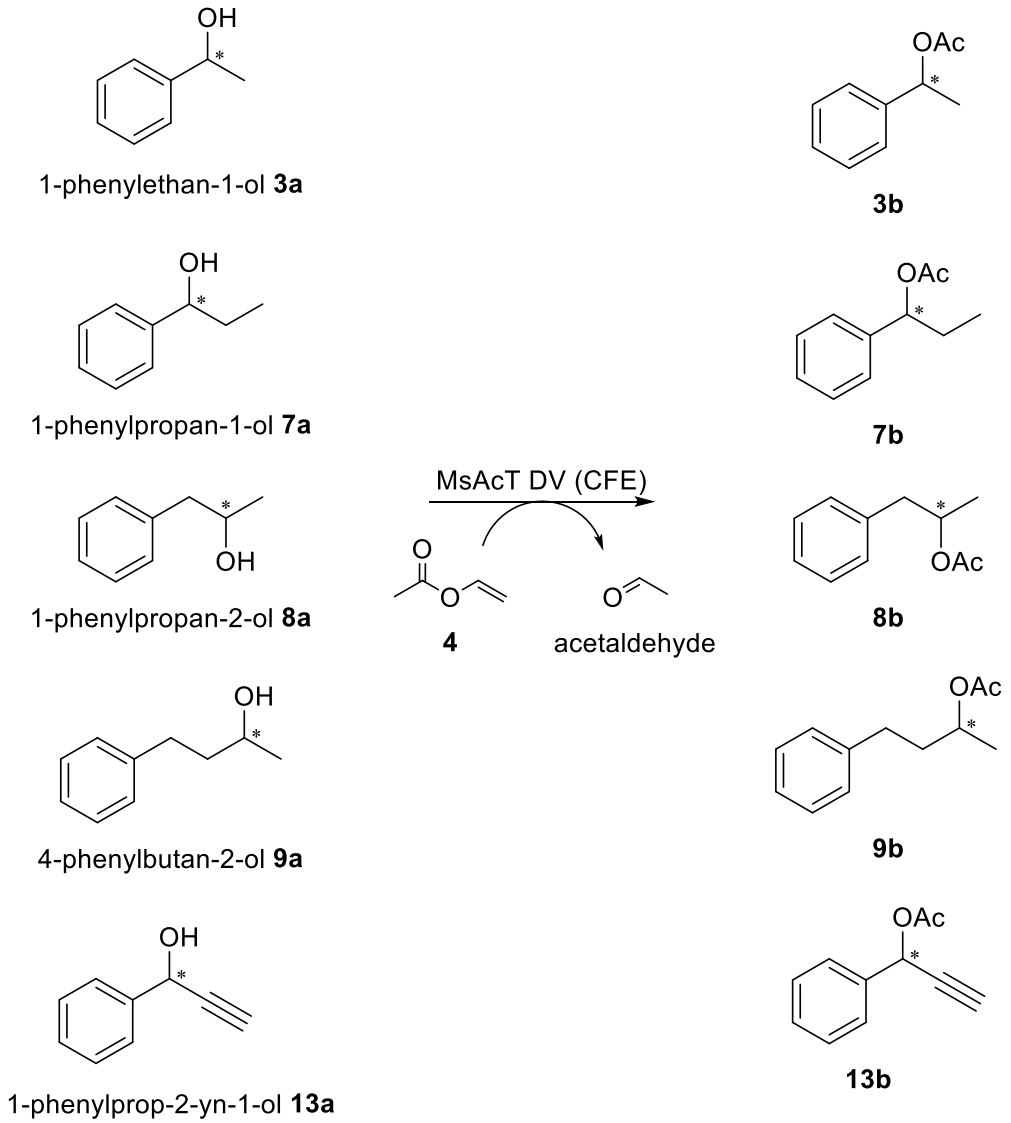


*Figure 33: Acetylation of different aromatic substrates **3a**, **7a**, **8a**, **9a**, **13a** (100 mM) with MsAcT F154A/F174A, F150A/F154A, F150A/F174A, F154V/F174V, F150V/F154V, F150V/F174V (CFE, 0.5 mg/mL) and the acyl donor **4** (1 M) at 30 °C for 5 h.*

Table 11: Acetylation of aromatic substrates.

substrate	***variant***	***conversion [%]***	***e.e.$_P$ [%]***	***E***
3a	F150A/F154A	15	99 (*R*)	>200 (201, *R*)
	F150A/F174A	4	90 (*R*)	20 (*R*)
	F154A/F174A	35	>99 (*R*)	>200 (2022, *R*)
	F150V/F154V	50	99 (*R*)	>200 (860, *R*)
	F154V/F174V	50	>99 (*R*)	>200 (2621, *R*)
	F150V/F174V	7	92 (*R*)	24 (*R*)

7a	F150A/F154A	n.d.	n.d.	n.d.
	F150A/F174A	n.d.	n.d.	n.d.
	F154A/F174A	n.d.	n.d.	n.d.
	F150V/F154V	29	98 (*R*)	119 (*R*)
	F154V/F174V	31	>99 (*R*)	>200 (508, *R*)
	F150V/F174V	n.d.	n.d.	n.d.
8a	F150A/F154A	n.d.	n.d.	n.d.
	F150A/F174A	n.d.	n.d.	n.d.
	F154A/F174A	25	74 (*R*)	8 (*R*)
	F150V/F154V	22	81 (*R*)	12 (*R*)
	F154V/F174V	55	81 (*R*)	70 (*R*)
	F150V/F174V	34	50 (*R*)	4 (*R*)
9a	F150A/F154A	48	97 (*R*)	174 (*R*)
	F150A/F174A	13	65 (*R*)	5 (*R*)
	F154A/F174A	45	97 (*R*)	136 (*R*)
	F150V/F154V	n.d.	n.d.	n.d.
	F154V/F174V	n.d.	n.d.	n.d.
	F150V/F174V	21	42 (*R*)	3 (*R*)
13a	F150A/F154A	49	>99 (*R*)	>200 (1711, *R*)
	F150A/F174A	3	94 (*R*)	32 (*R*)
	F154A/F174A	47	>99 (*R*)	>200 (666, *R*)
	F150V/F154V	49	99 (*R*)	>200 (749, *R*)
	F154V/F174V	50	99 (*R*)	>200 (643, *R*)
	F150V/F174V	4	90 (*R*)	19 (*R*)

*The reactions were performed with lyophilized cell free extract of MsAcT F154A/F174A, F150A/F154A, F150A/F174A, F154V/F174V, F150V/F154V, F150V/F174V and WT (CFE, 0.5 mg/mL), **3a**, **7a**, **8a**, **9a**, **13a** (100 mM) as a substrate and **4** (1 M) as an acyl donor in buffer (KPi, 200 mM, pH 7.5) at 30 °C for 5 h. The E-value was calculated from e.e.$_P$ and e.e.$_S$. Due to low conversions (<5%) of some variants E values and conversion could not be determined (n.d..). Values shown in the table are the average of three reactions.*

With the MsAcT double variants enantio-selectivity could be even further improved not only in comparison to the MsAcT wild type but also the previous best performing variant F154A (Figure 22, Table 7, Table 9). Excellent to good conversion were obtained for all substrates except for **8a** and good to full conversion was achieved for all substrates. Hence, the additional opening of the active site seems to vastly improve activity of the enzyme without compromising on the selectivity of the biocatalyst. The poor selectivity, towards substrates **8a** and **9a** could only be minimally improved with the double variants. The reason for the results of these

substrates might be the differing spatial orientation of the substrates in the active site due to the flexibility of the alkyl chains. This supports the theory that the chiral recognition of the enzymatic active site strongly depends on the residues in the immediate proximity of the chiral center of the substrate. The F154V/F174V variant was found to be the most promising candidate *in silico* and experimental data is supporting that the combination of mutations at positions Phe154 and Phe174 seem to be generally the most beneficial. However, depending on the substrate either substitution with Val or Ala in those positions worked best. The tendency seems to be, the bigger the substrate the better the Ala variants perform. Interestingly, for substrates **9a** and **13a** MsAcT F150A/F154A showed highest enantio-selectivity. Since, these two substrates are the most sterically demanding of the tested compounds it might be assumed that the combination of mutations on these positions is beneficial in the case of such bulky compounds. A clear trend for further usage of the variants could not be made and requires more testing. However, all tested double variants showed undoubtedly high enantio-preference towards the (*R*)-enantiomer of each substrate. The large opening of the active site in the "B-position" seems to strongly support this preference and thereby further proves *in silico* and experimental findings.

4 Conclusion

The general aim of this thesis was to optimize the acyl transferase derived from *Mycobacterium smegmatis* towards transesterification in aqueous medium and increased enantio-selectivity. Regarding the first project, enzyme engineering was performed in order to enhance the preference towards the acyl transfer reaction over hydrolysis in aqueous medium. Based on a computational study performed by M. Kazemi and F. Himo in the report of 11.06.2018 (unpublished results) 14 variants were found to have those improved properties due to a mutated loop in the surroundings of the active site. Those variants were successfully obtained, and the purified enzymes were tested for their activities towards acyl transfer in comparison hydrolysis. For neither of the transesterification reactions of the variants conversion was found. For the hydrolysis reaction with the variants low conversion was achieved only after 24 h. Hence, a drastic decrease in overall activity of the variants in comparison to the WT MsAcT was observed. In addition, the engineered MsAcT variants seem to have even lower affinity towards acyl transfer, since no conversion was observable for these reactions in comparison to the hydrolysis samples. The reason for that might be the drastic changes in those variants which could cause overall instability or decreased accessibility of the active site. Thus, further application of these variants does not appear to hold advantages in comparison to the WT MsAcT.

In the second enzyme engineering project of the MsAcT the focus was on improving the enantio-selectivity of this biocatalyst, since previous research showed that the MsAcT can perform enantio-selective acyl transfer on secondary alcohols, however selectivity was usually very low (E-values around 10) [51, 52]. Computational studies were performed to obtain variants of interest by M. Kazemi and F. Himo in the report of 17.08.2018 (unpublished results) and in literature [56]. In those studies, it was found out that there are mainly three positions in the active site of which two are highly relevant for chiral recognition. A decrease in bulk in "A-/C-position" would lead to a preference of (*S*)-**3a** and lower steric demand in "B-position" would enhance selectivity towards (*R*)-**3a**. The chosen variants were successfully obtained and tested. Several variants of the MsAcT with improved enantio-selectivity and activity were acquired, due to the adjustments in the sterics of the active site. Still the newly found variants keep their strong preference towards transesterification over hydrolysis which enables the performance of kinetic resolutions in water. This did not only widen the applicability of the MsAcT, but also gain more insights for future enzyme engineering of the biocatalyst. *Via* decreasing bulk in "C-position" (W16A) preference towards (*S*)-**3a** was observed and by opening the "B-position" (F154A, F154V etc.) selectivity towards (*R*)-**3a** was enhanced. Hence,

these findings support the *in silico* data and open the opportunity to switch the enantio-preference of the MsAcT. In general, the biggest factor for chiral recognition and activity seems to be the steric of both the active site and the substrate itself. This hypothesis is further supported by the results of the MsAcT double variant screening. Even higher enantio-selectivity and especially activity was observed, due to the vast decrease of bulk in the "B-position" and highly (*R*)-selective variants were obtained. The experimental data is again supported by computational studies performed by M. Kazemi and F. Himo in the report of 16.10.2019 (unpublished results) on the Val double variants. These findings do not only proof the origin of the enantio-preference of the MsAcT but might be used for further mutagenesis in the future. The newly found MsAcT variants were found to perform best on bulky, aromatic substrates, due to their optimization towards acyl transfer of benchmark substrate **3a**. In general, a well-defined chiral center with bulky residues in close proximity to it led to the highest chiral recognition, also for non-aromatic compounds. Overall, the experimental data in addition to the *in silico* study lead to a better understanding of the interaction of the substrate in the active site during the reaction. This might be applied for more educated predictions about stereo-selectivity of this specific biocatalyst on different substrates. Additionally, the same principal and strategy may be applied for different substrate groups in order to broaden the application spectrum of the MsAcT and kinetic resolution in water in general. For now, the vast improvement of enantio-selectivity, activity for a range of bulky, aromatic compounds already improves the applicability of the MsAcT greatly. This opens more possibilities for asymmetric synthesis in aqueous media and with that "greener" acyl transfer overall.

5 Experimental Procedures

5.1 Enzyme Sources

The acyltransferase from *Mycobacterium smegmatis* (pEG 32) is available in the in-house plasmid database and was cloned into a pET-28a(+) vector. The required variants of this acyltransferase were obtained either by ordering of the genes (Invitrogen by Thermo Fisher) and cloning them into pET-28a(+) vector or by site directed mutagenesis of the recombinant WT with specific primers (Eurofins). YADH1 from *Saccharomyces cerevisiae* (UniProt P00330) was ordered from Twist in a pET-28a(+) vector including an N-terminal His-tag with the restriction sites NdeI and XhoI. HL ADH from bovine liver (pEG 54) is available in the in house data bank already cloned into pET-28a(+) vector.

5.2 Chemical Sources

Compounds benzyl alcohol (**1a**), benzyl acetate (**1b**), *rac*-1-phenylethanol (**3a**), (*R*)-1-phenyl ethanol (**3a**), *rac*-1-phenyl-1-propanol (**7a**), *rac*-1-phenyl-2-propanol (**8a**), (*R*)-1-phenyl-2-propanol (**8a**), *rac*-4-phenyl-2-butanol (**9a**), (*S*)- 4-phenyl-2-butanol (**9a**), *rac*-4-phenyl-2-butyl acetate (**9b**), (*R*)-4-methyl-2-pentanol (**10a**), (*R*)-1-phenyl-2-propynol (**13a**), *rac*-4-pentyn-2-ol (**14a**), *rac*-3-pentyn-2-ol (**15a**), (*S*)-2-pentanol, *rac*-2-pentanol, *rac*-2-pentanyl acetate, ethanol, and 1-decanol were all purchased from Sigma Aldrich. Compounds *rac*-4-penten-2-ol (**11a**), *rac*-2-octanol (**12a**), *rac*-1-octyn-3-ol (**16a**) and isopropenyl acetate (**5**) were all purchased from Fluka. Compounds (*R*)-1-phenyl-1-propanol (**7a**) and *rac*-1-phenyl-2-propynol (**13a**) were purchased from TCI. Compounds (*R*)-4-penten-2-ol (**11a**) and phenyl acetate (**6**) were purchased from Alfa Aesar. Compound *rac*-1-phenylethyl acetate (**3b**) is purchased from Merck, *rac*-4-methyl-2-pentanol (**10a**) from Lancaster, ethyl acetate (**2**) from VWR, vinyl acetate (**4**) from Acros Organics. For the spectrophotometric assay co-factors NAD^+ (BASF) and $NADP^+$ (PanReac AppliChem) were used. All chemicals were purchased in the best quality available.

5.3 General, Kits and Instruments

For the cloning, synthetic DNA of the variants was ordered from Invitrogen by Thermo Fisher Scientific and restriction was performed using 10x Fast Digest Green Buffer, Fast Digest Xho I (10 U/µL) and Fast Digest Nde I (10 U/µL), as well as 10x T4 DNA Ligase Buffer and T4 DNA Ligase (5 U/µL), all from Thermo Scientific. Site-directed mutagenesis was performed using QuikChange II XL Site-Directed Mutagenesis Kit by Agilent Technologies. The primers used were designed according to the kit and were purchased from Eurofins. Colony PCR was performed implementing T7 Promotor (100 pmol/µL) and T7 Terminator (100 pmol/µL)

purchased from Eurofins and Dream Taq Green PCR Master Mix by Thermo Fisher Scientific. In order to express the desired proteins, transformations were performed with One Shot BL21 (DE3) chemically competent *E. coli* cells. The cloned genes were transformed in competent *E. coli* NEB 5-alpha (high efficiency) cells from New England Biolabs. The digested PCR product (QuikChange II Site Directed Mutagenesis Kit) was transformed in XL 10 Gold Ultracompetent *E. coli* cells by Agilent Technologies. Gel extraction was performed with QIAquick Gel Extraction Kit (250) from Qiagen following the protocol. Plasmid preparation was performed using QIAprep Spin Miniprep Kit (250) by Qiagen following their protocol. LB medium was prepared, using yeast extract (5 g), NaCl (5 g), tryptophan (10 g) and distilled water (1 L). Overnight cultures (ONC) were prepared from a single colony from an agar plate or from a glycerol stock (5 µL) in LB medium (10 mL) with kanamycin (10 µL, 0.05 mg/mL, Sigma Aldrich). Glycerol stocks were prepared using ONC medium (0.5 mL) and autoclaved glycerol (0.5 ml, 30 % final concentration in water).

The Eppendorf BioPhotometer plus was used for OD measurements, DNA concentration and protein concentration determination. The Eppendorf Mastercycler Nexus by was used to perform PCR reactions. The spectrophotometric assay was proceeded on the SpectraMax M2 Microplate Reader by Molecular Devices measuring at 340 nm. Cell disruption is carried out with a Branson Digital Sonifier.

His-tagged protein purification was performed utilizing His Trap Fast Flow (5 mL) columns from GE Healthcare on an Äkta pure system by GE Healthcare with following method: sample application at 3 mL/min, column wash at 5 mL/min (85 % washing buffer, 15 % elution buffer), elution at 5 mL/min with a linear gradient (15 %-100 % elution buffer), UV detection at 280 nm. Desalting was performed with PD-10 Desalting Columns by GE Healthcare. Small scale His-tagged protein purification was performed utilizing His SpinTrap TALON Spin Columns (GE Healthcare) following the protocol and desalting was performed using PD MiniTrap G-25 (GE Healthcare) columns, following the gravity protocol.

All GC measurements were performed with a 7890A Gas Chromatograph by Agilent Technologies using H_2 as a carrier gas and 250 °C injector temperature.

5.4 Analytics

5.4.1 Achiral GC-FID of 1a and 1b

GC-FID measurements were carried out in order to obtain activity of the transesterifications and hydrolysis performed by MsAcT using 1-decanol as an internal standard. The retention times of the obtained substances are listed in Table 12. Peaks were assigned with commercial reference substances of **1a** and **1b.**

A multipoint calibration with internal standard was done. Stock solutions of the substrates benzyl alcohol (**1a**) and benzyl acetate (**1b**) with varying concentrations (5 mM, 10 mM, 15 mM, 30 mM, 45 mM) were prepared in the EtOAc saturated buffer (KPi 200 mM, pH 7.5). All stock solutions were incubated (1.5 h, 30 °C, 120 rpm) and a portion of the stock solutions (1 mL) was transferred to a microcentrifuge vial. The analytes were extracted (2x400 µL, 1x200 µL MTBE including 20 mM IS 1-decanol) and dried (Na_2SO_4) prior to GC FID analysis.

GC measurements for substrates **1a** and **1b** were performed using the achiral Agilent HP-5 column (30 m, 0.32 mm, 0.25 µm).

GC temperature program: 2 µL injection; start 80°C, first ramp 50 °C/min to 120°C (6 min hold), second ramp 60 °C/min to 300 °C (1 min hold).

*Table 12: Retention times of **1a** and **1b** on achiral GC-FID.*

substance	retention time [min]
1a	1.9
1b	2.6
1-decanol (IS)	3.6

5.4.2 Chiral GC-FID and assignment of absolute configuration of substrates 3a/3b, 7a, 8a, 9a, 10a, 11a, 12a, 13a, 14a, 15a and 16a.

GC measurements for substrates **3a**/**3b**, **7a**, **8a**, **9a** and **12a** were performed on the chiral Agilent Chirasil DEX-CB column (25 m, 0.32 mm, 0.25 µm) and for substrates **10a**, **11a**, **13a**, **14a**, **15a** and **16a** the chiral RESTEK Rt-BDEXse column (30 m, 0.32 mm, 0.25 µm) was used with indicated methods for each substrate.

5.4.2.1 3a/3b and 7a/7b

GC temperature program: 2 µL injection volume; start 80°C (0.5 min hold), first ramp 2.5 °C/min to 100°C, second ramp 10 °C/min to 180 °C

The peaks were assigned with co-injection of commercially available authentic references of the corresponding racemic and optically pure compounds **3a**, **3b** and **7a** as well as synthesized reference compounds (*R*)-**3b** and **7b**.

*Table 13: Retention times of the enantiomers of **3a**, **3b**, **7a** and **7b** on chiral GC-FID.*

substance	retention time [min]	substance	retention time [min]
(*S*)-**3b**	11.7	(*S*)-**7b**	12.8
(*R*)-**3b**	12.2	(*R*)-**7b**	13.0
(*R*)-**3a**	12.9	(*R*)-**7a**	14.0
(*S*)-**3a**	13.1	(*S*)-**7a**	14.2

5.4.2.2 8a/8b

GC temperature program: 1 µL injection volume; start 80°C (0.5 min hold), first ramp 10 °C/min to 140°C (10 min hold), second ramp 10 °C/min to 180 °C

The peaks were assigned with co-injection of commercially available authentic references *rac*-**8a**, (*R*)-**8a** and synthesized reference compound **8b**.

*Table 14: Retention times of the enantiomers of **8a** and **8b** on chiral GC-FID.*

substance	retention time [min]	substance	retention time [min]
(*S*)-**8a**	8.0	(*S*)-**8b**	8.2
(*R*)-**8a**	8.1	(*R*)-**8b**	8.5

5.4.2.3 9a/9b

GC temperature program: 1 µL injection volume; start 80°C (0.5 min hold), first ramp 10 °C/min to 150°C (10 min hold), second ramp 10 °C/min to 180 °C

The peaks were assigned with co-injection of commercially available authentic references *rac*-**9a**, (*S*)-**9a** and **9b**.

*Table 15: Retention times of the enantiomers of **9a** and **9b** on chiral GC-FID.*

substance	retention time [min]	substance	retention time [min]
(*S*)-**9a**	9.4	(*S*)-**9b**	9.8
(*R*)-**9a**	9.5	(*R*)-**9b**	10.1

5.4.2.4 *12a/12b*

GC temperature program [52]: 2 µL injection; start 70°C (5 min hold), first ramp 15 °C/min to 110°C (3 min hold), second ramp 30 °C/min to 200 °C (1 min hold)

The peaks were assigned following the literature retention times [59] and with commercially available authentic reference **12a** and synthesized reference compound **12b**. Calibration was performed for the substrate **12a** for the determination of the E-value. Samples with varying concentrations of **12a** (10 mM, 50 mM, 75 mM, 100 mM, 150 mM) were prepared in buffer (900 µL, KPi 200 mM, pH 7.5) and vinyl acetate (100 µL). All samples were extracted (2x500 µL MTBE including 20 mM IS 1-decanol) and dried (Na_2SO_4) prior to GC FID analysis.

*Table 16: Retention times of the enantiomers of **12a** and **12b** on chiral GC-FID.*

substance	retention time [min]	substance	retention time [min]
12a	10.2	(*R*)-**12b**	11.3
(*S*)-**12b**	10.7	1-decanol	13.4

5.4.2.5 *10a/10b, 11a/11b, 14a/14b, 15a/15b and 16a/16b*

GC temperature program: 2 µL injection; start 40°C (5 min hold), first ramp 2.5 °C/min to 90°C (3 min hold), second ramp 20 °C/min to 200 °C (1.5 min hold)

The peaks were assigned with co-injection of commercially available authentic references of the corresponding racemic and optically pure compounds **10a**, **11a** and **16a** as well as synthesized reference compounds **10b**, **11b** and **16b**.

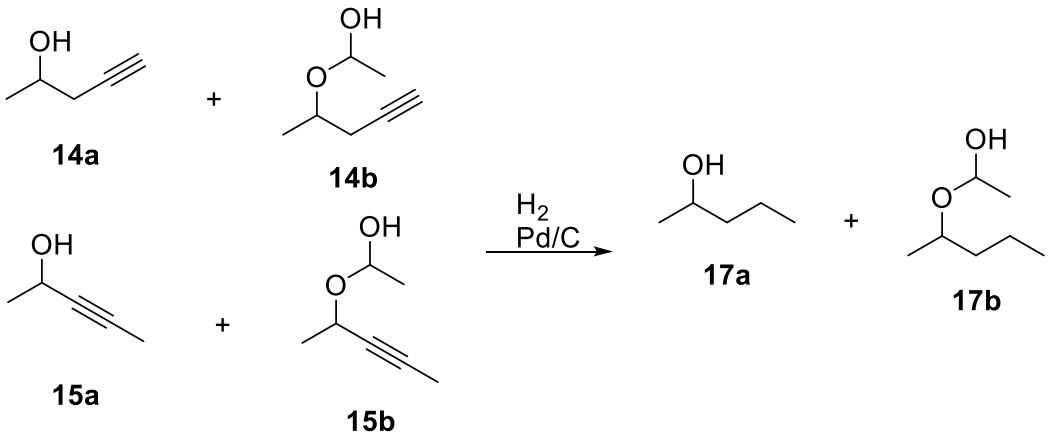


*Figure 34: Hydrogenation of the biotransformations with substrates **14a** and **15a** for assignment their absolute configuration via co-injection with (S)-2-pentanol, rac-2-pentanol and rac-2-pentanyl acetate on chiral GC-FID.*

Substrates **14a** and **15a** were assigned by converting them to 2-pentanol and 2-pentanyl acetate *via* Pd-catalyzed hydrogenation of the reaction mixture (Figure 34). Reactions were performed in a 50 mL round bottom flask with a septum lid. The worked up biotransformations (extraction with MTBE and drying over Na_2SO_4) of substrates **14a** or **15a** were mixed with Pd/C (20 mg) in solvent (ethanol, 1.5 mL) The flasks were flooded with H_2 and the reactions were stirred overnight under H_2 atmosphere. The reactions were then filtered through Celite 545 and directly measured on GC-FID. The reduced substrates and products were compared to commercially available authentic references *rac*-2-pentanol, *rac*-2-pentanyl acetate and (*S*)-2-pentanol and assigned with co-injection of optical pure authentic commercial reference (*S*)-2-pentanol.

*Table 17: Retention times of the enantiomers of **10a**, **10b**, **11a**, **11b**, **14a**, **14b**, **15a**, **15b**, **16a**, **16b**, 2-pentanol and 2-pentanyl acetate on chiral GC-FID.*

substance	retention time [min]	substance	retention time [min]
(*S*)-**10b**	16.5	(*R*)-**15a**	20.1
(*S*)-**10a**	17.8	(*S*)-**15b**	20.3
(*R*)-**10a**	18.0	(*R*)-**15b**	21.5
(*R*)-**10b**	21.3	(*S*)-**15a**	21.7
(*S*)-**11a**	12.8	(*S*)-**16a**	29.6
(*R*)-**11a**	13.0	(*R*)-**16b**	29.8
(*R*)-**11b**	15.2	(*R*)-**16a**	30.1
(*S*)-**11b**	20.6	(*S*)-**16b**	30.7
(*R*)-**14a**	15.8	(*R*)-2-pentanol	14.5
(*S*)-**14a**	16.0	(*S*)-2-pentanol	14.6
(*S*)-**14b**	18.7	(*S*)-2-pentanyl acetate	14.9
(*R*)-**14b**	21.9	(*R*)-2-pentanyl acetate	21.5

5.4.2.6 13a/13b

GC temperature program [52]: 2 µL injection; start 70°C (5 min hold), first ramp 15 °C/min to 110°C (3 min hold), second ramp 30 °C/min to 200 °C (1 min hold)

The peaks were assigned with co-injection of commercially available authentic references *rac*-**13a**, (*R*)-**13a** and synthesized reference compound **13b**.

*Table 18: Retention times of the enantiomers of **13a** and **13b** on chiral GC-FID.*

substance	retention time [min]	substance	retention time [min]
(*S*)-**13b**	15.8	(*R*)-**13a**	16.5
(*R*)-**13b**	16.3	(*S*)-**13a**	16.7

5.5 DNA Analysis and Purification

DNA analysis or purification was performed with agarose gels. Agarose gels were prepared with agarose (1 %, Invitrogen) in TAE buffer (Invitrogen) stained with SYBR Safe (Invitrogen). Samples were stained with 6x DNA Loading Dye (Thermo Fischer Scientific) and gel was run with Gene Ruler DNA Ladder Mix (Thermo Scientific) at 100 V.

5.6 Protein Analysis

The protein concentration was determined by Bradford protein assay. Bradford reagent was diluted 1:4 with water. The sample solution (20 µL) was pipetted into the reagent solution (980 µL) and incubated at room temperature for 5 min before measurement with the biophotometer at a wavelength of 595 nm. ExpressPlus PAGE Gels (GenScript) were used for protein analysis in MOPS buffer. Protein solutions (15 µg) were mixed with 2x Laemmli Sample Buffer and denatured (95 °C, 5 min). PageRuler Prestained Protein Ladder (Thermo Scientific) was used. Gels were run at 100 V and stained with Coomassie Brilliant Blue solution. Overexpression was checked *via* SDS PAGE. A small portion of the pellet (10-20 mg) was resuspended in water (1 mL). Cell lysis was performed *via* sonication on ice (15 sec, 40% amplitude, 0.1 sec on, 0.5 sec off, 1 min pause in between two sonications). The solution was centrifuged (5 min, 14000 rpm) to separate lysate and cell pellet. The pellet was dissolved in urea (6 M in water). Protein concentration was determined by Bradford and SDS PAGE was done of the lysate and the pellet.

5.7 Cloning

To introduce the mutations the according gene sequences were cloned into pET-28a(+) vector with an N-terminal His6-Tag. Restriction was performed in sterile microcentrifuge tubes. Fast Digest Green Buffer 10x (2 µL), sterile water (6 µL), Fast Digest XhoI (1 µL, 0.5 U/µL), Fast Digest NdeI (1 µL, 0.5 U/µL) and mutant DNA solution (10 µL, 10 ng/µL) were mixed and incubated for 1.5 h at 37°C. In sterile microcentrifuge tubes Fast Digest Green Buffer 10x (2 µL), sterile water (10 µL), Fast Digest XhoI (1 µL, 0.5 U/µL), Fast Digest NdeI (1 µL, 0.5 U/µL) and vector DNA solution (6 µL, 26 ng/µL) were mixed and incubated for 0.5 h at 37°C. The samples were put on ice after the incubation. All samples were purified on agarose

gel and the DNA was extracted from the excised bands with the QIAquick Gel Extraction kit. Ligation was performed after determination of the DNA concentration. In sterile microcentrifuge tubes DNA ligase buffer 10x (2 µL), T4 ligase (1 µL, 0.25 Weiss U/µL), DNA vector solution (30 ng, 5 kb), DNA insert solution (19 ng, 1 kb) and sterile water (to final volume 20 µL) were mixed. A religation sample was made, in the same way as the other samples, but with water instead of the insert. All samples were incubated at room temperature for 3 h. The ligase then was heat deactivated (65 °C, 10 min) and samples were put on ice. The samples were stored at -20 °C until transformation into *E. coli* NEB5α cells.

5.8 PCR Mutagenesis

The site-directed mutagenesis was done with the QuikChange II Site-Directed Mutagenesis Kit. The primer design was done according to the recommended guidelines of the QuikChange II Site-Directed Mutagenesis Kit. In thin-walled PCR tubes 10x reaction buffer (5 µL), MsAcT wild type plasmid DNA solution (1 µL, 13.6 ng), primer mix (2 µL, 125 ng of forward and reverse primer), dNTP mix (1 µL), sterile water (40 µL) and Pfu Ultra HF DNA polymerase (1 µL, 2.5 U) were mixed and reaction was performed in the Thermocycler (1: 95 °C for 30 sec; 2: 95 °C for 30 sec, 70 °C/ 72 °C for 1 min, 68 °C for 6 min, 18 repeats; 3: 68 °C for 2 min, 4: 4 °C storage). The digestion of nonmutated, parental DNA was done by addition of Dpn I restriction enzyme (1 µL, 10 U) to the amplification products, mixing of the samples, spinning down (1 min, 14800 rpm) and incubation at 37 °C for 1 h. The samples were stored at -20 °C until transformation into *E. coli* XL 10 Gold Ultracompetent Cells.

5.9 Colony PCR

After cloning or site-directed mutagenesis, pre-sequencing analysis was done *via* colony PCR of the transformed genes. At least 4 of the grown colonies were picked and singularization plates were done as well as colony PCR: The single colonies were dissolved in sterile water (15 µL), heated (95 °C, 15 min) and centrifuged (15 min, 14000 rpm). In sterile PCR tubes T7 Primer Mix (2.5 µL, 50 pmol/µL T7 terminator und promotor), Dream Taq Green PCR Master Mix 2x (12.5 µL) and the colony supernatant (10 µL) were mixed and PCR was performed on the Thermocycler (1: 95 °C for 15 min; 2: 94 °C for 30 sec, 55 °C for 30 sec, 72 °C for 10 min, 30 repeats; 3: 72 °C for 10 min, 4: 4 °C storage). Analysis was done on agarose gel. Positive hits were sent do sequencing and transformed into *E. coli* BL21 (DE3).

5.10 Transformation

5.10.1 NEB-5alpha

Transformation into *E. coli* NEB5α cells was performed for ligation samples after cloning and with digested PCR products (two step site-directed mutagenesis) according to the High efficiency Transformation Protocol of *E. coli* NEB5α cells. Competent cells were taken from -80 °C and placed on ice for 10 min. Plasmids (5 µL, ligation solution) were transferred into *E. coli* cells (100 µL). The samples were incubated on ice for 30 min, before gentle shaking, followed by shock heating to 42 °C for 30 sec and addition of SOC medium (250 µL). The samples were shaken at 300 rpm for 1 h at 37 °C. Cell solutions were plated (100 µL) on an agar plate (LB, kanamycin) and cultivated overnight at 37 °C. These cells were used for amplification of the plasmids prior to colony PCR and sequencing.

5.10.2 XL10 Gold

Transformation in *E. coli* XL 10 Gold Ultracompetent Cells was performed for digested PCR products (QuikChange II Site-Directed mutagenesis) according to protocol of *E. coli* XL 10 Gold Ultracompetent Cells. Competent cells were taken from -80 °C and placed on ice for 10 min. Cells (50 µL) were aliquoted in pre-cooled sterile microcentrifuge tubes, β-mercaptoethanol (2 µL) was added and the cells were incubated for 10 min, while being gently shaken. Plasmids (1 µL, digestion solution) were transferred into the prepared *E. coli* cells. The samples were incubated on ice for 30 min, and afterwards shock heated at 42 °C for 30 sec before SOC medium (250 µL) was added. The samples were shaken at 300 rpm for 1 h at 37 °C. The entire cell solutions were plated on an agar plate (LB, kanamycin) and cultivated overnight at 37 °C. These cells were used for amplification of the digested PCR products prior to colony PCR and sequencing.

5.10.3 BL21 (DE3)

For all samples that showed the correct gene sequence, transformation into the expression host *E. coli* BL21 (DE3) cells was performed according to High Efficiency Transformation Protocol. Competent cells were taken from -80 °C and placed on ice for 10 min. Plasmids (5 µL) were transferred into *E. coli* cells (100 µL). The samples were incubated on ice for 30 min, gently shaken, and then shock heated at 42 °C for 10 sec before LB medium (250 µL) was added. The samples were shaken with 300 rpm for 1 h at 37 °C. Cell solutions were plated (150 µL) on an agar plate (LB, kanamycin) and cultivated overnight at 37 °C.

5.11 Expression and Purification

5.11.1 Expression and purification of MsAcT-Variants that Target Increased Transesterification- *vs.* Hydrolysis-Activity

For the expression of recombinant MsAcT an ONC (100 µL) was used to inoculate LB-medium (20 mL) with kanamycin (20 µL, 0.05 mg/mL final concentration) in a 50 ml plastic tube. Incubation was performed at 30 °C with 120 rpm horizontally until an OD of approximately 0.6 was reached and expression of MsAcT was induced with addition of IPTG (10 µL, 0.5 mM final concentration). After incubation overnight at 20 °C and 120 rpm, cells were harvested by centrifugation (4500 rpm, 10 °C, 20 min) and washed with buffer (KPi, 200 mM, pH 7.5) in a subsequent centrifugation (14800 rpm, 4 °C, 30 min). The cell pellets (ca. 100 mg) were stored at 4 °C.

Small scale enzyme purification was performed with His SpinTrap TALON Spin Columns. The pellet was dissolved in lysis buffer (100 mg cells/mL buffer, KPi 100 mM, 300 mM NaCl, 10 mM imidazole, pH 7.5) and sonicated (1 sec, 40% amplitude, 0.1 sec on, 0.5 sec off, 1 min pause in between two sonications). The samples were centrifuged (14600 rpm, 30 min, 4 °C) and the lysate filtered (0.45 µm). The columns were equilibrated (2x600 µL lysis buffer followed by centrifugation, 300 rpm, 1 min). The sample (600 µL) was loaded, incubated for 5 min with gentle shaking and the flow-through was removed by centrifugation (300 rpm, 1 min). This step was repeated once. For washing, the lysis buffer (600 µL) was added, the supernatant in the column was resuspended by pipetting, and the wash-fraction removed by centrifugation (300 rpm, 1 min). This step was repeated once. Elution buffer (200 µL, KPi 100 mM, 300 mM NaCl, 500 mM imidazole, pH 7.5) was added, supernatant in the column was resuspended by pipetting, and the purified enzyme solution was eluted by centrifugation (300 rpm, 1 min). This step was repeated once. Efficiency of the purification was validated with SDS PAGE (Figure 35) analysis of all fractions. Desalting was done with PD MidiTrap G-25 according to gravity protocol: Columns were equilibrated with buffer (15 mL, KPi 200 mM, pH 7.5) and the flow through was discarded. Sample (400 µL) was loaded and allowed to enter the bed. Buffer (600 µL) was added and allowed to enter the bed completely. The desalted enzyme was eluted *via* addition of buffer (1.5 mL, KPi 200 mM, pH 7.5). The enzyme solution was stored at -20 °C.

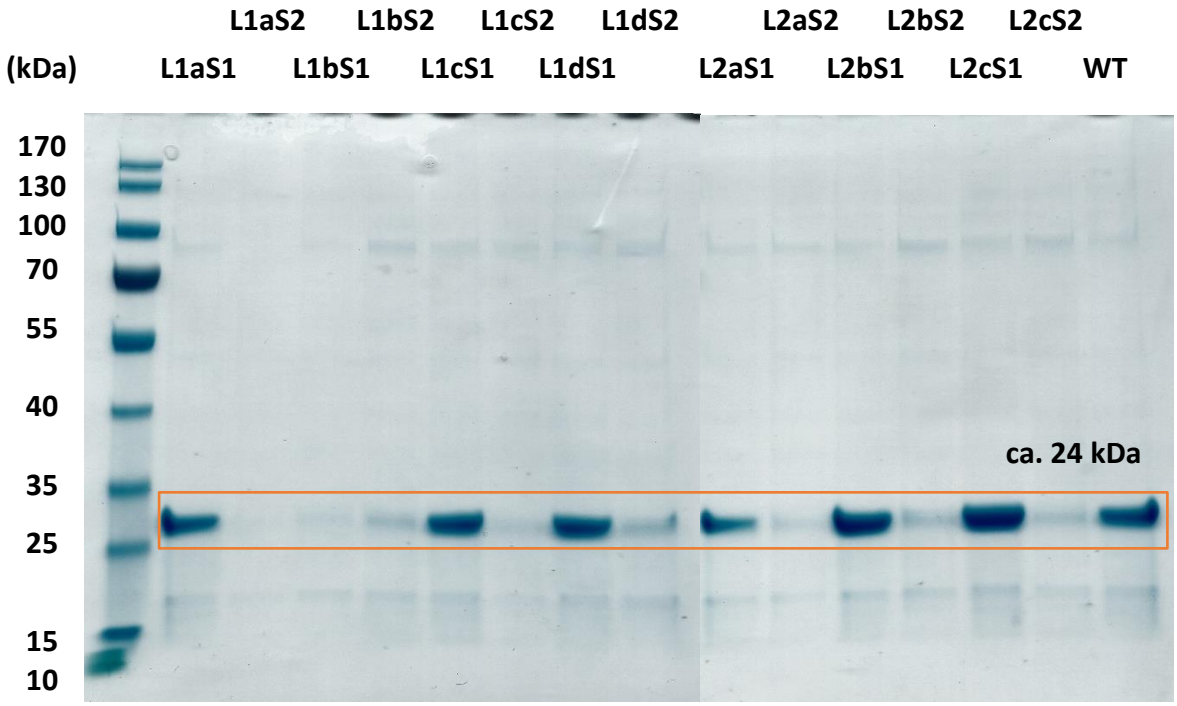


Figure 35: SDS PAGE of MsAcT WT and its variants (L1aS1, L1aS2, L1bS1, L1bS2, L1cS1, L1cS2, L1dS1, L1dS2, L2aS1, L2aS2, L2bS1, L2bS2, L2cS1, L1cS2) purified with His SpinTrap TALON Spin Column with Page Ruler Protein Ladder. The purified enzyme (ca. 24 kDa [16]*) is marked red.*

5.11.2 Expression and Purification of ADHs for Coupled Spectrophotometric Assay

The recombinant YADH1 was overexpressed in LB-medium (750 mL) with additional kanamycin (750 µL, 0.05 mg/mL final concentration) and inoculated with ONC (1.9 mL). Incubation was performed at 30 °C with 120 rpm in a baffled flask until an OD of approximately 0.6 was reached and expression of MsAcT was induced with addition of IPTG (375 µL, 0.5 mM final concentration). After incubation overnight at 20 °C with 120 rpm cells were harvested by centrifugation (5000 rpm, 4 °C, 20 min) and washed with buffer (KPi, 200 mM, pH 7.5) during subsequent centrifugation (5000 rpm, 4 °C, 20 min). The cell pellet was stored at 4 °C.

The recombinant HL ADH was overexpressed in LB-medium (400 mL) with additional kanamycin (400 µL, 0.05 mg/mL) and inoculated with ONC (1.2 mL). Incubation was performed at 25 °C with 120 rpm in a baffled flask until an OD of approximately 0.5 was reached and expression of MsAcT was induced with addition of IPTG (200 µL, 0.5 mM). After incubation overnight at 25 °C with 120 rpm, cells were harvested by centrifugation (4000 rpm, 4 °C, 20 min) and washed with buffer (KPi, 200 mM, pH 7.5) in a subsequent centrifugation (4000 rpm, 4 °C, 20 min). The cell pellet was stored at 4 °C.

For protein purification the cell pellets were resuspended in lysis buffer (KPi 100 mM, NaCl 300 mM, imidazole 10 mM, pH 7.5). Cell lysis was performed *via* sonication (5 min, 40% amplitude, 1.0 sec on, 4.0 sec off). The solution was centrifuged (20 min, 4800 rpm, 4 °C). The supernatant was filtered (0.45 µm). The lysate was loaded on a HisTrap FF histidine-tagged protein purification column. Washing was performed with washing buffer (KPi 100 mM, NaCl 300 mM, imidazole 10 mM, pH 7.5) and elution was induced with elution buffer (KPi 100 mM, NaCl 300 mM, imidazole 500 mM, pH 7.5). Efficiency of the purification was validated with SDS PAGE analysis (Figure 36). The purified enzyme solution was concentrated (10000 MCO, centrifuging for 40 min, 4800 rpm, 10 °C). The enzyme solution was desalted by loading on a PD-10 Desalting Column following gravity protocol. Columns were equilibrated with buffer (25 mL, KPi 200 mM, pH 7.5) and the flow through was discarded. The sample (2.5 mL) was loaded and was allowed to enter the bed. The desalted enzyme was eluted *via* addition of buffer (3.5 mL, KPi 200 mM, pH 7.5). The enzyme solution was stored at -20 °C.

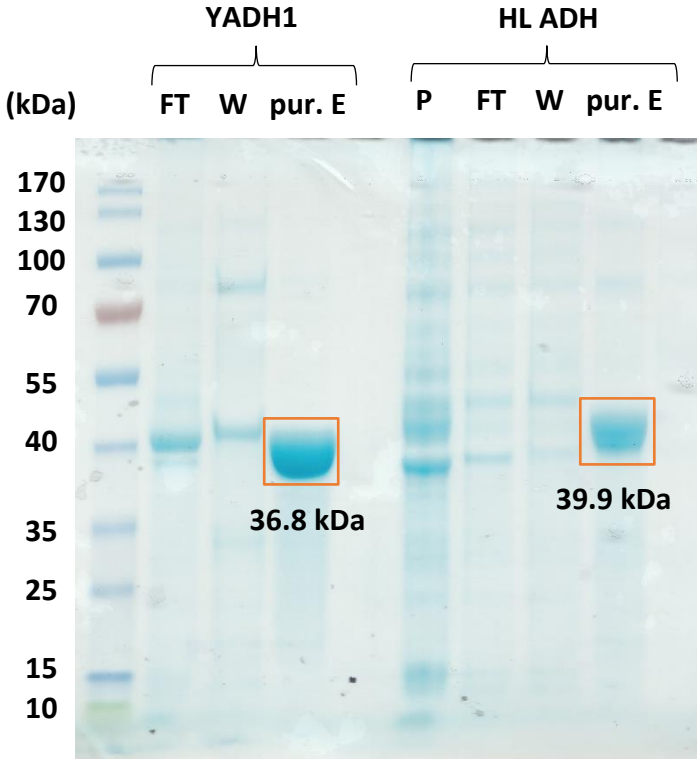


Figure 36: SDS PAGE of fractions from the purification using HisTrap FF histidine-tagged protein purification column on an Äkta chromatography system. From left to right: flow through of YADH1, wash of YADH1, purified YADH1, pellet of HL ADH, flow through of HL ADH, wash of HL ADH and purified HL ADH. Purified enzymes were marked in red.

5.11.3 Expression of MsAcT-Variants that Target Higher Enantio-Selectivity

The recombinant MsAcT and its variants were overexpressed in LB-medium (400 mL) with additional kanamycin (400 µL, 0.05 mg/mL final concentration) and inoculated with ONC

(1.0 mL). Incubation was performed at 30 °C with 120 rpm in a baffled flask until an OD of approximately 0.6 was reached and expression of MsAcT was induced with addition of IPTG (200 µL, 0.5 mM final concentration). After incubation overnight at 20 °C with 120 rpm cells were harvested by centrifugation (5000 rpm, 4 °C, 10 min) and washed with buffer (KPi, 200 mM, pH 7.5) in a subsequent centrifugation (5000 rpm, 4 °C, 10 min). The cell pellet (2-3 g) was stored at 4 °C. The cell pellets were resuspended in buffer (KPi 200 mM, pH 7.5). Cell lysis was performed *via* sonication (5 min, 40% amplitude, 1.0 sec on, 4.0 sec off). The solution was centrifuged (30 min, 15000 rpm, 4 °C). The cell free extract was lyophilized and stored at 4 °C.

5.12 Spectrophotometric Assay

The buffer (KPi 200 mM, pH 7.5) was saturated with **2** as this allows more precise control of the concentration of the volatile acetyl donor **2** for the coupled assay. **2** (15 mL) and buffer (15 mL, KPi 200 mM, pH 7.5) were therefore mixed in a 50 mL tube and incubated (10 min, 700 rpm). The aqueous phase of the resulting mixture was used for the reactions. Stock solutions of the substrates **1a** and **1b** (25 mM), co-factors ($NAD(P)^+$; 10 mM), purified YADH1 (3.7 mg/mL in KPi 200 mM, pH 7.5), purified HL ADH (0.3 mg/mL in KPi 200mM, pH7.5) and purified MsAcT (5.0 µg/mL) were prepared in buffer (KPi 200 mM, pH 7.5).

For measurement of the esterification, substrate stock **1a** (50 µL, 10 mM) and YADH1 solution (10 µL, 0.4 mg/mL) were mixed and the reaction was started with addition of co-factor stock solution (40 µL, 4 mM) and purified MsAcT solution (10 µL, 0.5 µg/mL).

For measurement of the hydrolysis, substrate stock **1b** (50 µL, 10 mM) and HL-ADH solution (10 µL, 25.0 µg/mL) were mixed and the reaction was started with addition of co-factor stock solution (40 µL, 4 mM) and purified MsAcT solution (10 µL, 0.5 µg/mL).

The spectrophotometric assay was proceeded on the SpectraMax M2 Microplate Reader by Molecular Devices measuring at 340 nm for 10 min at 30 °C with continuous shaking.

5.13 Synthesis of Reference Compounds

For the assignment of the peaks in the GC chromatograms esters (*R*)-**3b**, *rac*-**7b**, *rac*-**8b**, *rac*-**10b**, *rac*-**11b**, *rac*-**12b**, *rac*-**13b**, *rac*-**14b**, *rac*-**15b** and *rac*-**16b** to the corresponding alcohols (*R*)-**3a**, *rac*-**7a**, *rac*-**8a**, *rac*-**10a**, *rac*-**11a**, *rac*-**13a**, *rac*-**14a**, *rac*-**15a** and *rac*-**16a** were synthesized (Figure 37). [60]

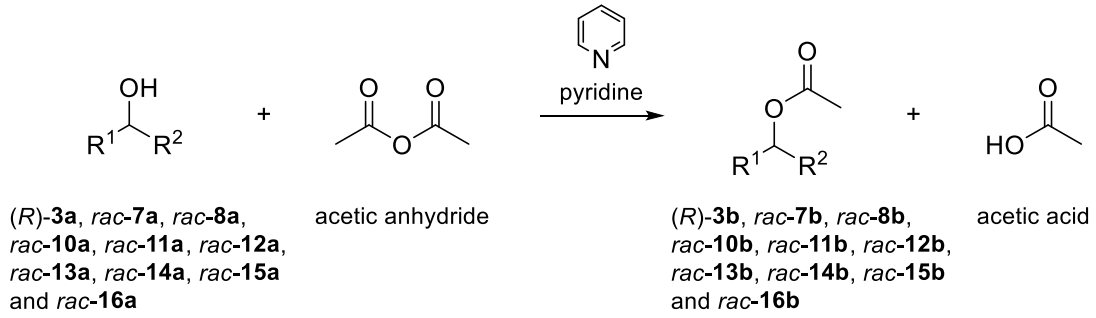


*Figure 37: Reaction scheme of the acetylation of various alcohols (R)-**3a**, rac-**7a**, rac-**8a**, rac-**10a**, rac-**11a**, rac-**12a**, rac-**13a**, rac-**14a**, rac-**15a** and rac-**16a** in order to obtain reference substances (R)-**3b**, rac-**7b**, rac-**8b**, rac- rac-**10b**, rac-**11b**, rac-**12b**, rac-**13b**, rac-**14b**, rac-**15b** and rac-**16b**.*

The reactions were performed in a 4 mL glass vial with a septum lid. The substrates (*R*)-**3a**, *rac*-**7a**, *rac*-**8a**, *rac*-**10a**, *rac*-**11a**, *rac*-**12a**, *rac*-**13a**, *rac*-**14a**, *rac*-**15a** and *rac*-**16a** (1 mmol), were mixed with acetic anhydride (150 µL, 1.5 mmol) and the reaction was started with addition of pyridine (100 µL, 1.25 mmol). The reactions were stirred overnight at room temperature and stopped with addition of water (1 mL) and acidified with aq. HCl (1 M). The reactions were worked up by extraction (3 x 5 mL diethyl ether), washing with HCl (2 x 5 mL, 1 M) and drying (Na_2SO_4). Solvent was removed under an air stream.

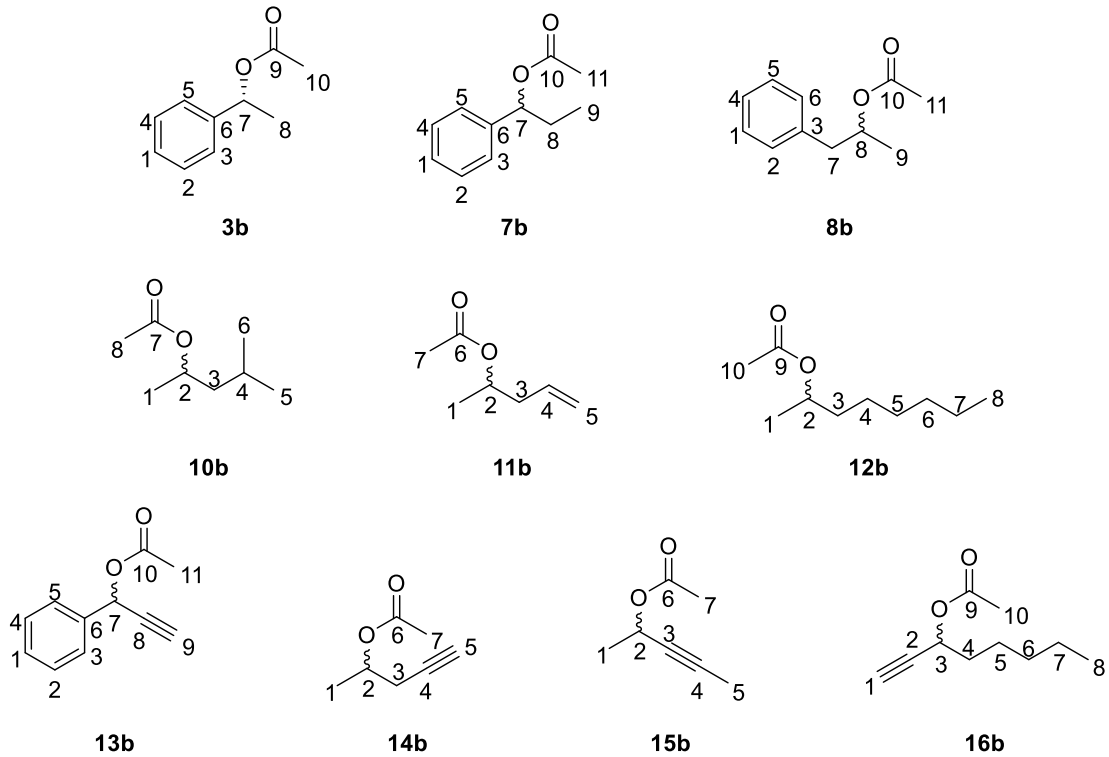


*Figure 38: Synthesized reference compounds (R)-**3b**, rac-**7b**, rac-**8b**, rac-**10b**, rac-**11b**, rac-**12b**, rac-**13b**, rac-**14b**, rac-**15b** and rac-**16b** with position labels for NMR assignment.*

(*R*)-**3b**:

Yield: 86 mg (0.5 mmol, 53%), [164.2 g/mol]

^{1}H NMR (300 MHz, $CDCl_3$) δ 7.48 – 7.24 (m, 5H, H-1, H-2, H-3, H-4, H-5), 5.91 (q, *J* = 6.6 Hz, 1H, H-7), 2.10 (s, 3H, H-10), 1.56 (d, *J* = 6.6 Hz, 3H, H-1).

^{13}C NMR (75 MHz, $CDCl_3$) δ 170.35 (C-9), 141.66 (C-6), 128.49 (C-4, C-2), 127.86 (C-1), 126.09 (C-3, C-5), 72.32 (C-7), 22.22 (C-8), 21.37 (C-10).

rac-**7b**:

Yield: 75 mg (0.4 mmol, 41%), [178.2 g/mol]

^{1}H NMR (300 MHz, $CDCl_3$) δ 7.41 – 7.26 (m, 5H, H-1, H-2, H-3, H-4, H-5), 5.69 (t, *J* = 6.9 Hz, 1H, H-7), 2.10 (s, 3H, H-11), 2.03 – 1.75 (m, 2H, H-8), 0.90 (t, *J* = 7.4 Hz, 3H, H-9).

^{13}C NMR (75 MHz, $CDCl_3$) δ 170.44 (C-10), 140.53 (C-6), 128.37 (C-1), 127.81 (C-2, C-4), 126.56 (C-3, C-5), 77.36 (C-7), 29.29 (C-8), 21.28 (C-11), 9.92 (C-9).

rac-**8b**:

Yield: 101 mg (0.6 mmol, 57%), [178.2 g/mol]

^{1}H NMR (300 MHz, $CDCl_3$) δ 7.36 – 7.14 (m, 5H, H-1, H-2, H-3, H-4, H-5), 5.13 (h, *J* = 6.4 Hz, 1H, H-8), 2.86 (ddd, *J* = 53.8, 13.6, 6.6 Hz, 2H, H-7), 2.02 (s, 3H, H-11), 1.24 (d, *J* = 6.3 Hz, 3H, H-9).

^{13}C NMR (75 MHz, $CDCl_3$) δ 170.56 (C-10), 137.60 (C-6), 129.41 (C-2, C-4), 128.32 (C3, C-5), 126.46 (C-1), 71.47 (C-8), 42.23 (C-7), 21.33 (C-9), 19.45 (C-11).

rac-**10b**:

Yield: 59 mg (0.4 mmol, 40%), [144.2 g/mol]

^{1}H NMR (300 MHz, $CDCl_3$) δ 5.06 – 4.94 (m, 1H, H-2), 2.04 (s, 3H, H-8), 1.72 – 1.50 (m, 2H, H-3), 1.34 – 1.24 (m, 1H, H-4), 1.22 (t, *J* = 4.8 Hz, 3H, H-1), 0.91 (dd, *J* = 6.5, 2.4 Hz, 6H, H-5, H-6).

^{13}C NMR (75 MHz, $CDCl_3$) δ 170.82 (C-7), 69.47 (C-2), 45.09 (C-3), 24.72 (C-4), 22.86 (C-5), 22.35 (C-6), 21.40 (C-8), 20.46 (C-1).

rac-**11b**:

Yield: 110 mg (0.9 mmol, 80%), [128.2 g/mol]

^{1}H NMR (300 MHz, $CDCl_3$) δ 5.77 (ddt, *J* = 17.3, 10.3, 7.1 Hz, 1H, H-6), 5.10 (ddd, *J* = 11.2, 6.8, 1.4 Hz, 2H, H-5), 4.97 (h, *J* = 6.3 Hz, 1H, H-4), 2.41 – 2.22 (m, 2H, H-3), 2.04 (s, 3H, H-7), 1.23 (d, *J* = 6.3 Hz, 3H, H-1).

^{13}C NMR (75 MHz, $CDCl_3$) δ 170.66 (C-6), 133.67 (C-4), 117.66 (C-5), 70.04 (C-2), 40.25 (C-3), 21.32 (C-7), 19.45 (C-1).

rac-**12b**:

Yield: 8.9 g (51.7 mmol, 82%), [172.3 g/mol]

^{1}H NMR (300 MHz, $CDCl_3$) δ 4.96 – 4.84 (m, 1H, H-2), 2.04 (s, 3H, H-10), 1.67 – 1.23 (m, 10H, H-3, H-4, H-5, H-6, H-7), 1.21 (t, *J* = 5.2 Hz, 3H, H-1), 0.89 (t, *J* = 6.7 Hz, 3H, H-8).

^{13}C NMR (75 MHz, $CDCl_3$) δ 170.81 (C-9), 71.08 (C-2), 35.92 (C-3), 31.73 (C-6), 29.11 (C-4), 25.36 (C-5), 22.57 (C-7), 21.39 (C-10), 19.95 (C-1), 14.05 (C-8).

rac-**13b**:

Yield: 98 mg (0.6 mmol, 53%), [174.2 g/mol]

^{1}H NMR (300 MHz, $CDCl_3$) δ 7.59 – 7.51 (m, 2H, H-3, H-5), 7.47 – 7.35 (m, 3H, H-1, H-2, H-4), 6.48 (d, *J* = 2.3 Hz, 1H, H-7), 2.68 (d, *J* = 2.3 Hz, 1H, H-9), 2.13 (d, *J* = 5.3 Hz, 3H, H-11).

^{13}C NMR (75 MHz, $CDCl_3$) δ 169.72 (C-10), 136.42 (C-6), 129.10 (C-1), 128.71 (C-2, C-4), 127.70 (C-3, C-5), 80.24 (C-8), 75.39 (C-9), 65.30 (C-7), 21.04 (C-11).

rac-**14b**:

Yield: 94 mg (0.7 mmol, 70%), [126.2 g/mol]

^{1}H NMR (300 MHz, $CDCl_3$) δ 5.09 – 4.95 (m, 1H, H-2), 2.55 – 2.39 (m, 2H, H-3), 2.07 (s, 3H, H-7), 2.03 (t, *J* = 2.7 Hz, 1H, H-5), 1.35 (d, *J* = 6.3 Hz, 3H, H-1).

^{13}C NMR (75 MHz, $CDCl_3$) δ 170.46 (C-6), 79.70 (C-4), 70.44 (C-5), 68.53 (C-2), 25.52 (C-3), 21.21 (C-7), 19.03 (C-1).

rac-**15b**:

Yield: 98 mg (0.8 mmol, 73%), [126.2 g/mol]

^{1}H NMR (300 MHz, $CDCl_3$) δ 5.42 (qq, *J* = 6.5, 2.1 Hz, 1H, H-2), 2.08 (s, 3H, H-7), 1.85 (d, *J* = 2.1 Hz, 3H, H-5), 1.46 (d, *J* = 6.6 Hz, 3H, H-1).

^{13}C NMR (75 MHz, $CDCl_3$) δ 170.07 (C-6), 81.08 (C-3), 77.73 (C-4), 60.83 (C-2), 21.67 (C-7), 21.14 (C-1), 3.57 (C-5).

rac-**16b**:

Yield: 139 mg (0.8 mmol, 80%), [168.2 g/mol]

^{1}H NMR (300 MHz, $CDCl_3$) δ 5.36 (td, *J* = 6.7, 2.1 Hz, 1H, H-3), 2.46 (d, *J* = 2.2 Hz, 1H, H-1), 2.10 (s, 3H, H-10), 1.79 (ddd, *J* = 12.2, 7.8, 3.9 Hz, 2H, H-4), 1.54 – 1.40 (m, 2H, H-5), 1.39 – 1.25 (m, 4H, H-6, H-7), 0.91 (dd, *J* = 8.9, 4.9 Hz, 3H, H-8).

^{13}C NMR (75 MHz, $CDCl_3$) δ 169.97 (C-9), 81.32 (C-2), 73.36 (C-1), 63.82 (C-3), 34.51 (C-4), 31.23 (C-6), 24.53 (C-5), 22.45 (C-7), 20.99 (C-10), 13.94 (C-8).

5.14 Biotransformations With MSAcT-WT and its Variants

5.14.1 Activity Assay for Engineered Variants of MsAcT that target Increased Transesterification- *vs*. Hydrolysis-Activity

Biotransformations were performed for the determination of enzyme activities towards acyl transfer and towards hydrolysis. The buffer (KPi 200 mM, pH 7.5) was saturated with **2** as this allows precise control of the concentration of the volatile acetyl donor **2** for the coupled assay. EtOAc (15 mL) and buffer (15 mL, KPi 200 mM, pH 7.5) were mixed in a 50 mL tube, shaken (10 min, 700 rpm) and the aqueous phase was used for the reactions. Stock solutions of the substrates benzyl alcohol (**1a**) and benzyl acetate (**1b**, 10.2 mM) were prepared in the EtOAc-saturated buffer. All stock solutions were incubated (1.5 h, 30 °C), and then a portion of the substrate stocks **1a** or **1b** (980 µL, 10 mM) was transferred in a 1 mL screw top glass vial. The reactions were started with the addition of purified MsAcT solution (20 µL, 0.2 µg/mL) and always run as triplicates. Blank reactions were prepared with buffer (20 µL, KPi 200 mM, pH 7.5) instead of enzyme solution. The biotransformations were incubated at 30 °C on a tabletop shaker (500 rpm) for the indicated time (10 min, 30 min, 50 min, 24 h or in case of the time study: 10 min, 30 min, 50 min, 2 h, 3.5 h, 5 h, 6.5 h, 8 h, 10 h, 24 h and 26 h). The reactions were stopped with extraction (2x400 µL, then 1x200 µL MTBE including 20 mM IS 1-decanol), dried (Na_2SO_4) and subjected to GC-FID analysis.

For the further tests of the variants on acyl transfer reactions two different reaction conditions were tested. Substrate **1a** (10 µL, 100 mM), EtOAc (100 µL, 1 M), purified enzyme solution of variants L2bS1 and L2cS1 (30 µg/mL or 6 µg/mL) and buffer (KPi 200 mM, pH 7.5, 1 mL final volume) were added into a 1 mL screw top glass vial. Biotransformations were performed as triplicates for each variant and each reaction condition and incubated at 30 °C for 24h on a tabletop shaker (500 rpm). Reactions were stopped with extraction (2x400 µL, then 1x200 µL MTBE including 20 mM IS 1-decanol), dried (Na_2SO_4) and subjected to GC-FID analysis.

5.14.2 Screening of the MsAcT Variant Library Targeting Higher Enantio-selectivity

Biotransformations were performed to screen the entire single and double mutant library for the enantio-selectivity of the MsAcT. Stock solutions of the substrates **3a**/**3b** (1 M) in the acyl donor EtOAc and enzyme solutions with the lyophilized CFE (10 mg/mL) in buffer (KPi, 200 mM, pH 7.5) were prepared. The substrate stocks **3a**/**3b** (100 µL, 100 mM) were mixed with buffer (870 µL, KPi, 200 mM, pH 7.5) in a 1 mL screw top glass vial. The reactions were started with the addition of CFE solution (30 µL, 0.3 mg/mL). All biotransformations were run as triplicates. Blank reactions were prepared with buffer (30 µL, KPi 200 mM, pH 7.5) instead of enzyme solution. The biotransformations were incubated at 30 °C on a tabletop shaker (500 rpm) for 5 h. Reactions were stopped with extraction (2x500 µL MTBE including 20 mM IS 1-decanol), dried (Na_2SO_4) and subjected to GC-FID analysis.

5.14.3 Reaction Engineering

Different reaction conditions were tested to optimize activity and selectivity of the MsAcT WT. A stock solution of the substrate **3a** (1 M) in the acyl donor EtOAc and enzyme solution with the lyophilized CFE (50 mg/mL) in buffer (KPi, 200 mM, pH 7.5) were prepared. The substrate stock **3a** (100 µL, 100 mM) was mixed with buffer (final volume 1 mL, KPi, 200 mM, pH 7.5) in a 1 mL screw top glass vial. The reactions were started with the addition of CFE solution (6 µL/0.3 mg/mL, 10 µL/0.5 mg/mL, 20 µL/1.0 mg/mL, 30 µL/1.5 mg/mL, 40 µL/2.0 mg/mL, 60 µL/3.0mg/mL or 80 µL/4 mg/mL). All biotransformations were run as triplicates. Blank reactions were prepared with buffer (KPi 200 mM, pH 7.5) instead of enzyme solution. The biotransformations were incubated at 30 °C on a tabletop shaker (500 rpm) for 5 h or 24 h. Reactions were stopped with extraction (2x500 µL MTBE including 20 mM IS 1-decanol), dried (Na_2SO_4) and subjected to GC-FID analysis.

5.14.4 Acyl-Donor Screening

Biotransformations were performed to screen different acyl donors with MsAcT WT and the variants F154A, F174A. Stock solutions of enzymes were prepared with lyophilized CFE

(50 mg/mL) in buffer (KPi, 200 mM, pH 7.5). The substrate **3a** (10 µL, 80 mM), enzyme solution (10 µL, 0.5 mg/mL) and buffer (880 µL, KPi, 200 mM, pH 7.5) were mixed in a 1 mL screw top glass vial. The reactions were started with the addition of the acyl donors **2**, **4**, **5**, **6** (100 µL). All biotransformations were run as triplicates. Blank reactions were prepared with buffer (10 µL, KPi 200 mM, pH 7.5) instead of enzyme solution. The biotransformations were incubated at 30 °C on a tabletop shaker (500 rpm) for 5 h. Reactions were stopped with extraction (2x500 µL MTBE including 20 mM IS 1-decanol), dried (Na_2SO_4) and subjected to GC-FID analysis.

5.14.5 Substrate Screening

Different substrates were screened with selected MsAcT variants (F154V, F154A, F154L, F174V, F174A) and WT. Stock solutions of the substrates **3a**, **7a**, **8a**, **9a**, **10a**, **11a**, **12a**, **13a**, **14a**, **15a**, **16a** (1 M) in the acyl donor **4** and enzyme solutions with the lyophilized CFE (50 mg/mL) in buffer (KPi, 200 mM, pH 7.5) were prepared. The substrate stocks (100 µL, 100 mM) were mixed with buffer (890 µL, KPi, 200 mM, pH 7.5) in a 1 mL screw top glass vial. The reactions were started with the addition of CFE solution (10 µL, 0.5 mg/mL). All biotransformations were run as triplicates. Blank reactions were prepared with buffer (10 µL, KPi 200 mM, pH 7.5) instead of enzyme solution. The biotransformations were incubated at 30 °C on a tabletop shaker (500 rpm) for 5 h. Reactions were stopped with extraction (2x500 µL MTBE including 20 mM IS 1-decanol), dried (Na_2SO_4) and subjected to GC-FID analysis.

6 Literature

[1] K. Faber, *Pure Appl. Chem.*, 69, 1613–1632, 1997.

[2] R. N. Patel, *Green Biocatalysis*. Hoboken, NJ: John Wiley & Sons, Inc, 2016.

[3] P. Grunwald, *Industrial Biocatalysis*. Singapore: Jenny Stanford Publishing, 2014.

[4] P. Hoyos, V. Pace, M. Hernaiz, and A. Alcantara, *Curr. Green Chem.*, 1, 155–181, 2013.

[5] N. J. Turner and E. O'Reilly, *Nat. Chem. Biol.*, 9, 285–288, 2013.

[6] B. M. Nestl, S. C. Hammer, B. A. Nebel, and B. Hauer, *Angew. Chemie Int. Ed.*, 53, 3070–3095, 2014.

[7] A. Goswami; and J. D. Stewart, Eds., *Organic Synthesis Using Biocatalysis*. Amsterdam: Elsevier, 2015.

[8] L. Hilterhaus, A. Liese, U. Kettling, and G. Antranikian, Eds., *Applied Biocatalysis: From Fundamental Science to Industrial Applications*. Weinheim, Germany: Wiley-VCH Verlag GmbH & Co. KGaA, 2016.

[9] A. S. Bommarius and B. R. Riebel, *Biocatalysis: Fundamentals and applications*, 1st ed., Weinheim: Wiley-VCH, 1–18, 2004.

[10] R. A. Sheldon, *Chem. Soc. Rev.*, 41, 1437–1451, 2012.

[11] J. C. Anastas and P.T. Warner, *Oxford Univ. Press*, 30, 1998.

[12] R. A. Sheldon and J. M. Woodley, *Chem. Rev.*, 118, 801–838, 2018.

[13] M. T. Reetz, *J. Am. Chem. Soc.*, 135, 12480–12496, 2013.

[14] F. H. Arnold, *Angew. Chemie Int. Ed.*, 57, 4143–4148, 2018.

[15] K. Faber, *Biotransformations in Organic Chemistry*, 7th ed., Berlin, Heidelberg: Springer Berlin Heidelberg, 31–139, 2018.

[16] I. Mathews, M. Soltis, M. Saldajeno, G. Ganshaw, R. Sala, W. Weyler, M. A. Cervin, G. Whited and R. Bott, *Biochemistry*, 46, 8969–8979, 2007.

[17] R. A. Sheldon, *Chem. Ind.*, 23, 903–906, 1992.

[18] C.G. Brundtland, "Report of the World Commission on Environment and Development: Our Common Future," Oxford, 1987.

[19] K. Faber, *Biotransformations in Organic Chemistry*, 7th ed., Springer International Publishing, 407–414, 2018.

[20] D. E. Edmondson and G. Gadda, *Protein Engineering Handbook*, vol. 1, Eds. Weinheim, Germany: Wiley-VCH Verlag GmbH & Co. KGaA, 1–13, 2011.

[21] G. Winter, A. R. Fersht, A. J. Wilkinson, M. Zoller and M. Smith, *Nature*, 299, 756–758, 1982.

[22] M. Smith, *Bioscience Rep.*, 14, 51–66, 1994.

[23] U. T. Bornscheuer, G. W. Huisman, R. J. Kazlauskas, S. Lutz, J. C. Moore, and K. Robins, *Nature*, 485, 185–194, 2012.

[24] S. Lutz and S. M. Iamurri, *Protein Engineering Methods and Protocols*, Humana Press, New York, NY, 1–12, 2018.

[25] F. H. Arnold, *Accounts Chem. Res.*, 31, 125–131, 1998.

[26] W. P. C. Stemmer, *Nature*, 370, 389–391, 1994.

[27] W. P. Stemmer, *P. Natl. Acad. Sci.*, 91, 10747–10751, 1994.

[28] M. T. Reetz, L.-W. Wang, and M. Bocola, *Angew. Chemie Int. Ed.*, 45, 1236–1241, 2006.

[29] S. Lutz, *Curr. Opin. Biotech.*, 21, 734–743, 2010.

[30] I. V. Korendovych, *Protein Engineering Methods and Protocols*, vol. 1685, Humana Press, New York, NY, 15–23, 2018.

[31] M. T. Reetz, *P. Natl. Acad. Sci.*, 101, 5716–5722, 2004.

[32] V. L. Schramm, *Arch. Biochem. Biophys.*, 433, 13–26, 2005.

[33] C. F. Butler, C. Peet, A. E. Mason, M. W. Voice, D. Leys and A. W. Munro, *J. Biol. Chem.*, 288, 25387–25399, 2013.

[34] G. Kiss, N. Çelebi-Ölçüm, R. Moretti, D. Baker and K. N. Houk, *Angew. Chemie Int. Ed.*, 52, 5700–5725, 2013.

[35] L. Brady *et al.*, *Nature*, 343, 767–770, 1990.

[36] D. Blow, *Nature*, 343, 694–695, 1990.

[37] J. D. Schrag, Y. Li, S. Wu, and M. Cygler, *Nature*, 351, 761–764, 1991.

[38] J. L. Sussman, M. Harel, F. Frolow, C. Oefner, A. Goldmant, L. Toker, I. Silman, *Science*, 253, 1991.

[39] B. C. Chen and C. J. Sih, *Angew. Chemie Int. Ed.*, 28, 695–707, 1989.

[40] C. J. Sih and S. Wu, *Top. Stereochem.*, 19, 63–125, 1989.

[41] C. Gruber, I. Lavandera, K. Faber and W. Kroutil, *Adv. Synth. Catal.*, 348, 1789–1805, 2006.

[42] S. Martin, S. S. Woodard, T. Katsuki, Y. Yamada, M. Ikeda and K. B. Sharpless, *J. Am. Chem. Soc.*, 103, 6237–6240, 1981.

[43] G. Bredig and K. Fajans, *Ber. Dtsch. Chem. Ges.*, 41, 752–763, 1908.

[44] R. E. Gawley, *J. Org. Chem.*, 71, 2411–2416, 2006.

[45] A. J. J. Straathof and J. J.A., *Enzyme Microb. Tech.*, 21, 559–571, 1997.

[46] C. S. Chen, S. H. Wu, G. Girdaukas and C. J. Sih, *J. Am. Chem. Soc.*, 109, 2812–2817, 1987.

[47] G. Langrand, J. Baratti, G. Buono and C. Triantaphylides, *Biocatalysis*, 1, 231–248, 1988.

[48] U. T. Bornscheuer and R. J. Kazlauskas, *Hydrolases in Organic Synthesis*, 2nd ed. Wiley, 2006.

[49] M. Paravidino, P. Böhm, H. Gröger and U. Hanefeld, *Enzyme Catalysis in Organic Synthesis*, vol. 1, Weinheim, Germany: Wiley-VCH Verlag GmbH & Co. KGaA, 249–362, 2012.

[50] K. Faber, *Biotransformations in Organic Chemistry*, 7th ed., Berlin, Heidelberg: Springer Berlin Heidelberg, 315–355, 2018.

[51] L. Wiermans, S. Hofzumahaus, C. Schotten, L. Weigand, M. Schallmey, A. Schallmey and P. Domínguez de María, *ChemCatChem*, 5, 3719–3724, 2013.

[52] N. de Leeuw, G. Torrelo, C. Bisterfeld, V. Resch, L. Mestrom, E. Straulino, L. Van der Weel and U. Hanefeld, *Adv. Synth. Catal.*, 360, 242–249, 2018.

[53] M. Finnveden, S. Semlitsch, O. He and M. Martinelle, *Catal. Sci. Technol.*, 9, 4920–4927, 2019.

[54] M. Kazemi, X. Sheng, W. Kroutil and F. Himo, *ACS Catal.*, 8, 10698–10706, 2018.

[55] L. Mestrom, J. G. R. Claessen and U. Hanefeld, *ChemCatChem*, 11, 2004–2010, 2019.

[56] M. Kazemi, X. Sheng and F. Himo, *Chem –Eur. J.*, 25, 11945–11954, 2019.

[57] O. De Smidt, J. C. Preez and J. Albertyn, *FEMS Yeast Res.*, 8, 967–978, 2008.

[58] P. Nikolova and P. Ward, *Biotechnol. Bioeng.*, 39, 870–876, 1992.

[59] W. Stampfer, B. Kosjek, C. Moitzi, W. Kroutil and K. Faber, *Angew. Chemie Int. Ed.*, 41, 1014–1017, 2002.

[60] R. Beckert, E. Fanghänel, W. D. Habicher, H.-J. Knölker, P. Metz, and K. Schwetlick, *Organikum*, 24th ed., Weinheim, Germany: Wiley-VCH Verlag GmbH & Co. KGaA, 468–637, 2015.

[61] R. A. Sheldon, *Pure Appl. Chem.*, 72, 1233–1246, 2000.

7 Abbreviations

ADH ..... Alcohol dehydrogenase (EC 1)

C ..... Conversion of a reaction

CFE..... Cell free extract

DFT ..... Density-Functional Theory

DNA ..... Deoxyribonucleic acid

DV ..... Double variant

E ..... Enantiomeric ratio

e.e. ..... Enantiomeric excess

EC 1..... Enzyme Commission number of oxidoreductases

EC 3..... Enzyme Commission number of hydrolases

EtOAc..... Ethyl acetate

GC(-FID/MS) ..... Gas chromatography (with flame ionization detector/ mass spectrometry detector)

His6-Tag..... Amino acid motif in proteins consisting of six His-residues at the N-/ C-terminus of a protein for affinity purification of recombinant proteins

HL ADH..... Horse liver alcohol dehydrogenase

IPTG ..... Isopropyl-β-D-thiogalactopyranosid

IS ..... Internal standard for calibration

K ..... Reaction equilibrium constant

KPi-buffer..... Potassium phosphate buffer

LB-medium ..... Lysogeny broth medium

MOPS buffer ..... 3-(N-Morpholino)propansulfonsäure

MsAcT..... Acyl transferase derived from *Mycobacterium smegmatis*

MTBE..... Methyl-tert-butylether

NAD(P)$^+$/ NAD(P)H ..... Nicotinamide adenine dinucleotide (phosphate)/ reduced form

NMR..... Nuclear magnetic resonance spectroscopy

OD ..... Optical density measurement

ONC ..... Overnight culture

PCR ..... Polymerase chain reaction

SDS PAGE ..... sodium dodecyl sulfate–polyacrylamide gel electrophoresis

SGNH ..... Enzyme superfamily of serine esterases that are characterized by their fold (similar to flavo proteins) consisting of a three-layer alpha/ beta/ alpha structure, where the beta-sheets are composed of five parallel strands

SOC medium ..... Super optimal broth medium for cell transformations

TAE-buffer ..... Buffer solution containing Tris base, acetic acid and EDTA (Ethylenediaminetetraacetic acid)

TS ..... Transition state

UV ..... Ultraviolet radiation

Vis ..... Visible light

YADH1 ..... Alcohol dehydrogenase derived from *Saccharomyces cerevisiae* (PDB Code P00330)

WT ..... Wild type enzyme

XRD ..... X-ray diffraction

8 Appendix

8.1 Agarose Gels

Two characteristic agarose gels are depicted in Figure 39 and Figure 40.

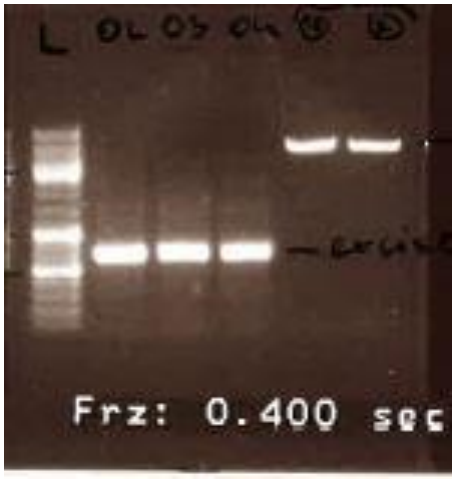


Figure 39: Agarose gel of the restriction ETJ-EA-046-08 with the variants of MsAcT of the correct size (622 bp) and the vector (5369 bp), from left to right: Gene Ruler DNA Ladder Mix, restricted L1cS1, restricted L2cS1, restricted L2cs2, restricted pET 28a(+) vector

Figure 40: Agarose gel of the colony PCR ETJ-EA-039-38 with variant at the correct size (936 bp), from left to right: Gene Ruler DNA Ladder Mix, colonies 1-4 of A55S, one colony of V125F, and colonies 1-4 of F174L

8.2 Sequences

Restriction Sites Nde I, Xho I, start-codon, stop-codon and mutations are highlighted.

8.2.1 Wild Type MsAcT (pEG 32)

gtgccgcgcggcagccatATGGCAAAACGTATTCTGTGTTTTGGTGATAGCCTGACCTGGGGTTGG
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TTAGTCCGCCTCCGCTGGCACCGATGCCGCATCCGTGGTTTCAGCTGATTTTTGAAGGTGG
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GCCGTTTTTTGATGCAGGTAGCGTTATTAGCACCGATGGTGTTGATGGTATTCACTTTACC
GAAGCCAATAATCGTGATCTGGGTGTTGCACTGGCCGAACAGGTTCGTAGCCTGCTGTAA
ctcgagccactgagatccggc

8.2.2 Variants of MsAcT that Target Increased Transesterification- vs. Hydrolysis-Activity

L1aS1

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L1aS2

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L1bS1

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L1bS2

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L1cS1

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L1cS2

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L1dS1

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L2cS1

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L2cS2

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8.2.3 Variants of MsAcT that Target Higher Enantio-Selectivity

A55S

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A55V

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S54M

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V125L

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V125F

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F150V

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8.3 Primer

A55S

(DNA template: WT MsAcT)

Forward: ATTGAAGAAGGTCTGAGCTCACGTACCACCAATATTG

Reverse: CAATATTGGTGGTACGTGAGCTCAGACCTTCTTCAAT

A55V

(DNA template: WT MsAcT)

Forward: TGAAGAAGGTCTGAGCGTACGTACCACCAATATTG

Reverse: CAATATTGGTGGTACGTACGCTCAGACCTTCTTCA

S54M

(DNA template: WT MsAcT)

Forward: AGATTTTGAAGTTATTGAAGAAGGTCTGATGGCACGTACCACCAAT

Reverse: ATTGGTGGTACGTGCCATCAGACCTTCTTCAATAACTTCAAAATCT

V125L

(DNA template: WT MsAcT)

Forward: CAGTGCCGGTGGTCTTGGCACCACCTA

Reverse: TAGGTGGTGCCAAGACCACCGGCACTG

V125F

(DNA template: WT MsAcT)

Forward: CCAGTGCCGGTGGTTTTGGCACCACCTAT

Reverse: ATAGGTGGTGCCAAAACCACCGGCACTGG

F150V

(DNA template: WT MsAcT)

Forward: GATGCCGCATCCGTGGGTTCAGCTGATTTTTGAA

Reverse: TTCAAAAATCAGCTGAACCCACGGATGCGGCATC

F150A

(DNA template: WT MsAcT)

Forward: CCGATGCCGCATCCGTGGGCTCAGCTGATTTTTGAAGG

Reverse: CCTTCAAAAATCAGCTGAGCCCACGGATGCGGCATCGG

F154V

(DNA template: WT MsAcT)

Forward: CCGTGGTTTCAGCTGATTGTTGAAGGTGGTGAACAG

Reverse: CTGTTCACCACCTTCAACAATCAGCTGAAACCACGG

F154A

(DNA template: WT MsAcT)

Forward: GCCGCATCCGTGGTTTCAGCTGATTGCTGAAGGTGGTGAA

Reverse: TTCACCACCTTCAGCAATCAGCTGAAACCACGGATGCGGC

F174V

(DNA template: WT MsAcT)

Forward: ATAGCGCACTGGCAAGCGTTATGAAAGTGCCGTTT

Reverse: AAACGGCACTTTCATAACGCTTGCCAGTGCGCTAT

F174A

(DNA template: WT MsAcT)

Forward: GTTTATAGCGCACTGGCAAGCGCTATGAAAGTGCCGTTTTTTGA

Reverse: TCAAAAAACGGCACTTTCATAGCGCTTGCCAGTGCGCTATAAAC

I153A

(DNA template: WT MsAcT)

Forward: GCATCCGTGGTTTCAGCTGGCTTTTGAAGGTGGTGAACAG

Reverse: CTGTTCACCACCTTCAAAAGCCAGCTGAAACCACGGATGC

I153M

(DNA template: WT MsAcT)

Forward: CCGTGGTTTCAGCTGATGTTTGAAGGTGGTGAACA

Reverse: TGTTCACCACCTTCAAACATCAGCTGAAACCACGG

F154W

(DNA template: WT MsAcT)

Forward: CATCCGTGGTTTCAGCTGATTTGGGAAGGTGGTGAACAGAAAAC

Reverse: GTTTTCTGTTCACCACCTTCCCAAATCAGCTGAAACCACGGATG

F154L

(DNA template: WT MsAcT)

Forward: CATCCGTGGTTTCAGCTGATTTTAGAAGGTGGTGAAC

Reverse: GTTCACCACCTTCTAAAATCAGCTGAAACCACGGATG

F150W

(DNA template: WT MsAcT)

Forward: CGATGCCGCATCCGTGGTGGCAGCTGATTTTTGAAGGT

Reverse: ACCTTCAAAAATCAGCTGCCACCACGGATGCGGCATCG

F150L

(DNA template: WT MsAcT)

Forward: GATGCCGCATCCGTGGTTACAGCTGATTTTTGAAG

Reverse: CTTCAAAAATCAGCTGTAACCACGGATGCGGCATC

F174W

(DNA template: WT MsAcT)

Forward: CATCAAAAAACGGCACTTTCATCCAGCTTGCCAGTGCGCTATAAAC

Reverse: GTTTATAGCGCACTGGCAAGCTGGATGAAAGTGCCGTTTTTTGATG

F174L

(DNA template: WT MsAcT)

Forward: AGCGCACTGGCAAGCTTAATGAAAGTGCCGTTTT

Reverse: AAAACGGCACTTTCATTAAGCTTGCCAGTGCGCT

I153F

(DNA template: WT MsAcT)

Forward: CATCCGTGGTTTCAGCTGTTTTTTGAAGGTGGTGAAC

Reverse: GTTCACCACCTTCAAAAAACAGCTGAAACCACGGATG

W16F

(DNA template: WT MsAcT)

Forward: TAGCCTGACCTGGGGTTTCGTTCCTGTTGAAGATGG

Reverse: CCATCTTCAACAGGAACGAAACCCCAGGTCAGGCTA

W16V

(DNA template: WT MsAcT)

Forward: GTGATAGCCTGACCTGGGGTGTGGTTCCTGTTGAAGATG

Reverse: CATCTTCAACAGGAACCACACCCCAGGTCAGGCTATCAC

W16A

(DNA template: WT MsAcT)

Forward: GATAGCCTGACCTGGGGTGCGGTTCCTGTTGAAGA

Reverse: TCTTCAACAGGAACCGCACCCCAGGTCAGGCTATC

F150A/F154A

(DNA template: MsAcT F150A)

Forward: CATCCGTGGGCTCAGCTGATTGCTGAAGGTGGTGAACA

Reverse: GTAGGCACCCGAGTCGACTAACGACTTCCACCACTTGT

F150A/F174A

(DNA template: MsAcT F174A)

Forward: CCGATGCCGCATCCGTGGGCTCAGCTGATTTTTGAAGG

Reverse: CCTTCAAAAATCAGCTGAGCCCACGGATGCGGCATCGG

F154A/F174A

(DNA template: MsAcT F174A)

Forward: GCCGCATCCGTGGTTTCAGCTGATTGCTGAAGGTGGTGAA

Reverse: TTCACCACCTTCAGCAATCAGCTGAAACCACGGATGCGGC

F150V/F154V

(DNA template: MsAcT F150V)

Forward: CGTGGGTTCAGCTGATTGTTGAAGGTGGTGAACA

Reverse: GCACCCAAGTCGACTAACAACTTCCACCACTTGT

F150V/F174V

(DNA template: MsAcT F150V)

Forward: ATAGCGCACTGGCAAGCGTTATGAAAGTGCCGTTT

Reverse: AAACGGCACTTTCATAACGCTTGCCAGTGCGCTAT

F154V/F174V

(DNA template: MsAcT F154V)

Forward: ATAGCGCACTGGCAAGCGTTATGAAAGTGCCGTTT

Reverse: AAACGGCACTTTCATAACGCTTGCCAGTGCGCTAT

8.4 NMR

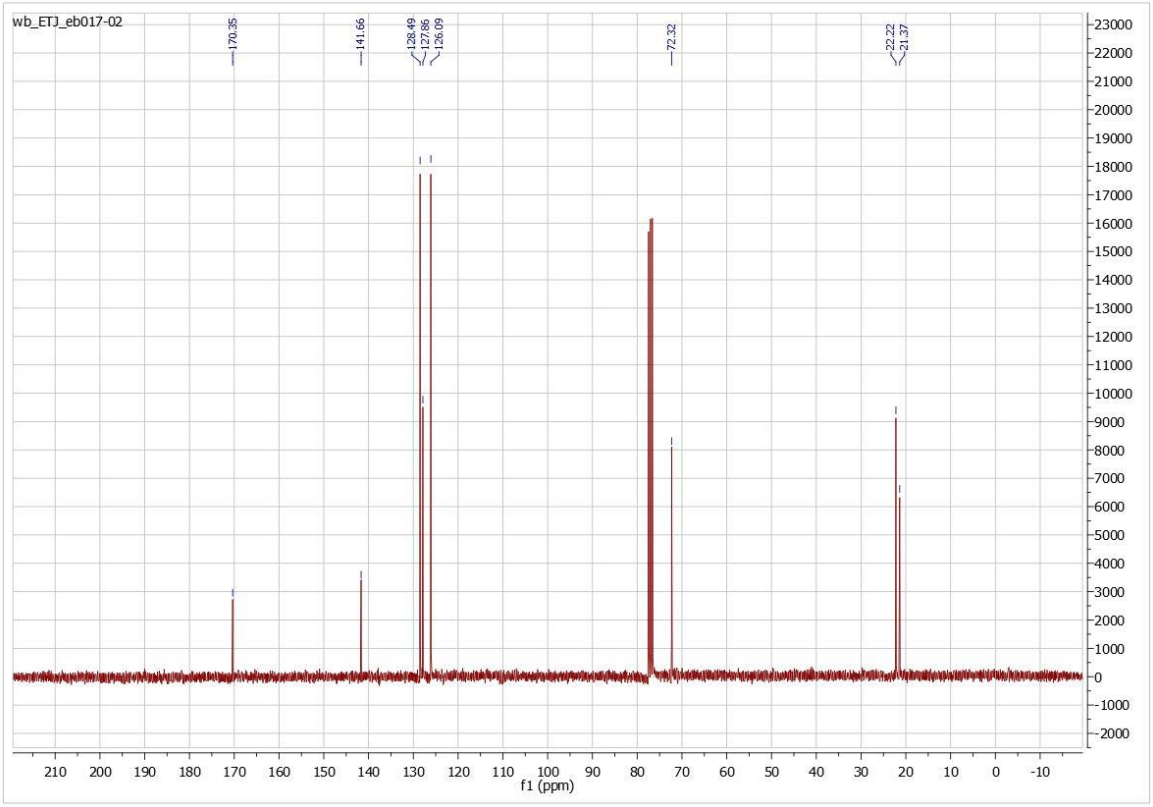

Figure 41: ^{13}C-NMR (300 MHz, CCl_3D -d_1) of reference compound (*R*)-**3b**

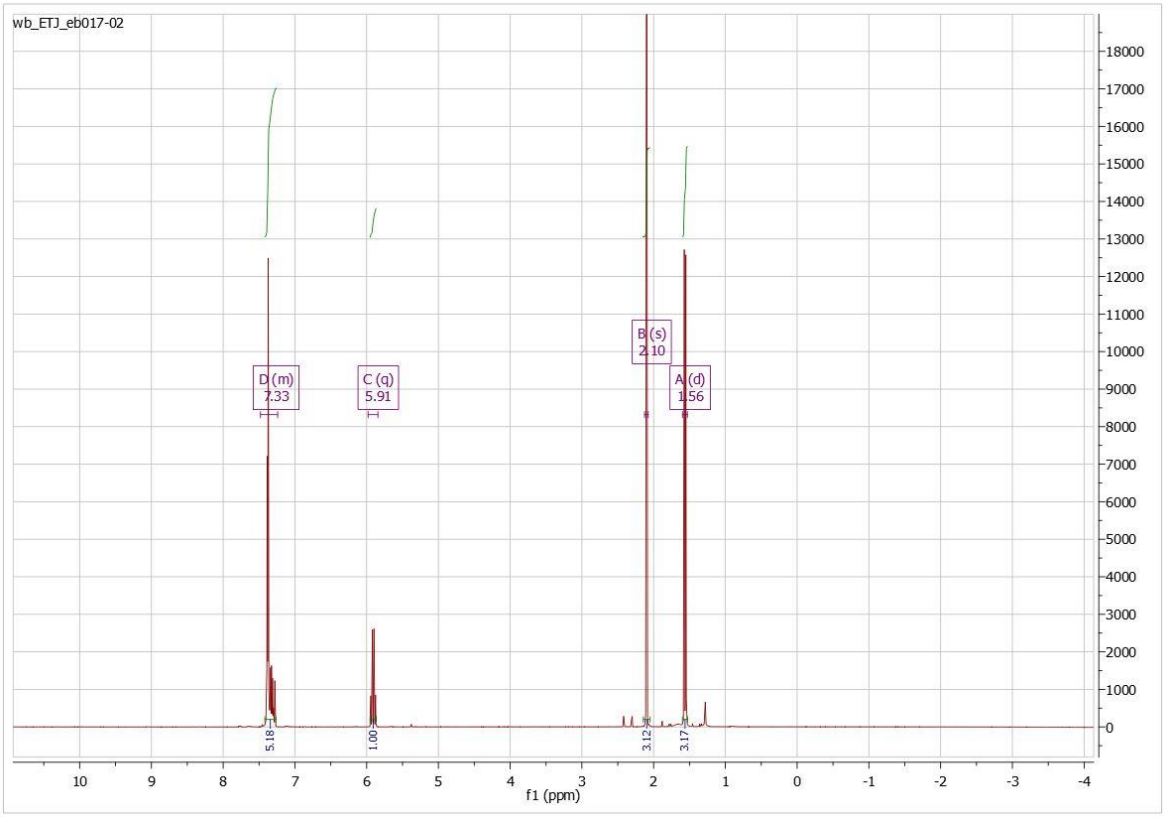

Figure 42: ^{1}H-NMR (300 MHz, CCl_3D -d_1) of reference compound (*R*)-**3b**

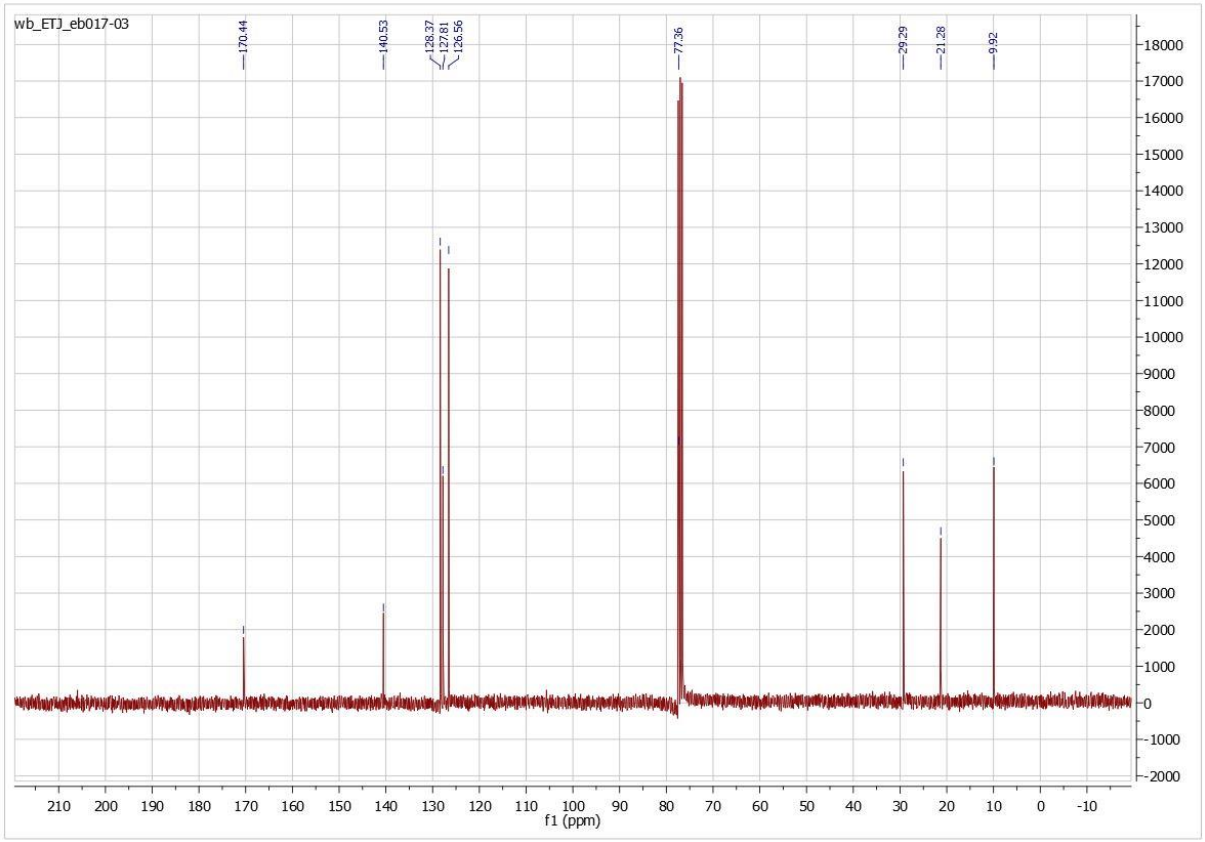

Figure 43: ^{13}C-NMR (300 MHz, CCl_3D -d_1) of reference compound (*rac*)-**7b**

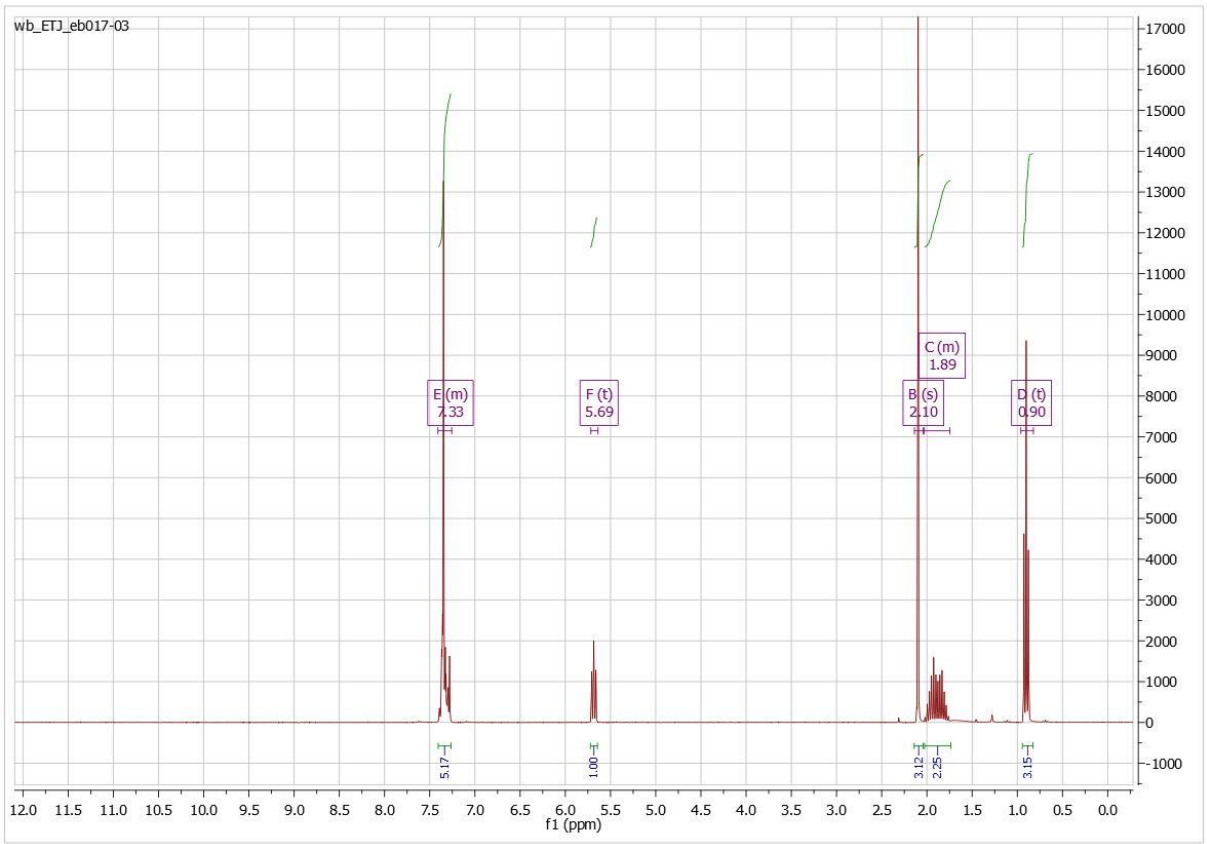

Figure 44: ^{1}H-NMR (300 MHz, CCl_3D -d_1) of reference compound (*rac*)-**7b**

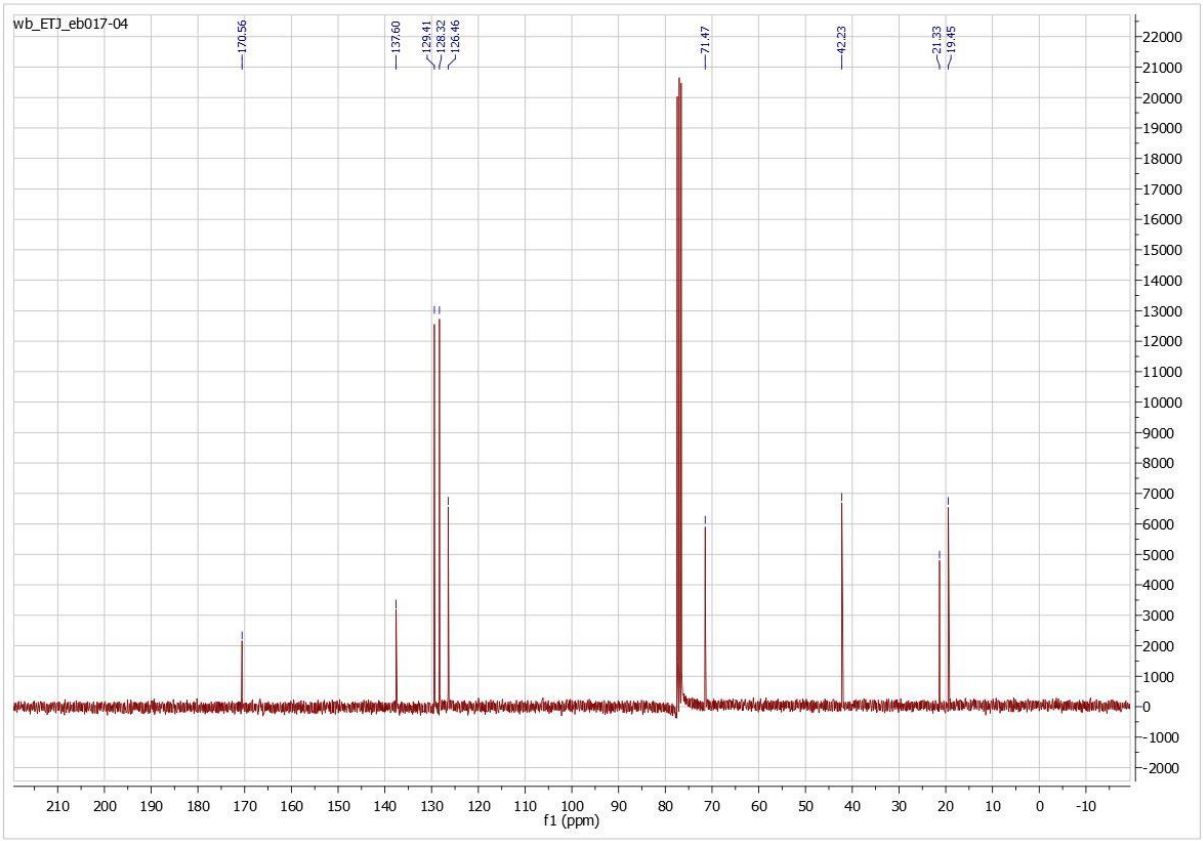

Figure 45: ^{13}C-NMR (300 MHz, CCl_3D -d_1) of reference compound (*rac*)-**8b**

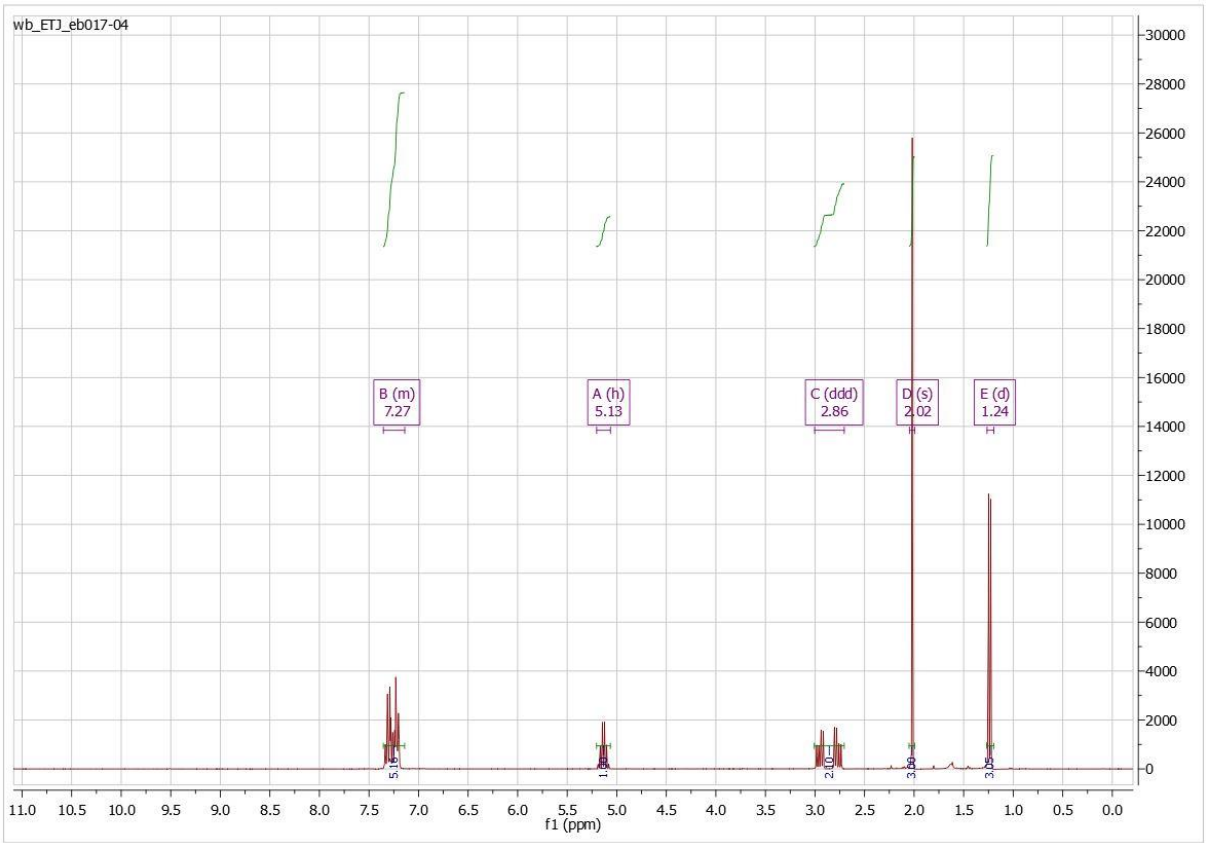

Figure 46: ^{1}H-NMR (300 MHz, CCl_3D -d_1) of reference compound (*rac*)-**8b**

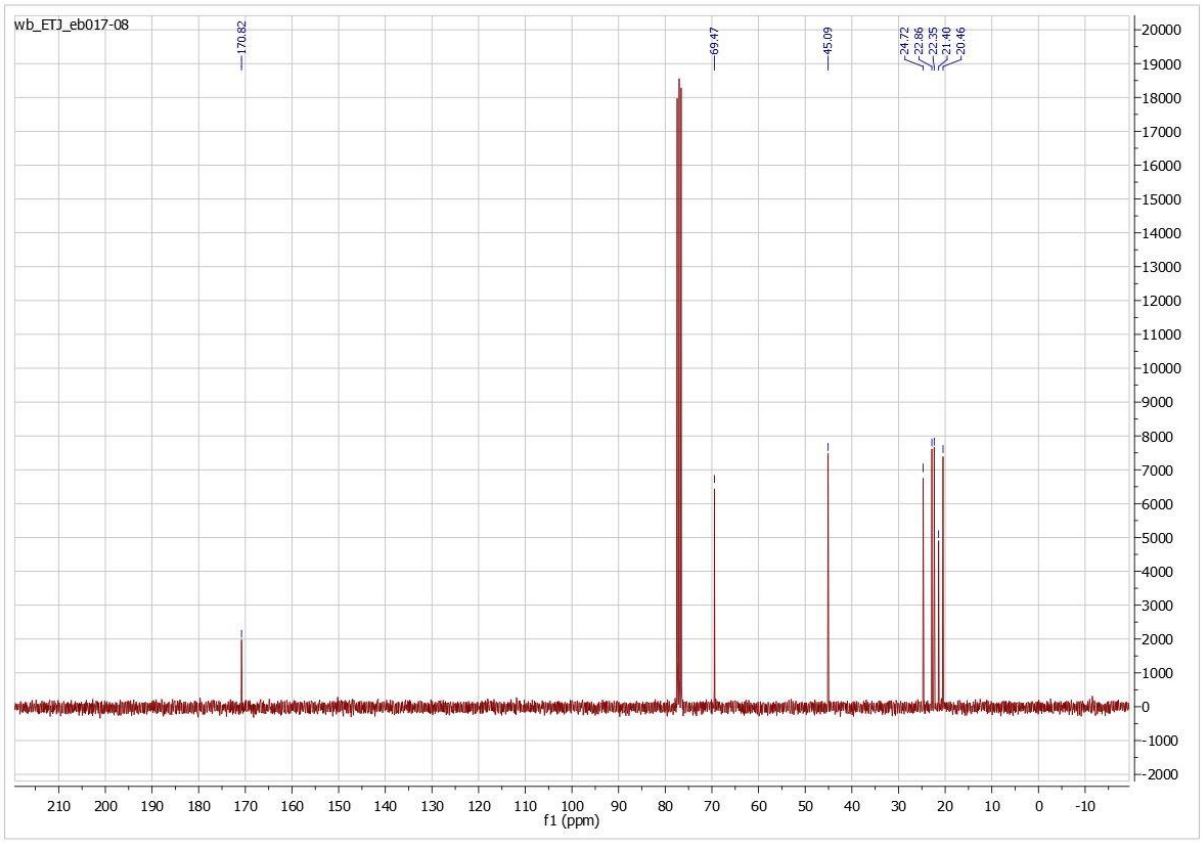

Figure 47: ^{13}C-NMR (300 MHz, CCl_3D -d_1) of reference compound (*rac*)-**10b**

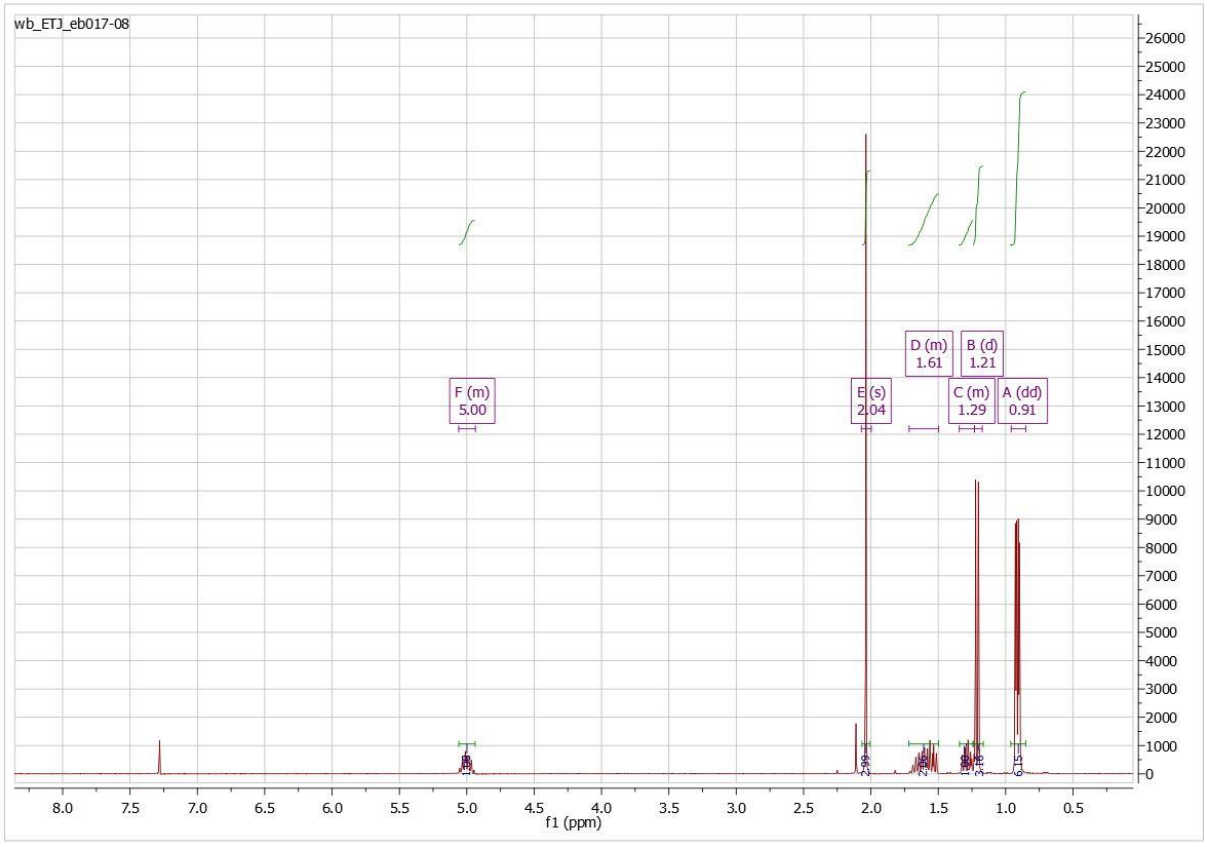

Figure 48: ^{1}H-NMR (300 MHz, CCl_3D -d_1) of reference compound (*rac*)-**10b**

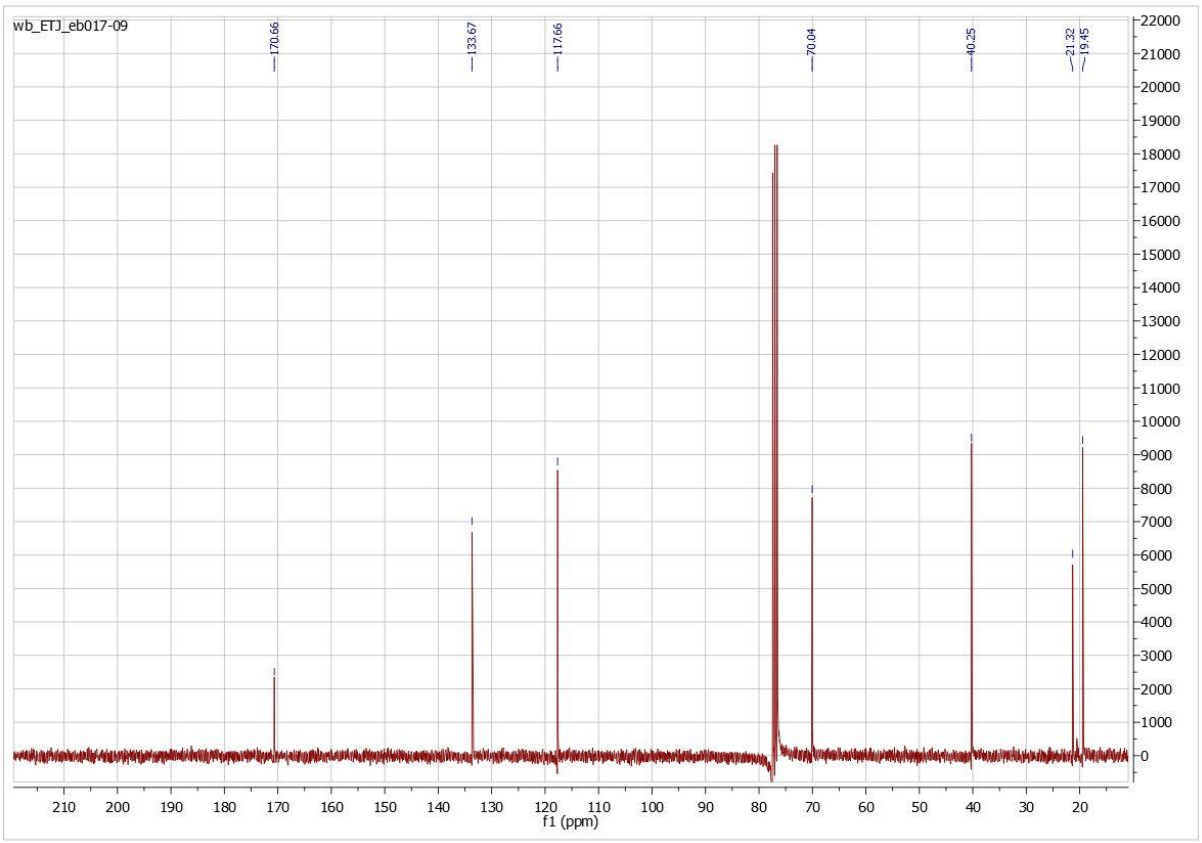

Figure 49: ^{13}C-NMR (300 MHz, CCl_3D -d_1) of reference compound (*rac*)-**11b**

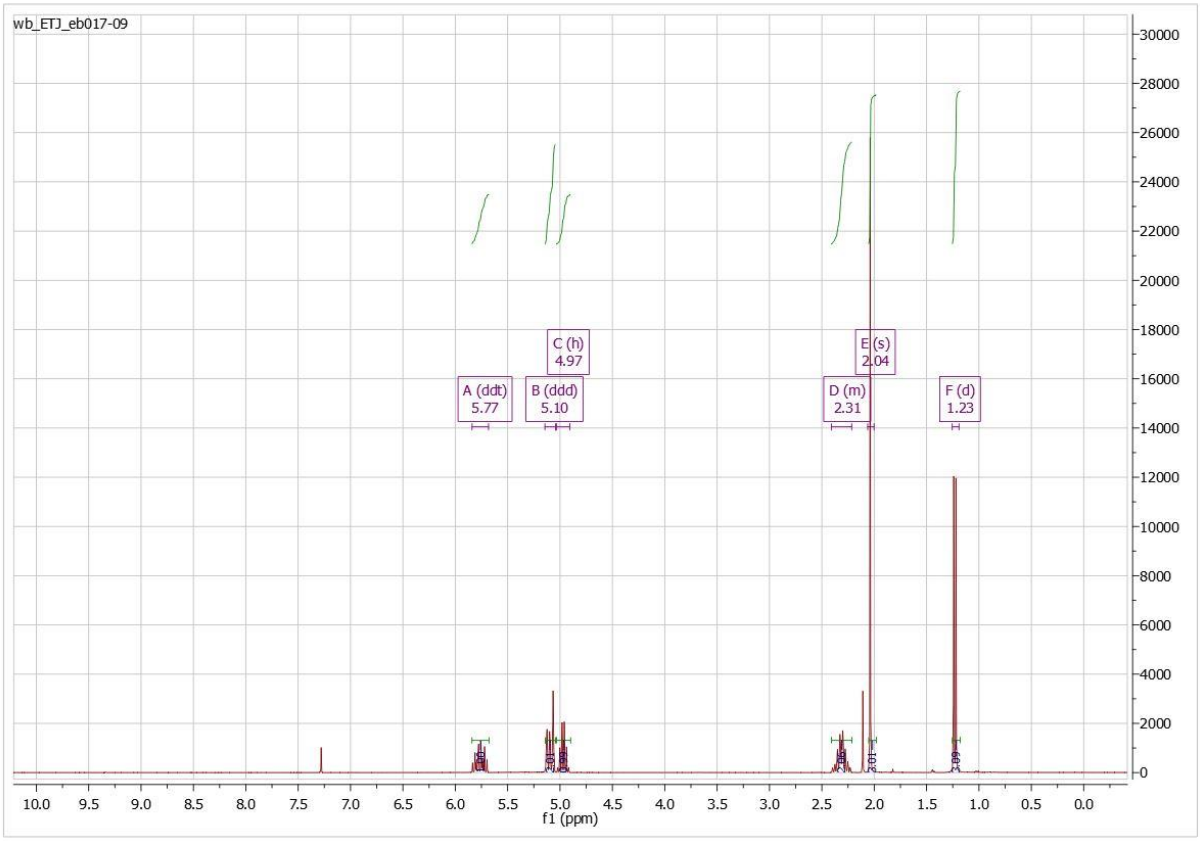

Figure 50: ^{1}H-NMR (300 MHz, CCl_3D -d_1) of reference compound (*rac*)-**11b**

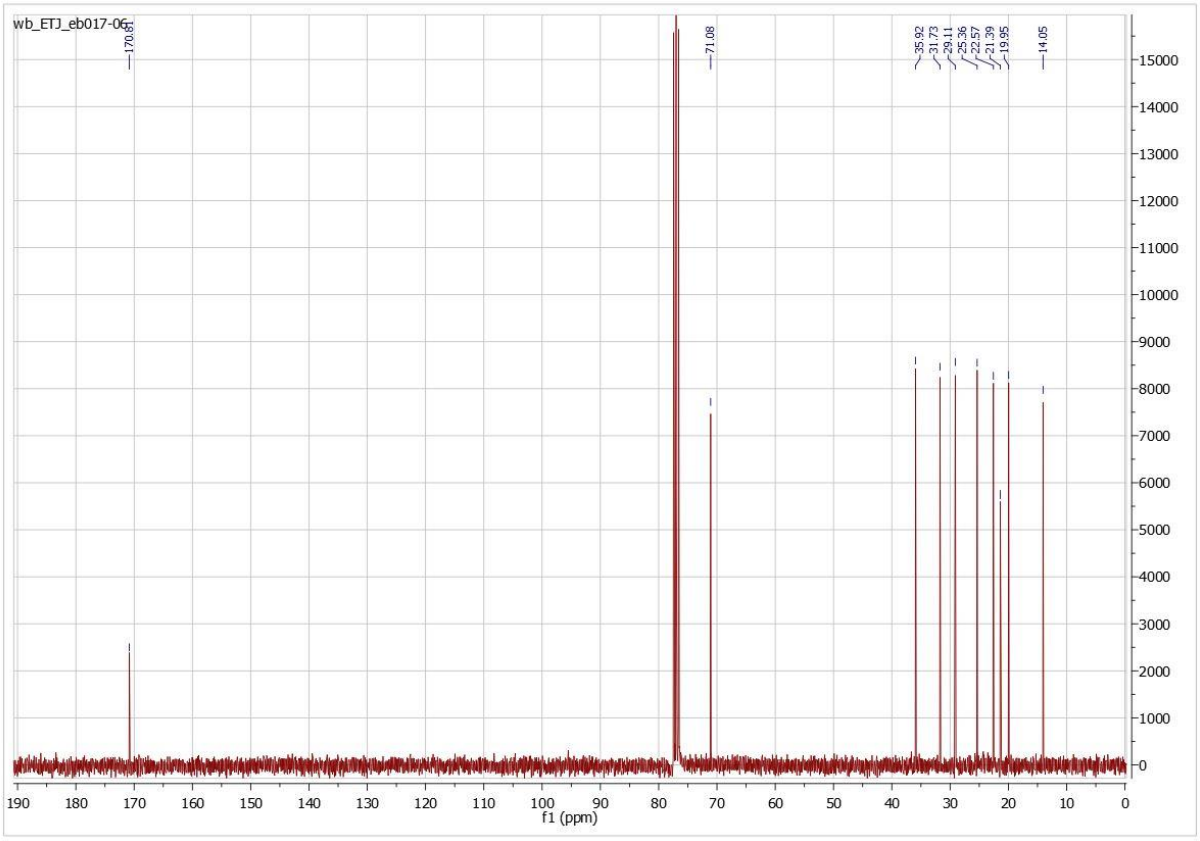

Figure 51: ^{13}C-NMR (300 MHz, CCl_3D -d_1) of reference compound (*rac*)-**12b**

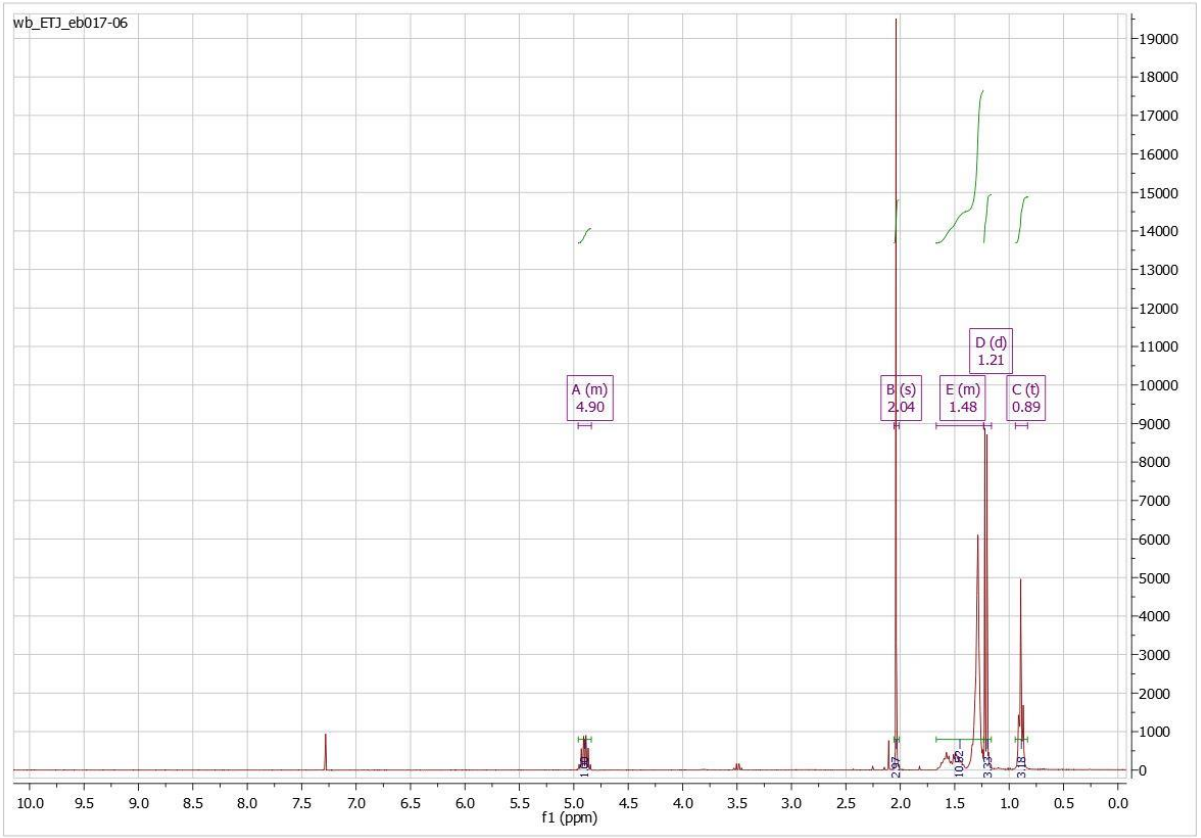

Figure 52: ^{1}H-NMR (300 MHz, CCl_3D -d_1) of reference compound (*rac*)-**12b**

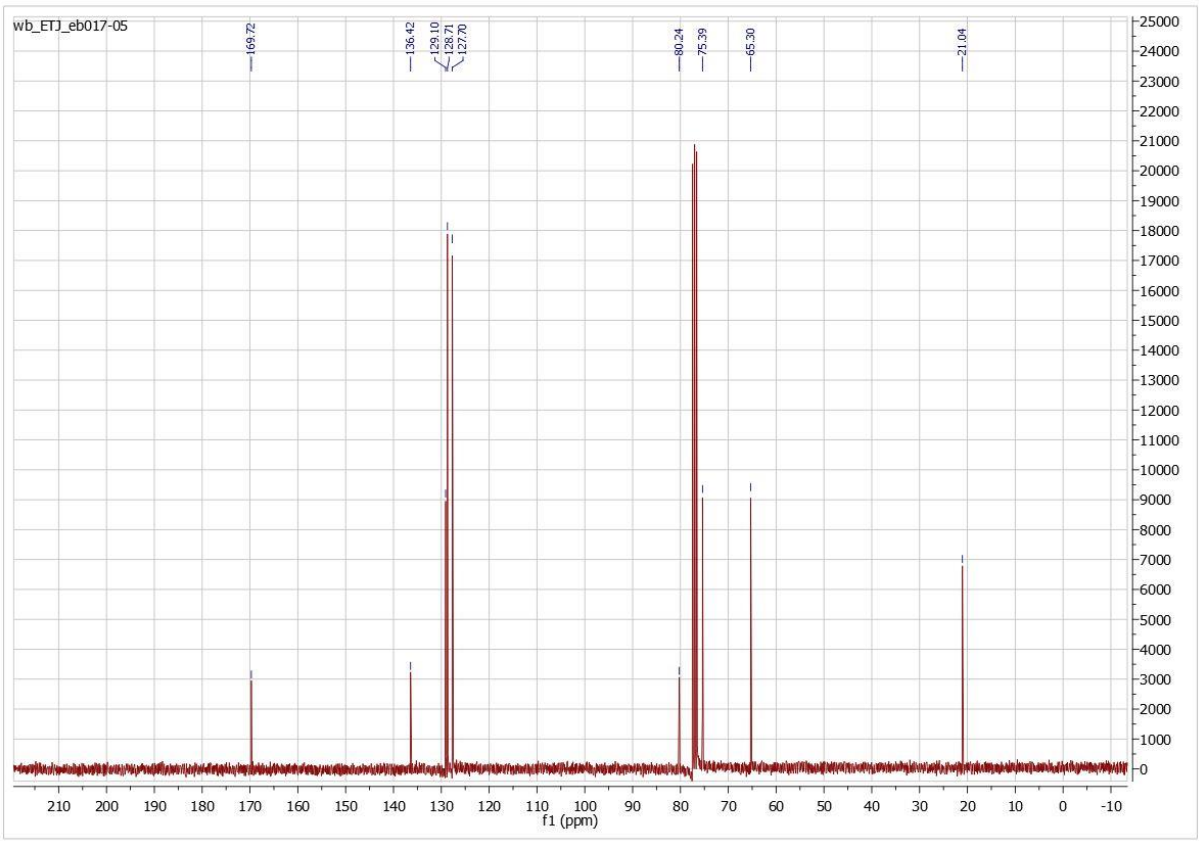

Figure 53: ^{13}C-NMR (300 MHz, CCl_3D -d_1) of reference compound (*rac*)-**13b**

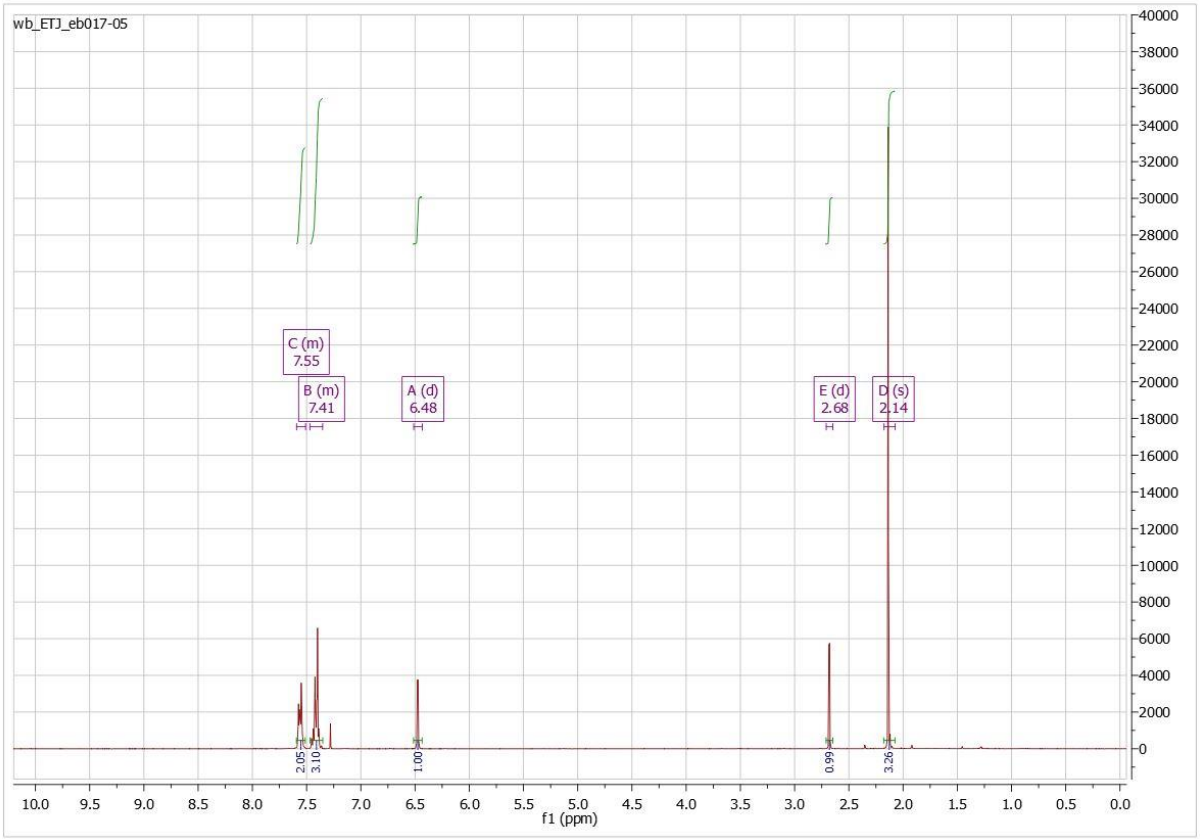

Figure 54: ^{1}H-NMR (300 MHz, CCl_3D -d_1) of reference compound (*rac*)-**13b**

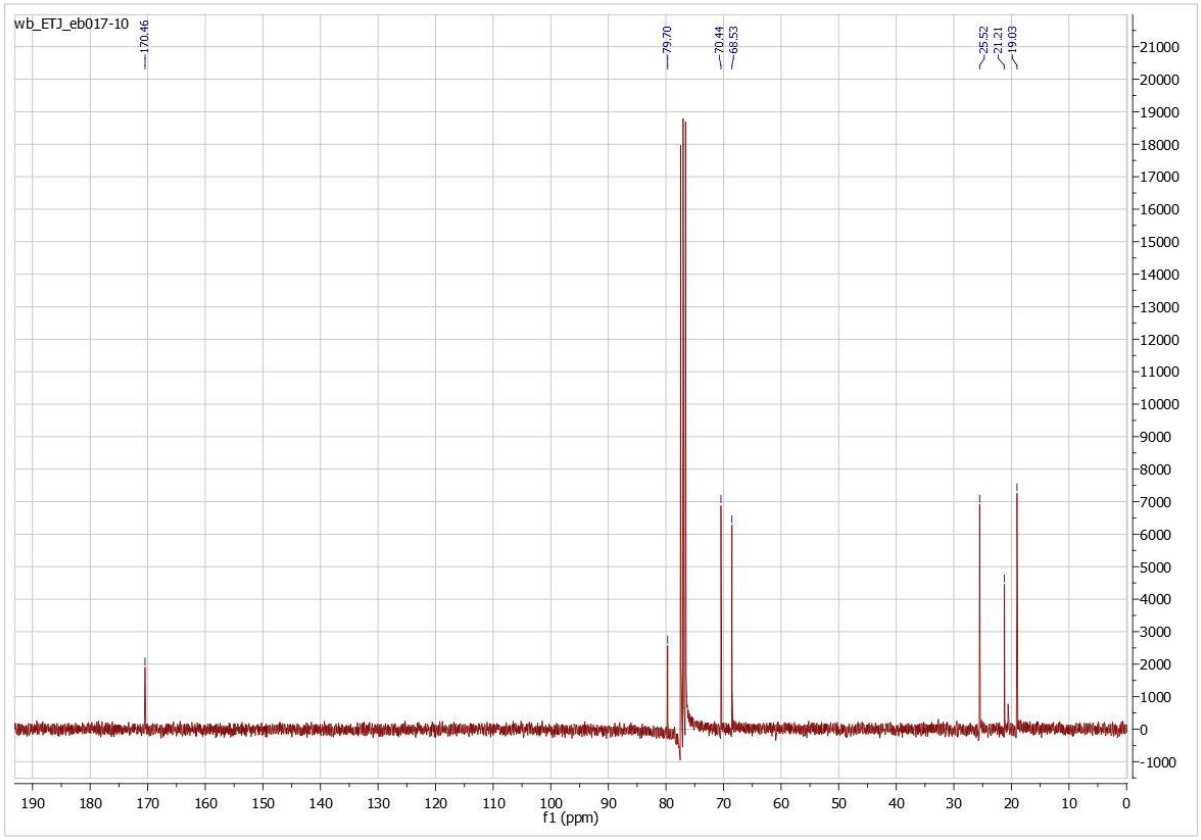

Figure 55: ^{13}C-NMR (300 MHz, CCl_3D -d_1) of reference compound (*rac*)-**14b**

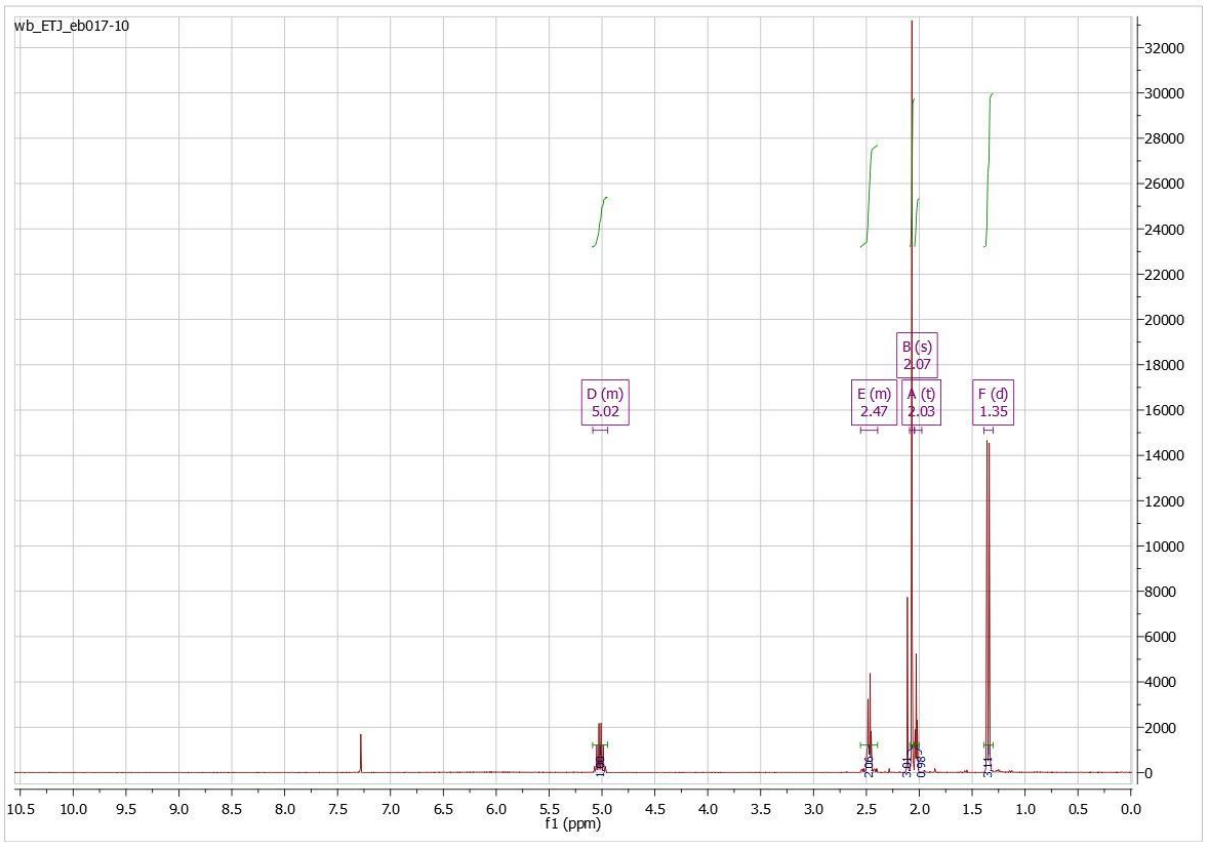

Figure 56: ^{1}H-NMR (300 MHz, CCl_3D -d_1) of reference compound (*rac*)-**14b**

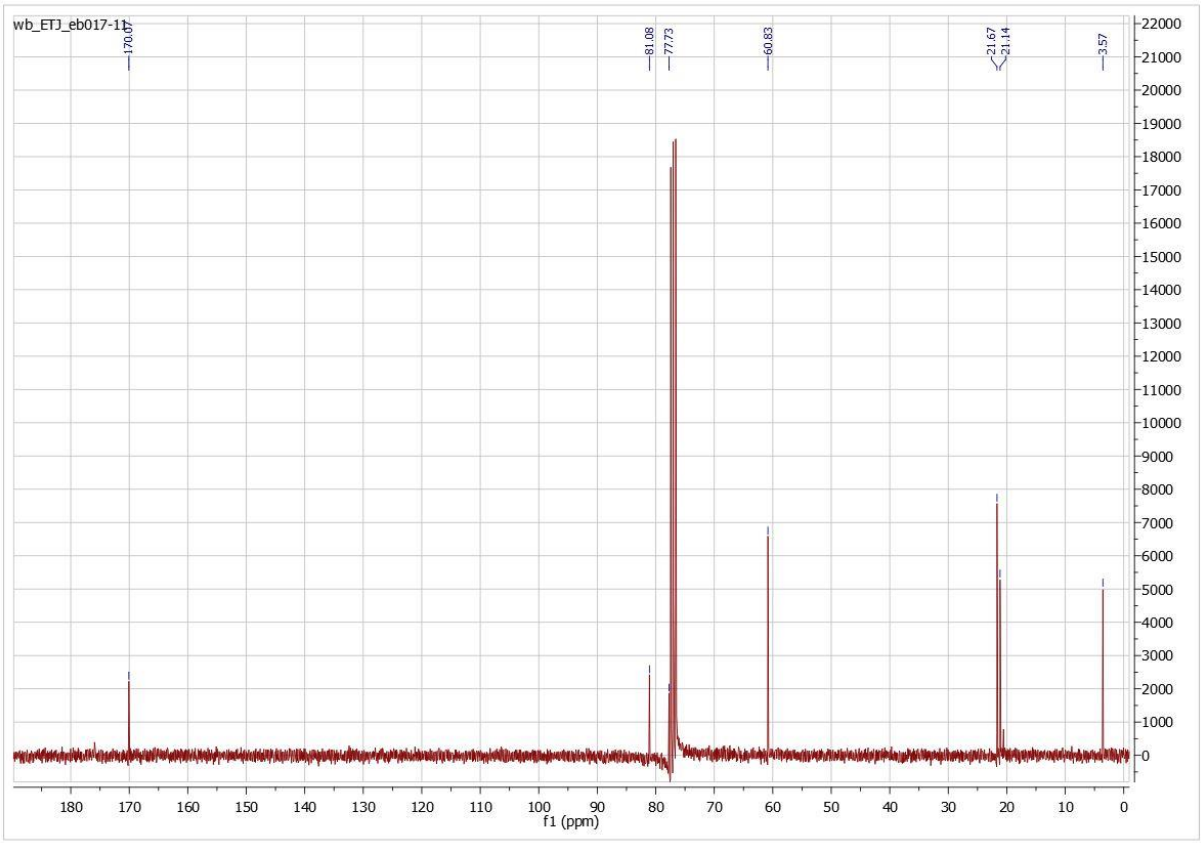

Figure 57: ^{13}C-NMR (300 MHz, CCl_3D -d_1) of reference compound (*rac*)-**15b**

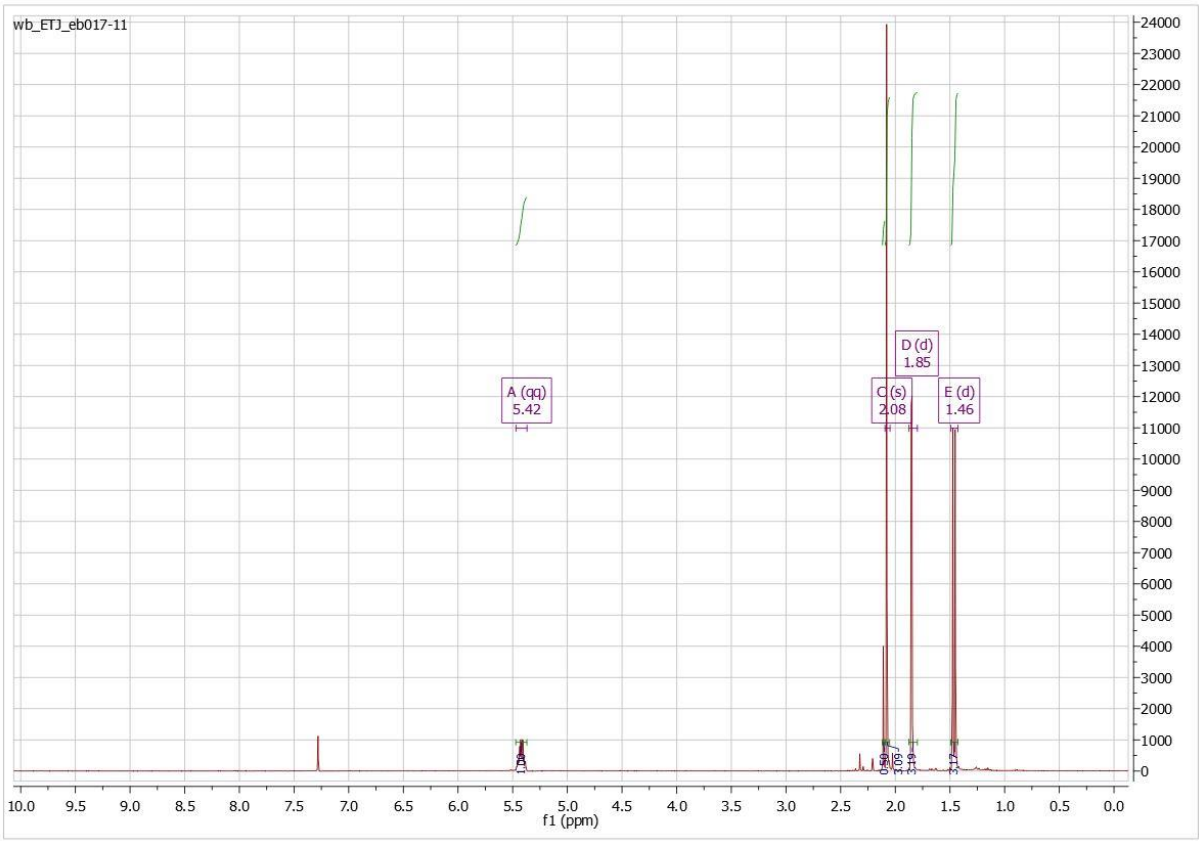

Figure 58: ^{1}H-NMR (300 MHz, CCl_3D -d_1) of reference compound (*rac*)-**15b**

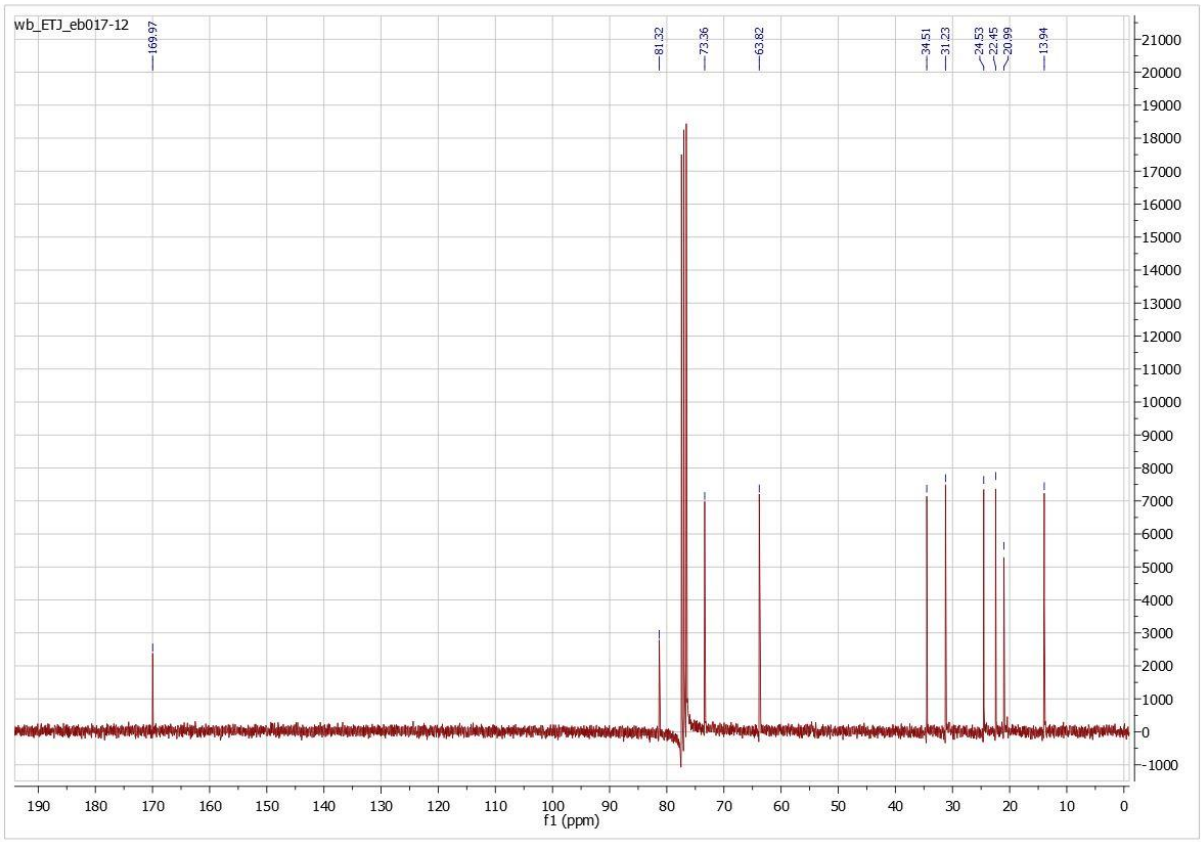

Figure 59: ^{13}C-NMR (300 MHz, CCl_3D -d_1) of reference compound (*rac*)-**16b**

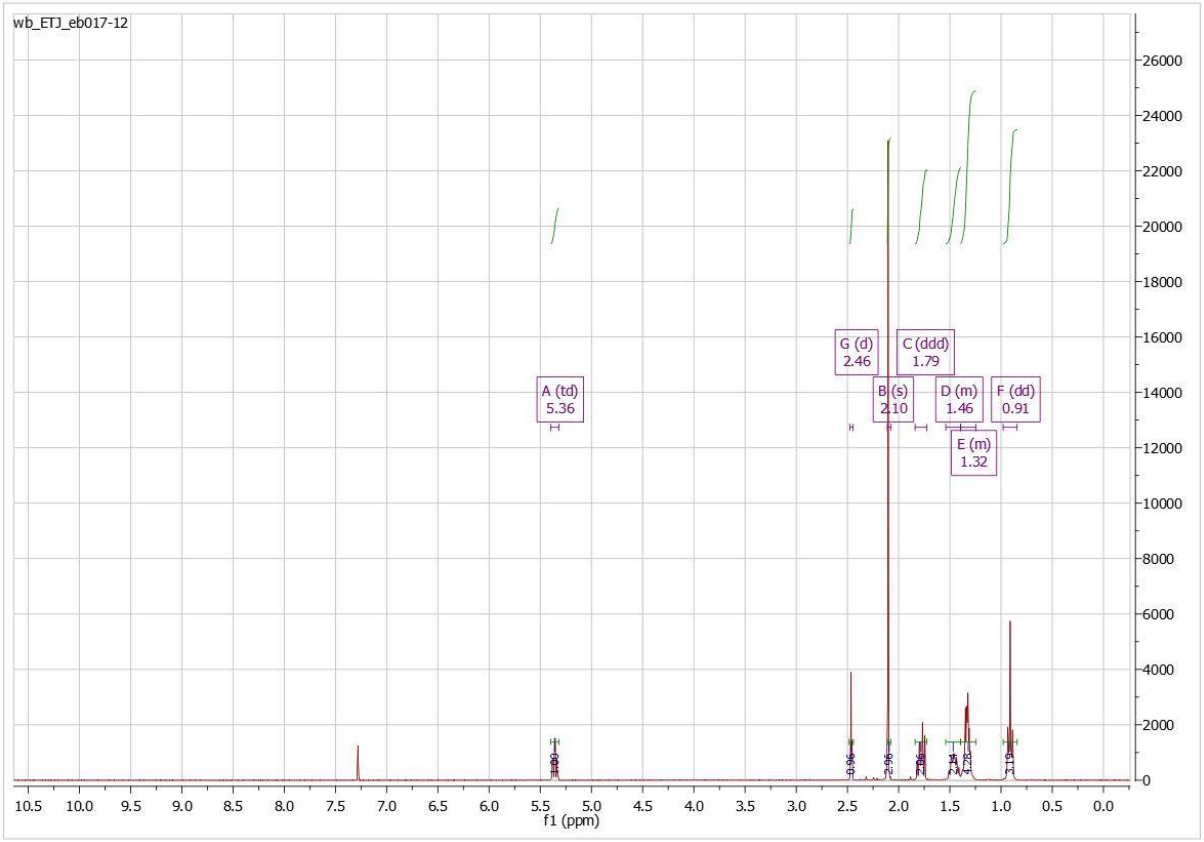

Figure 60: ^{1}H-NMR (300 MHz, CCl_3D -d_1) of reference compound (*rac*)-**16b**

8.5 GC-FID Chromatograms

8.5.1 GC-FID Chromatograms of the Enzyme Engineering Project of MsAcT that Target Increased Transesterification- vs. Hydrolysis-Activity

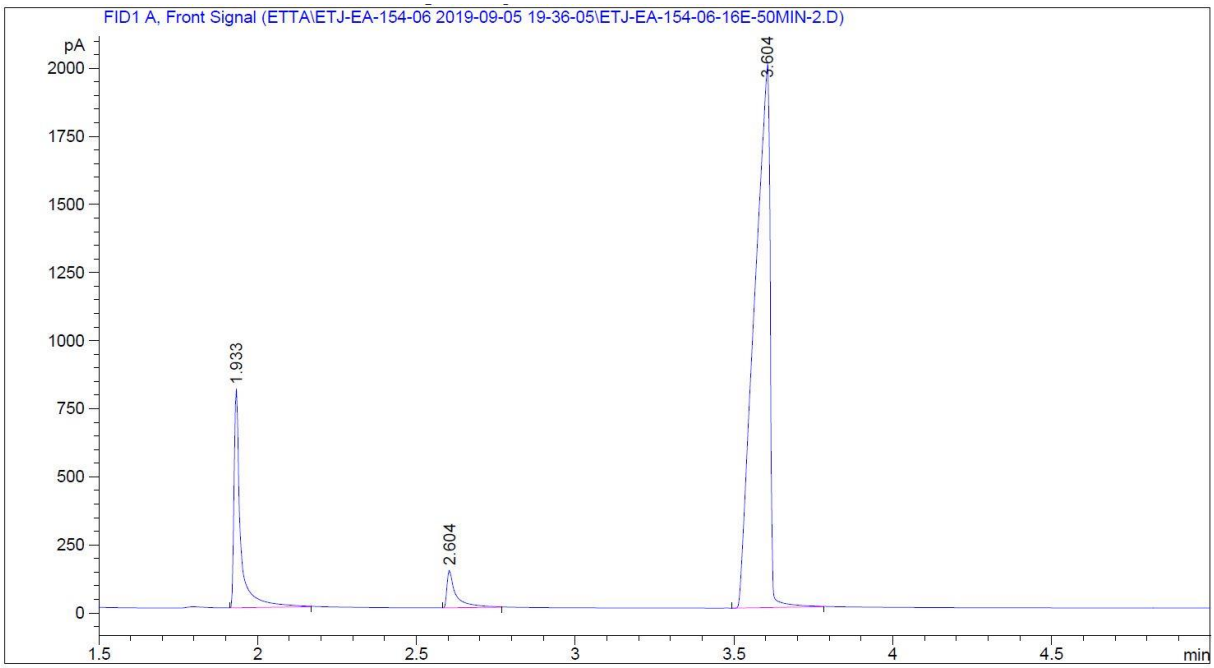

*Figure 61: GC-FID chromatogram of an acyl transfer reaction with compound **1a** with internal standard 1-decanol (RT 3.58 min).*

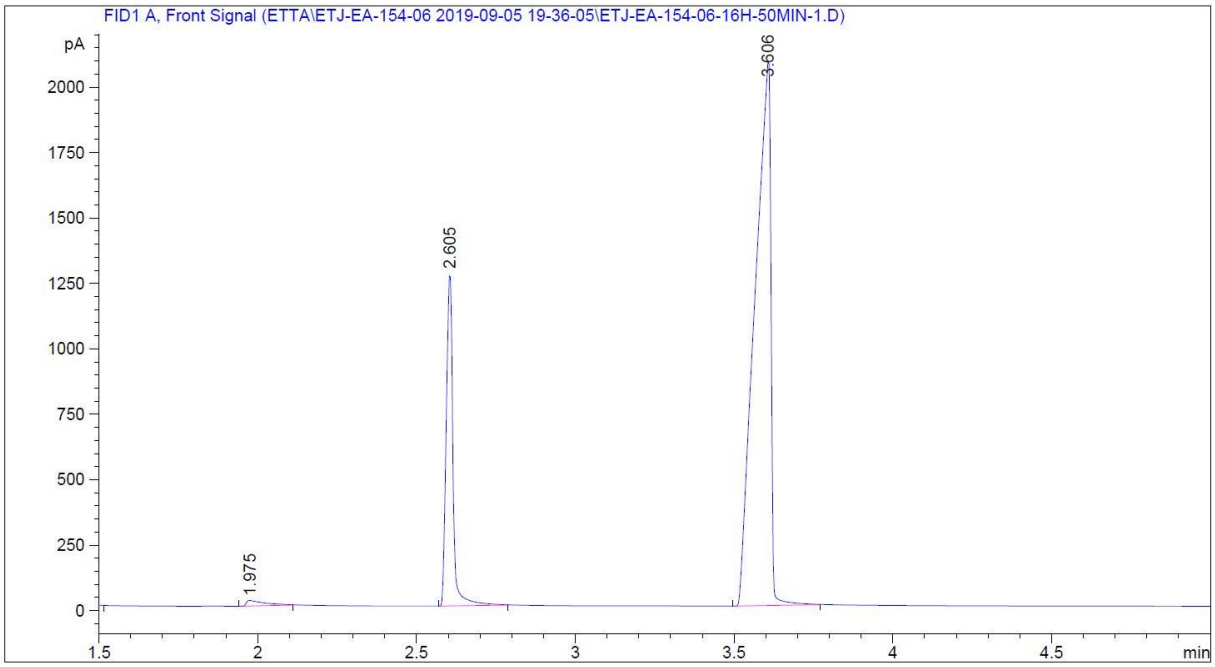

*Figure 62: GC-FID chromatogram of a hydrolysis reaction with compound **1b** with internal standard 1-decanol (RT 3.58 min).*

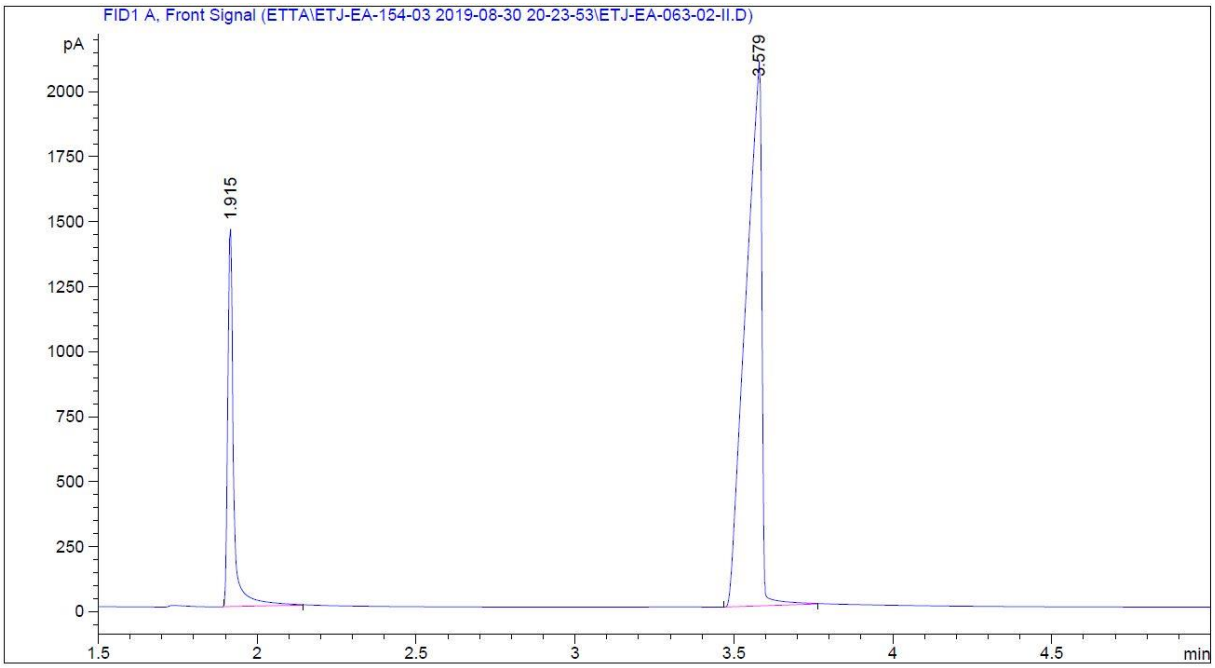

*Figure 63:GC-FID chromatogram of reference compound **1a** with internal standard 1-decanol (RT 3.58 min).*

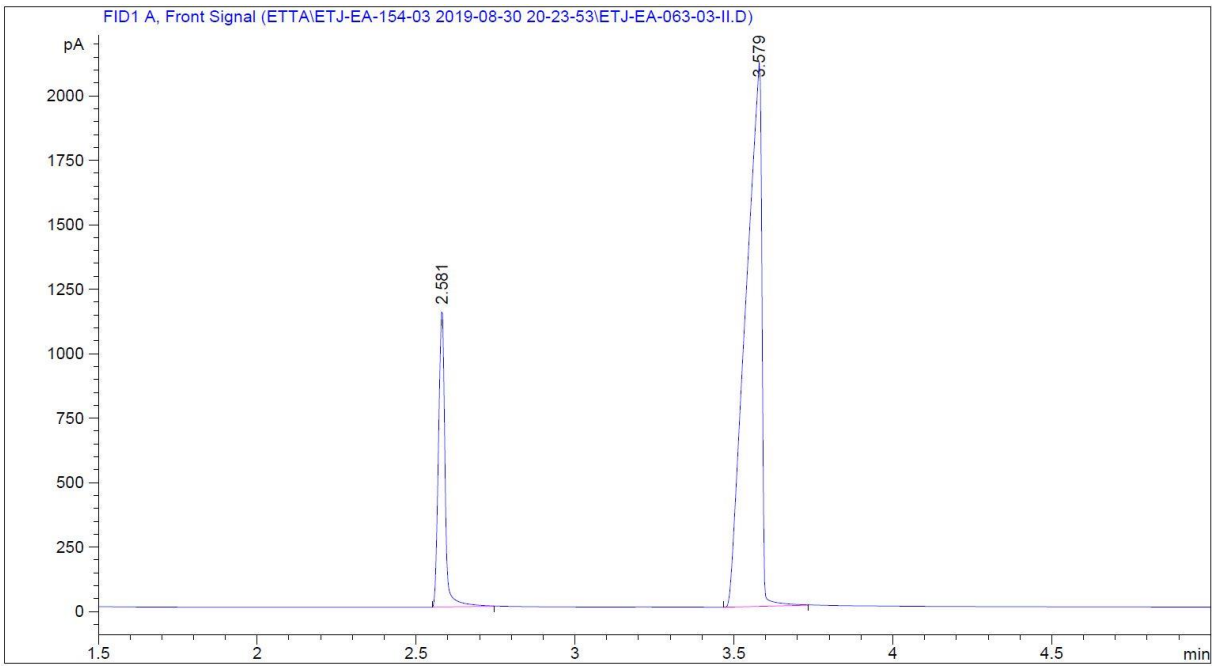

*Figure 64: GC-FID chromatogram of reference compound **1b** with internal standard 1-decanol (RT 3.58 min).*

8.5.2 GC-FID Chromatograms of the Enzyme Engineering Project of MsAcT that Target Higher Enantio-Selectivity

*8.5.2.1 Substrate **3a/3b***

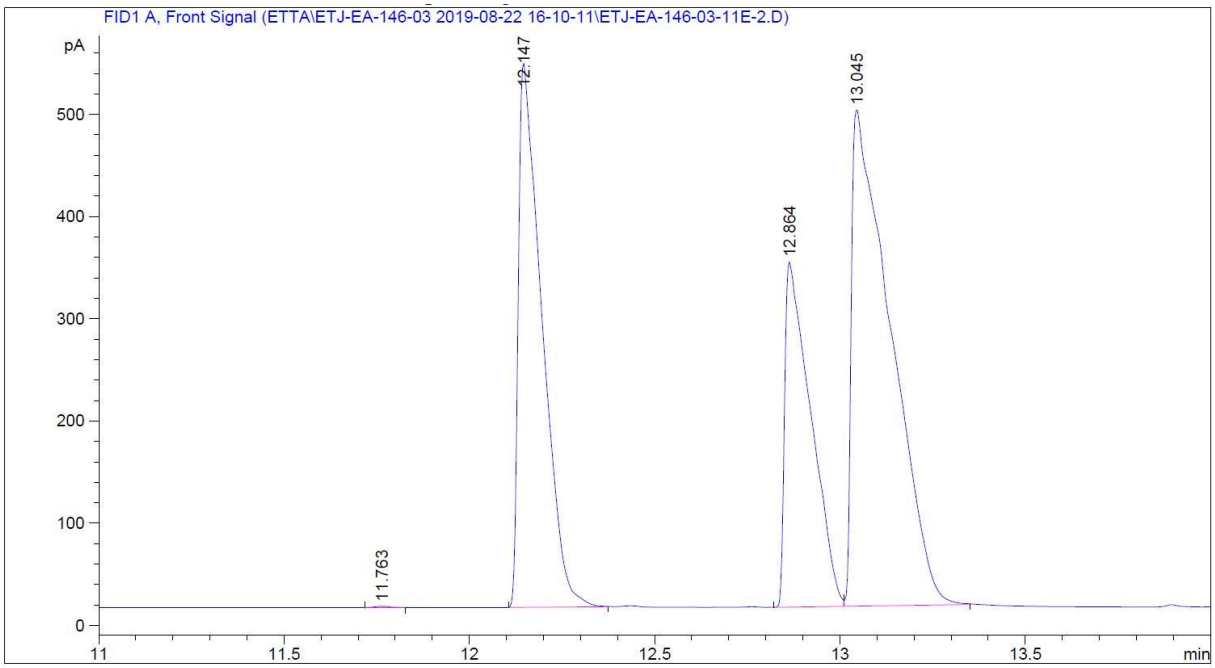

*Figure 65: GC-FID chromatogram of an acyl transfer reaction with substrate **3a**.*

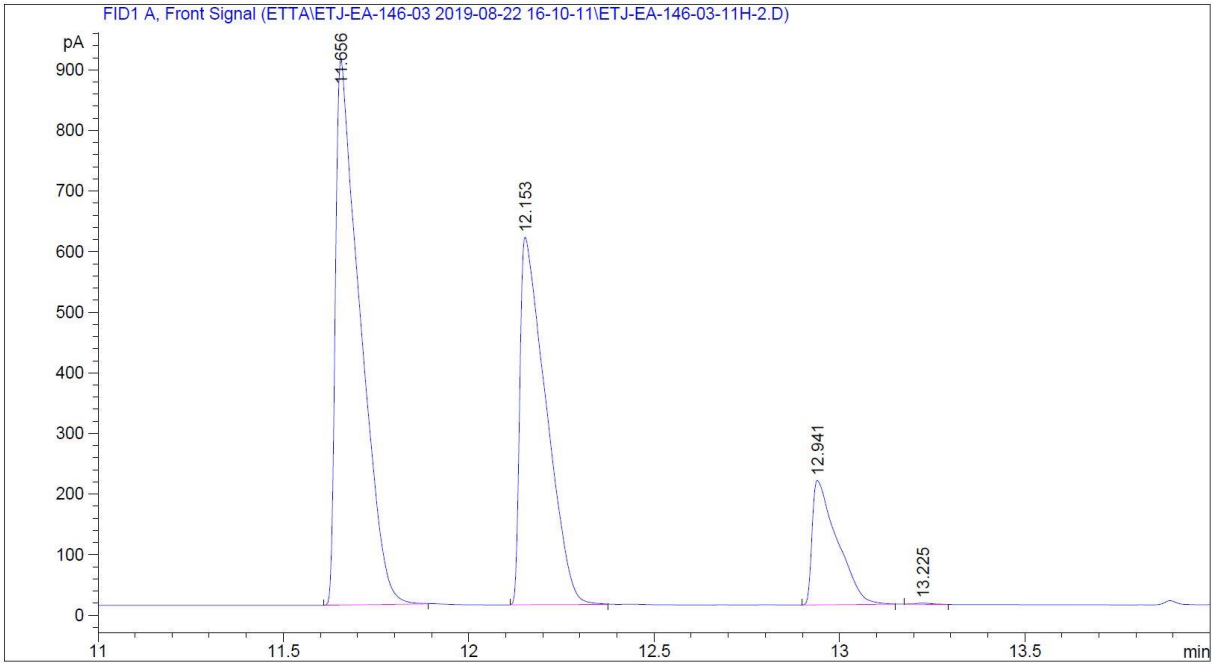

*Figure 66: GC-FID chromatogram of a hydrolysis reaction of substrate **3b**.*

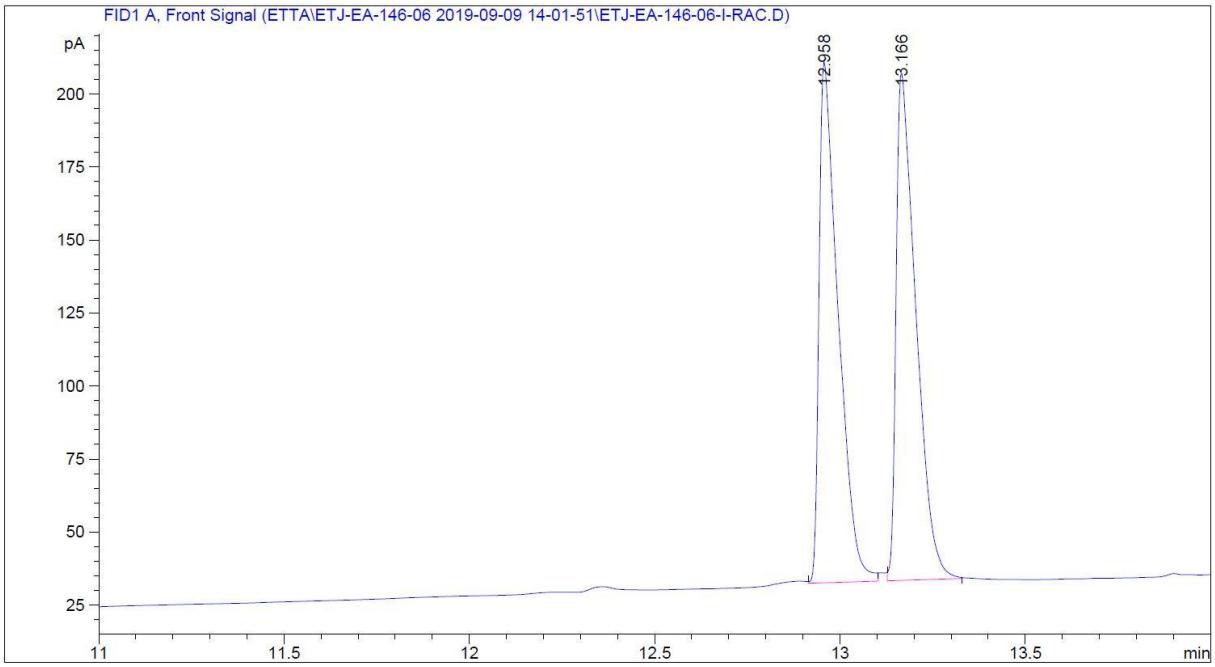

*Figure 67: GC-FID chromatogram of reference compound rac-**3a**.*

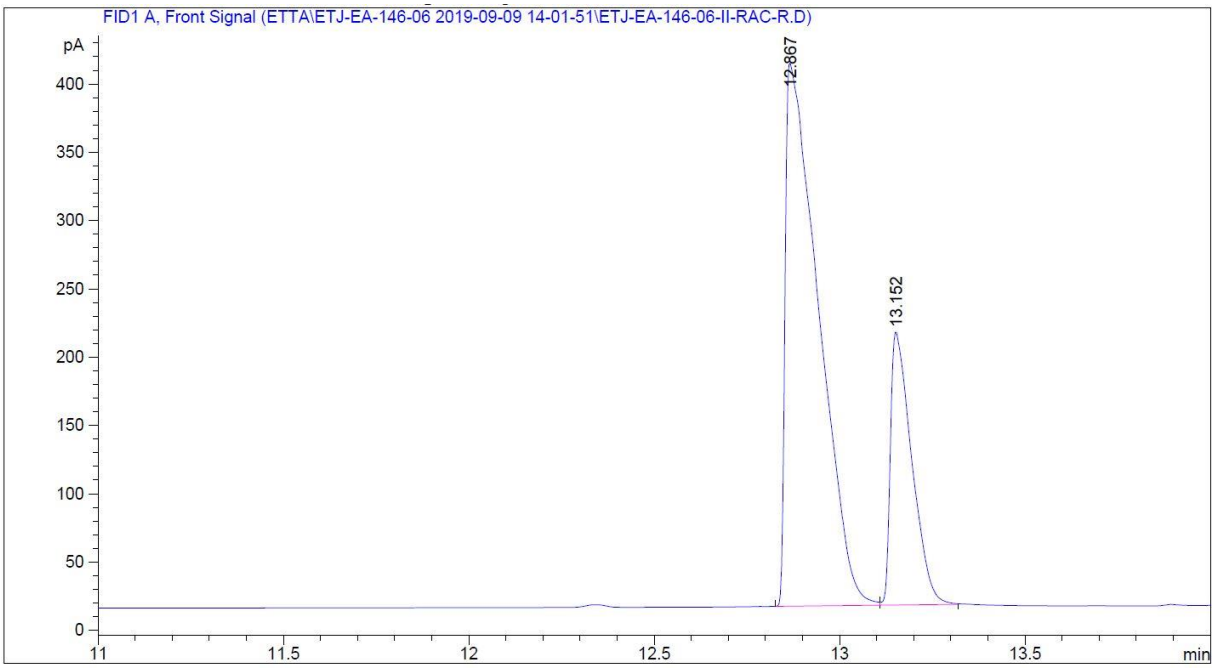

*Figure 68: GC-FID chromatogram of reference compound rac-**3a** with co-injection of (R)-**3a**.*

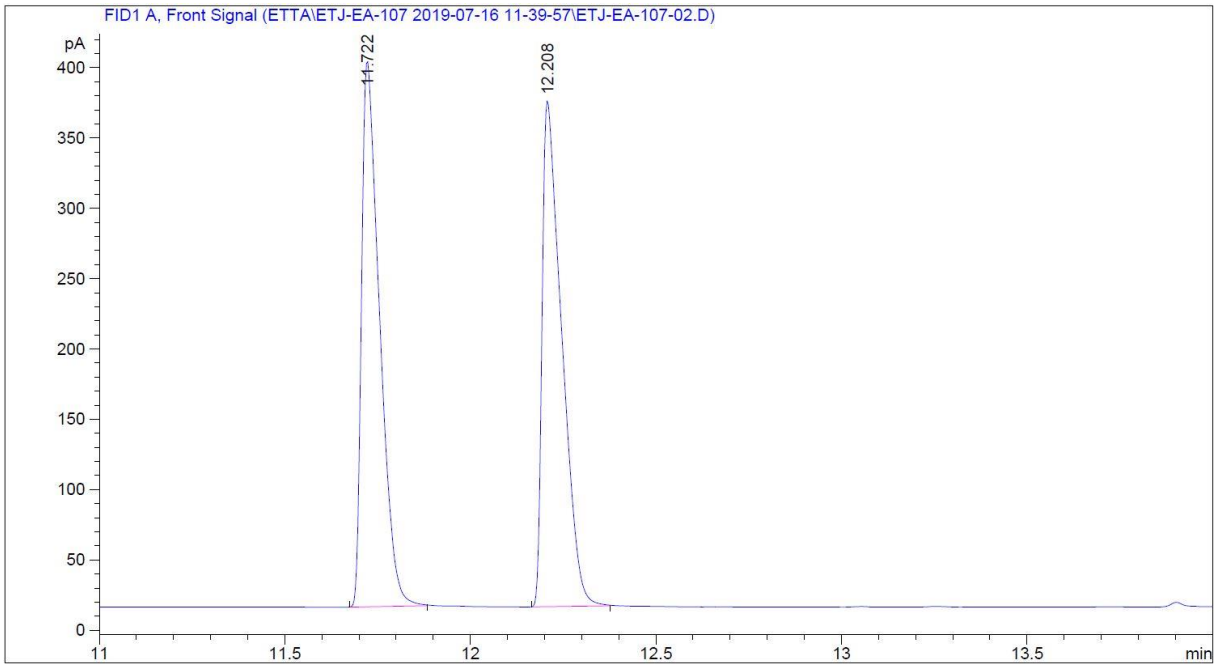

*Figure 69: GC-FID chromatogram of reference compound rac-**3b**.*

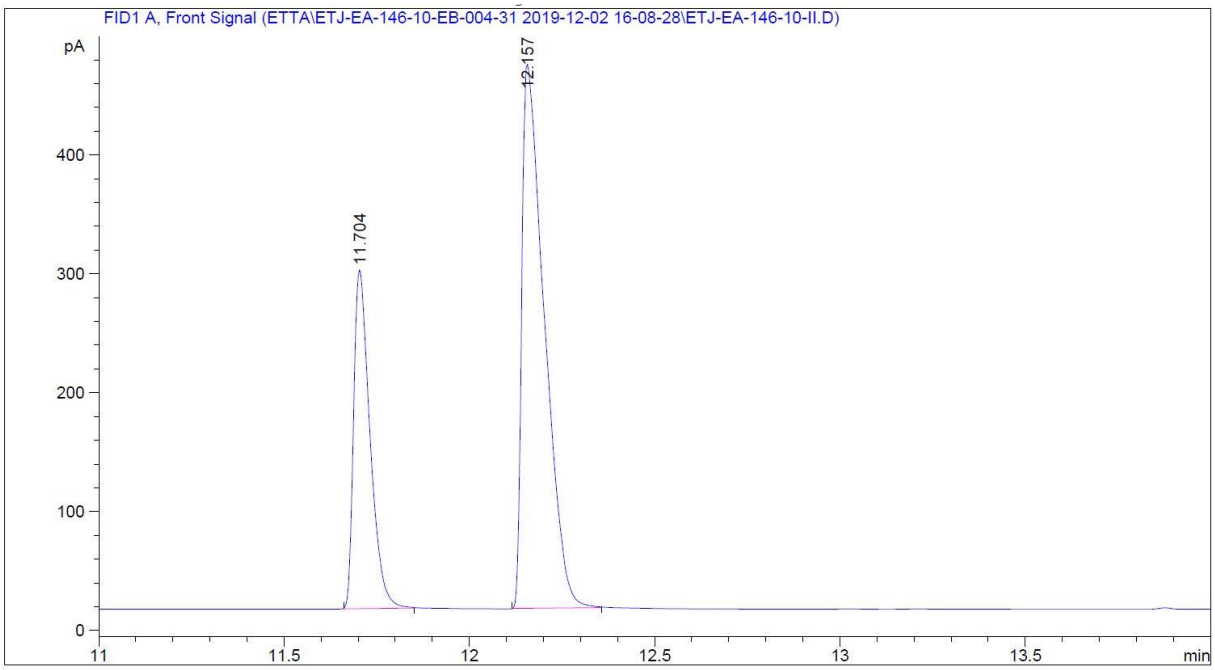

*Figure 70: GC-FID chromatogram of reference compound rac-**3b** with co-injection of (R)-**3b**.*

8.5.2.2 Substrate **7a/7b**

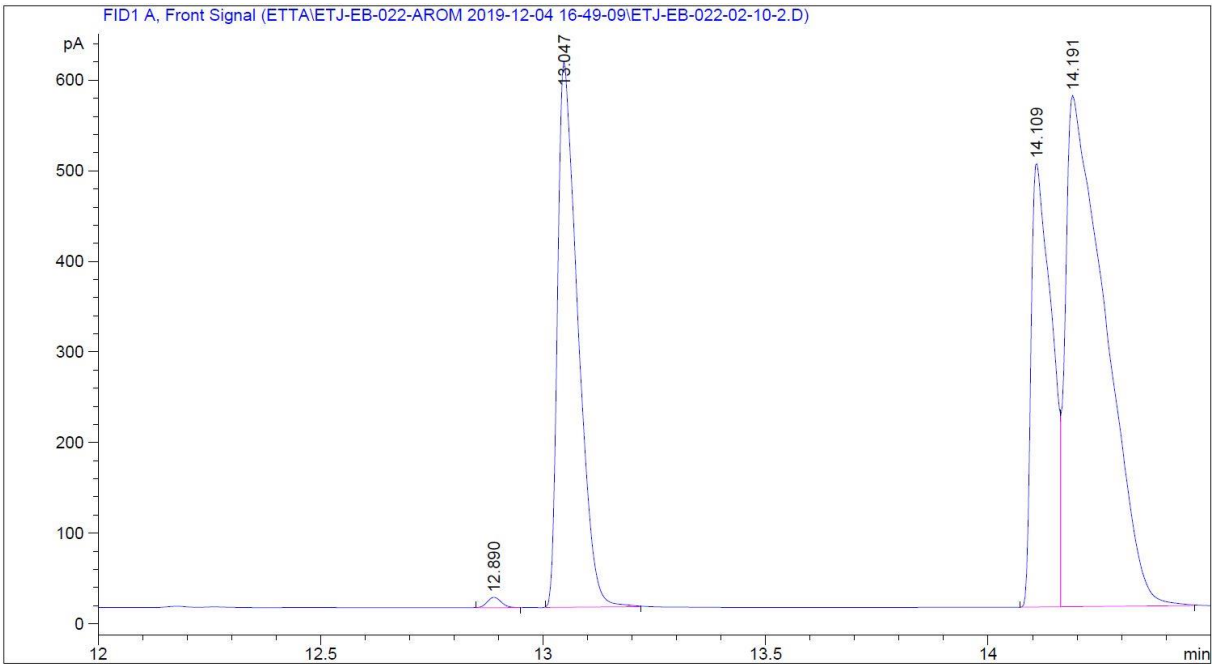

*Figure 71: GC-FID chromatogram of an acyl transfer reaction with substrate **7a**.*

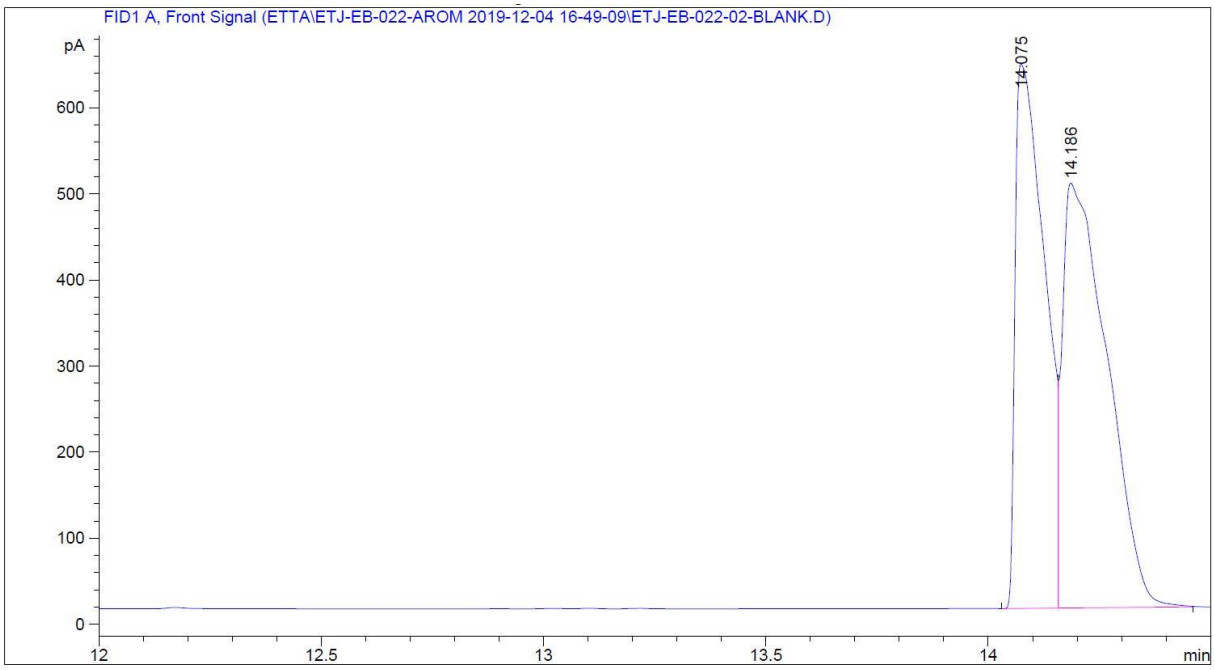

*Figure 72: GC-FID chromatogram of reference compound rac-**7a**.*

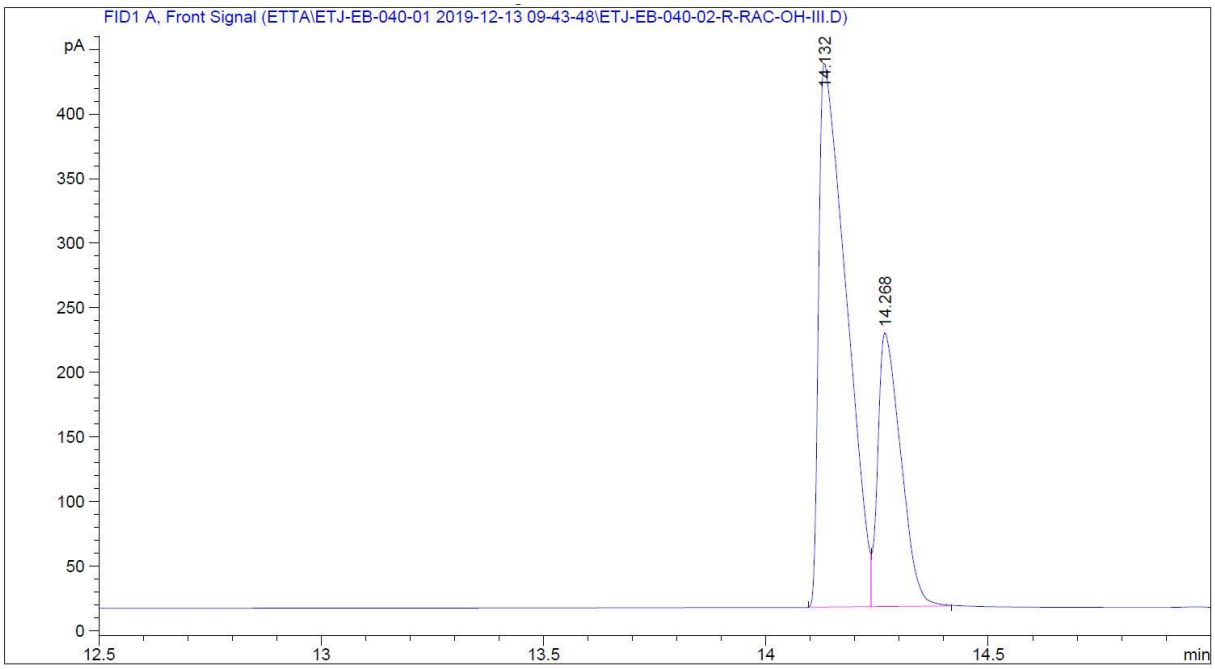

*Figure 73: GC-FID chromatogram of reference compound rac-**7a** with co-injection of (R)-**7a**.*

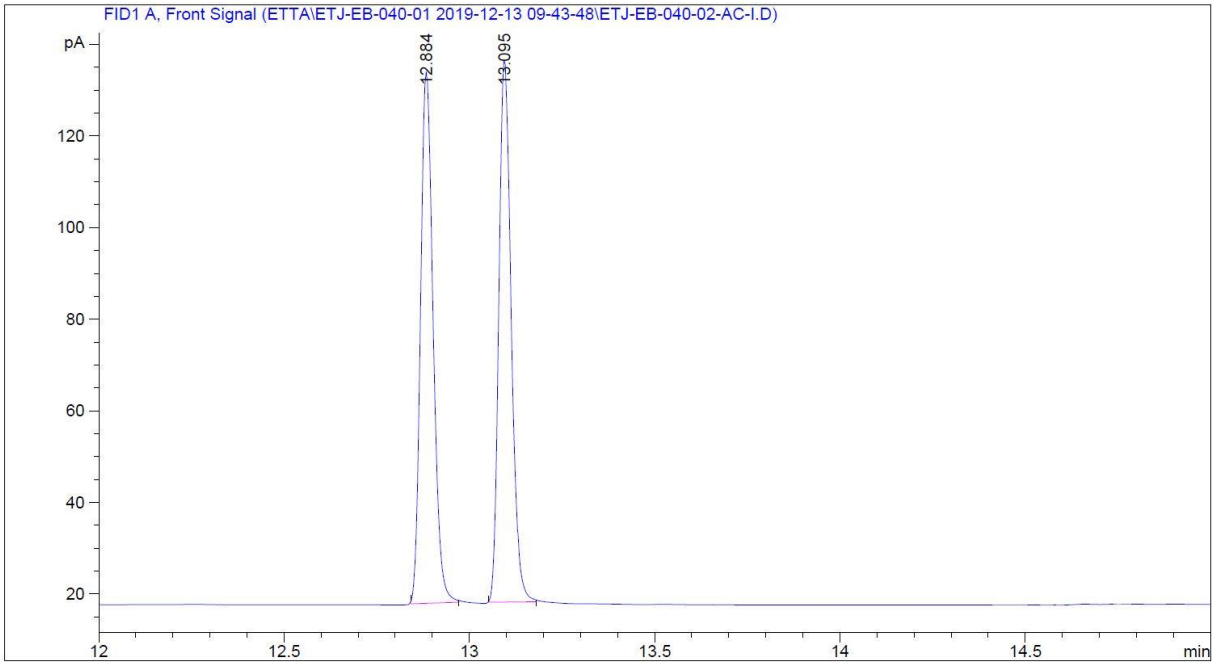

*Figure 74: GC-FID chromatogram of reference compound rac-**7b**.*

8.5.2.3 Substrate **8a/8b**

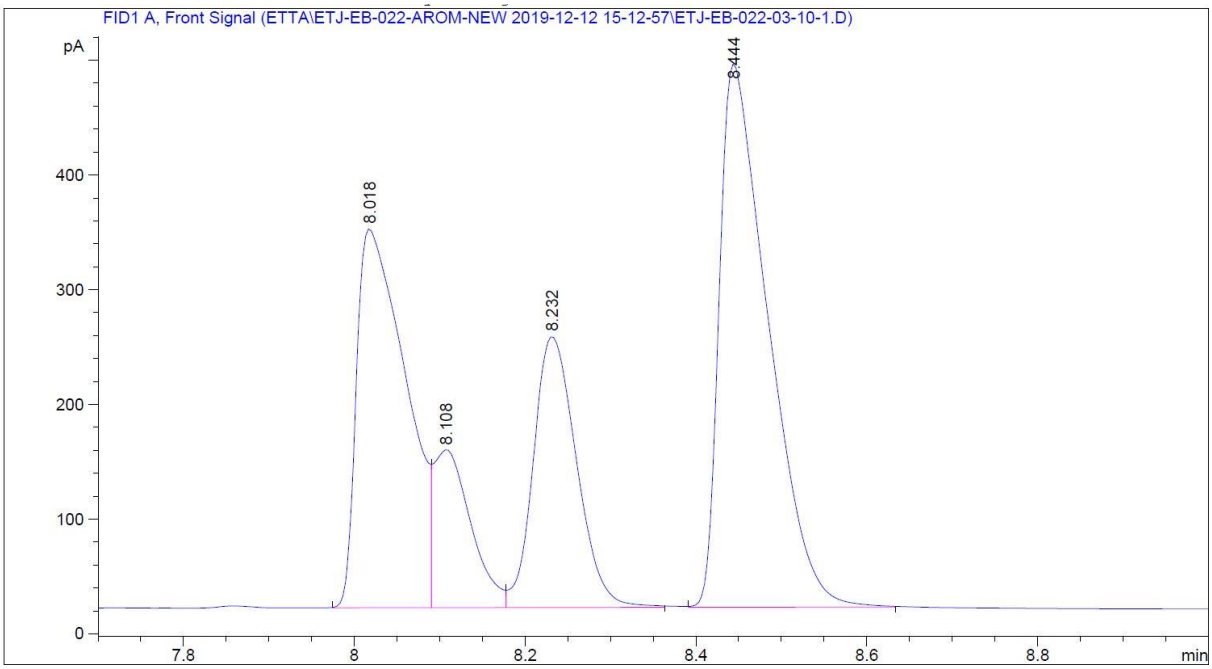

*Figure 75: GC-FID chromatogram of an acyl transfer reaction with substrate **8a**.*

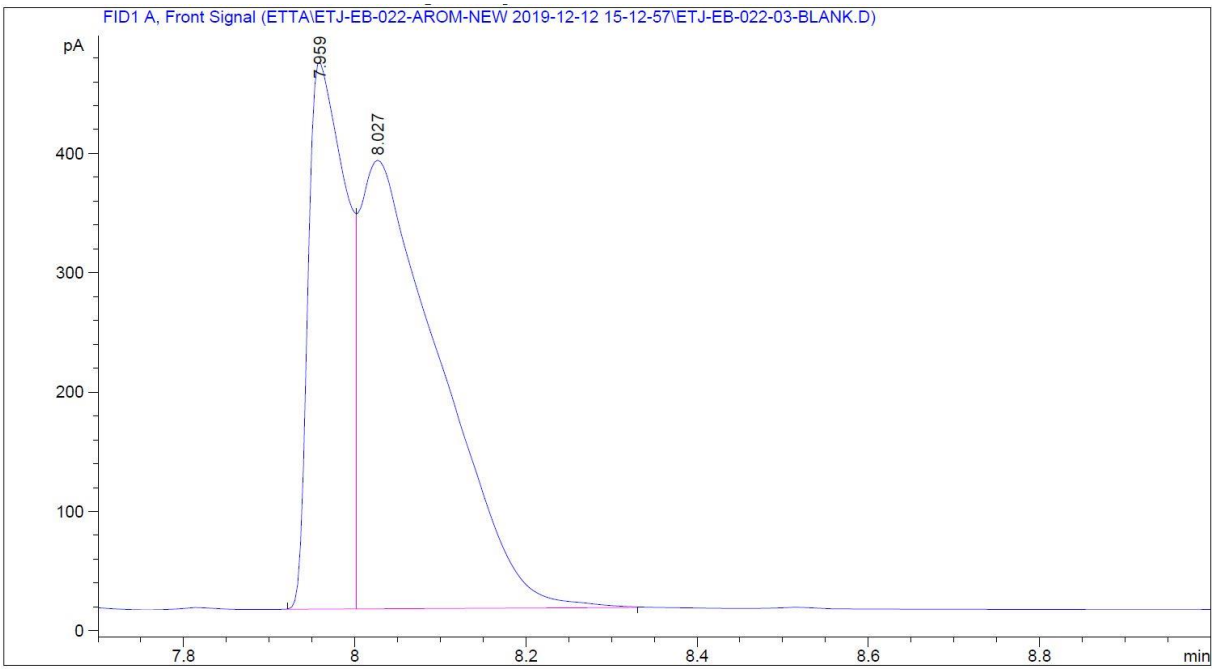

*Figure 76: GC-FID chromatogram of reference compound rac-**8a**.*

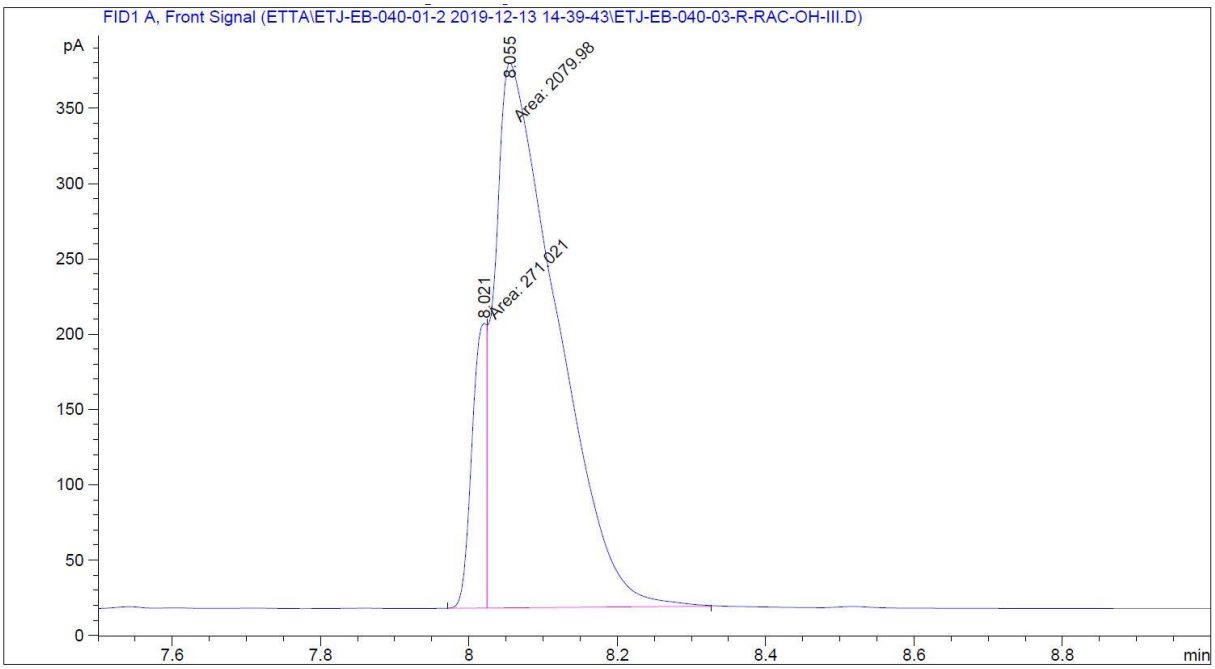

*Figure 77: GC-FID chromatogram of reference compound rac-**8a** with co-injection of (R)-**8a**.*

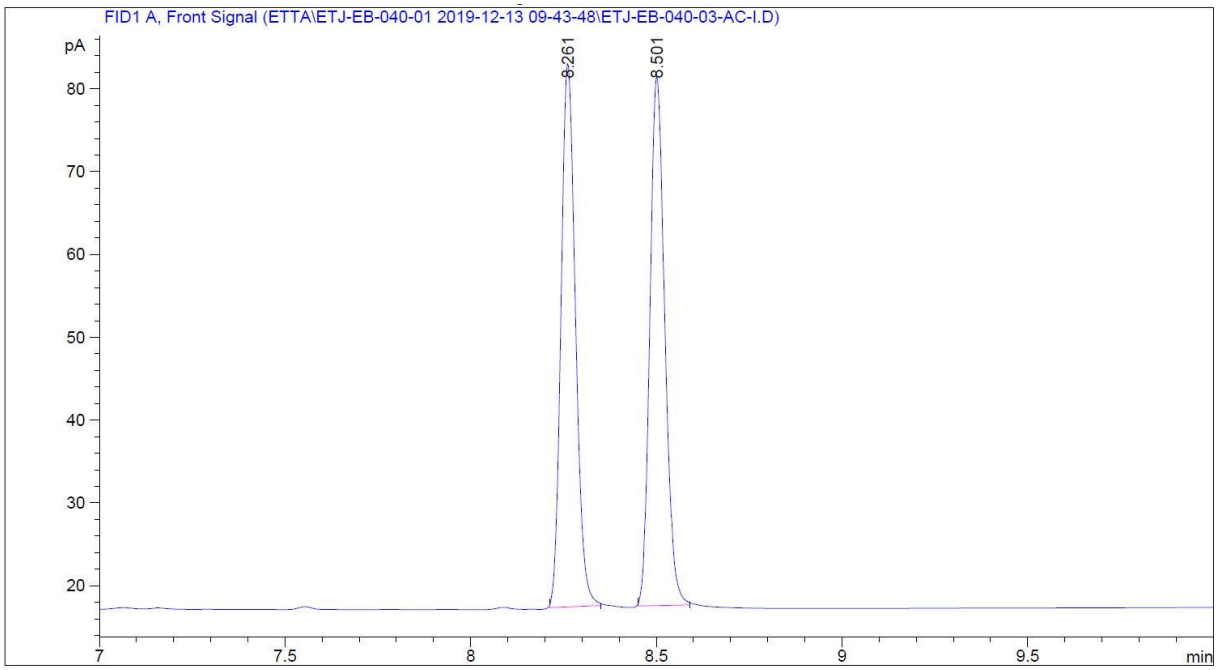

*Figure 78: GC-FID chromatogram of reference compound rac-**8b**.*

8.5.2.4 Substrate **9a/9b**

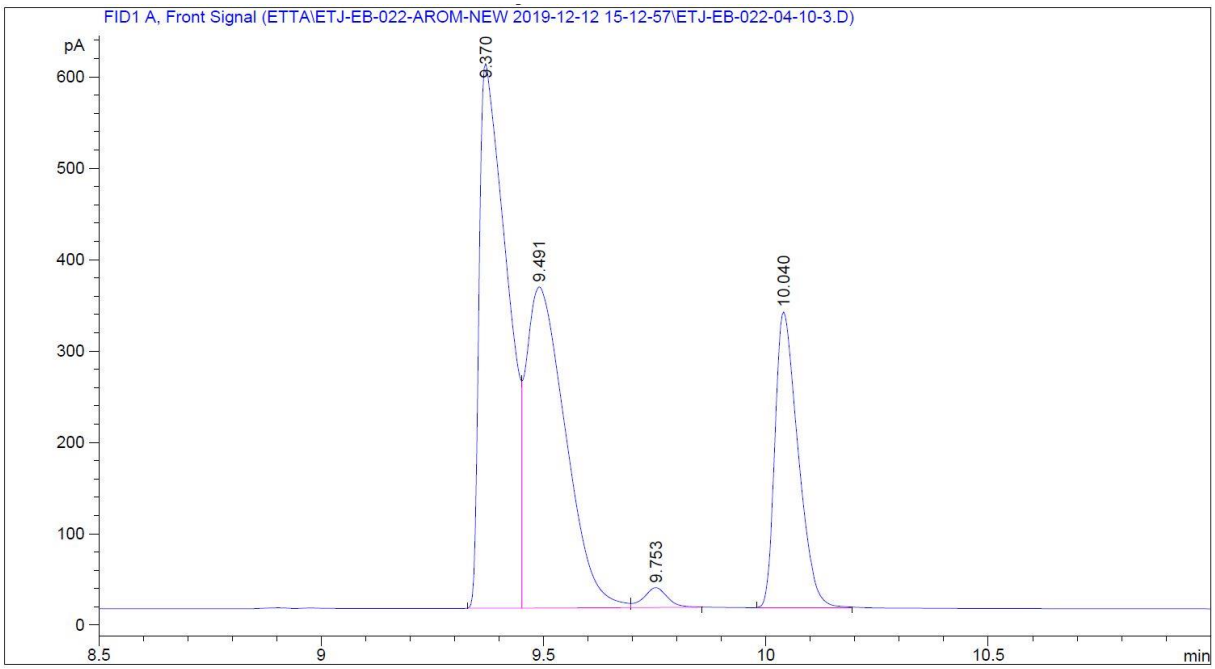

*Figure 79: GC-FID chromatogram of an acyl transfer reaction with substrate **9a**.*

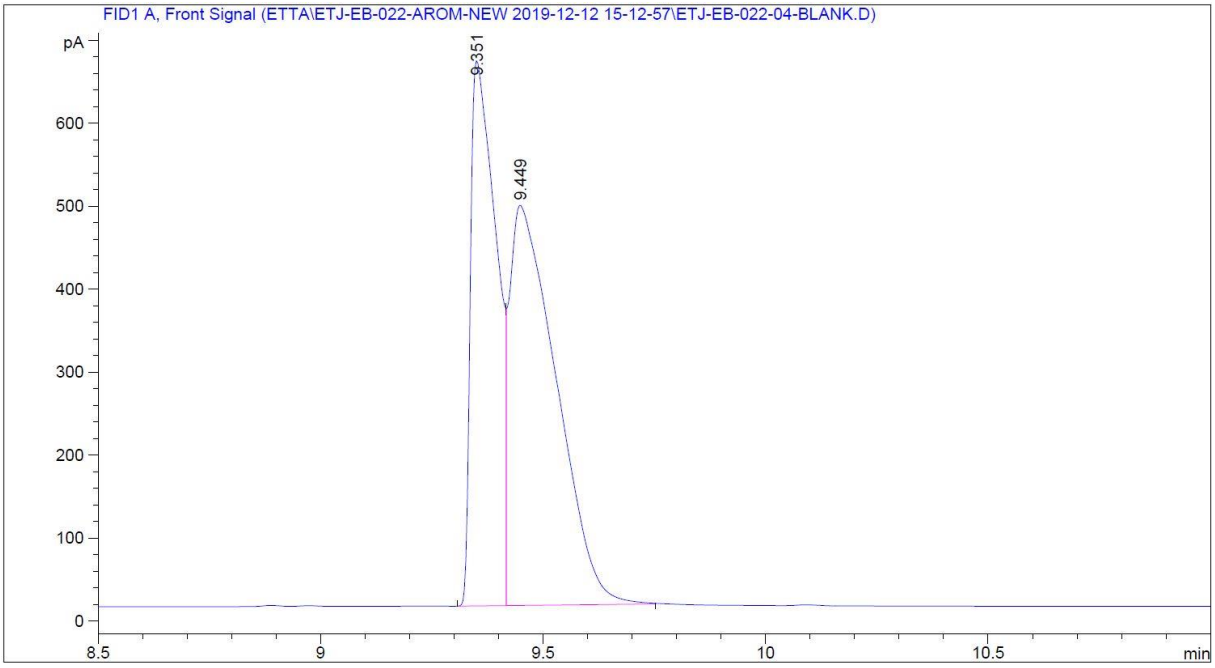

*Figure 80: GC-FID chromatogram of reference compound rac-**9a**.*

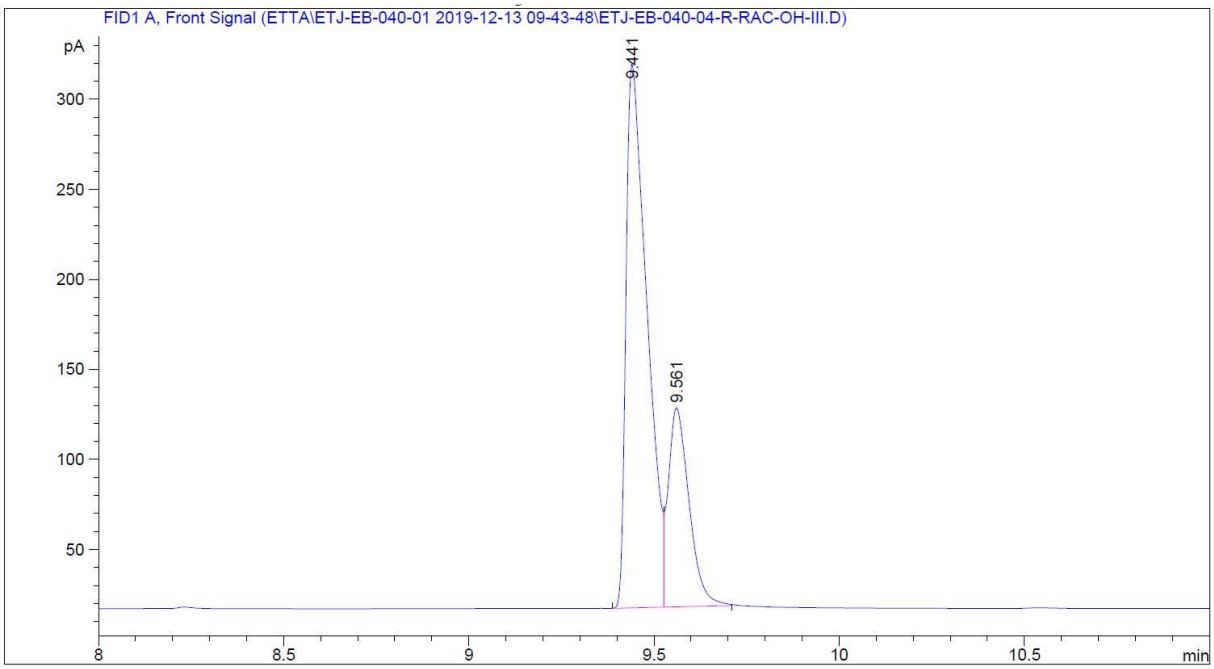

*Figure 81: GC-FID chromatogram of reference compound rac-**9a** with co-injection of (S)-**9a**.*

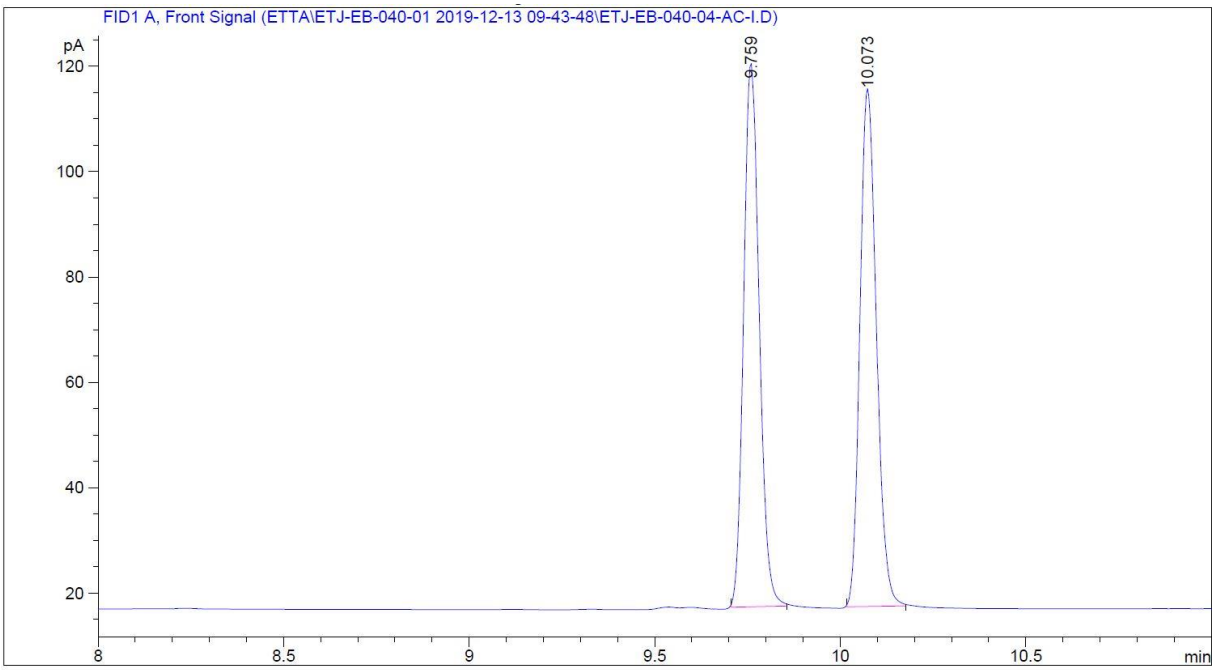

*Figure 82: GC-FID chromatogram of reference compound rac-**9b**.*

8.5.2.5 Substrate **10a/10b**

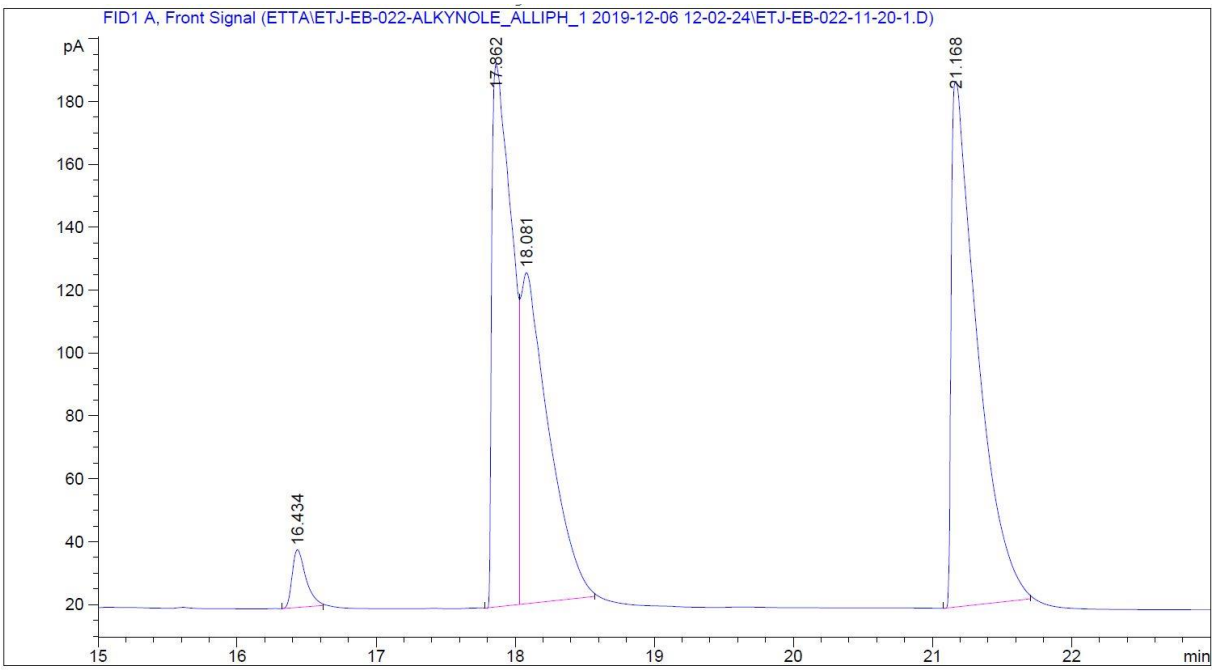

*Figure 83: GC-FID chromatogram of an acyl transfer reaction with substrate **10a**.*

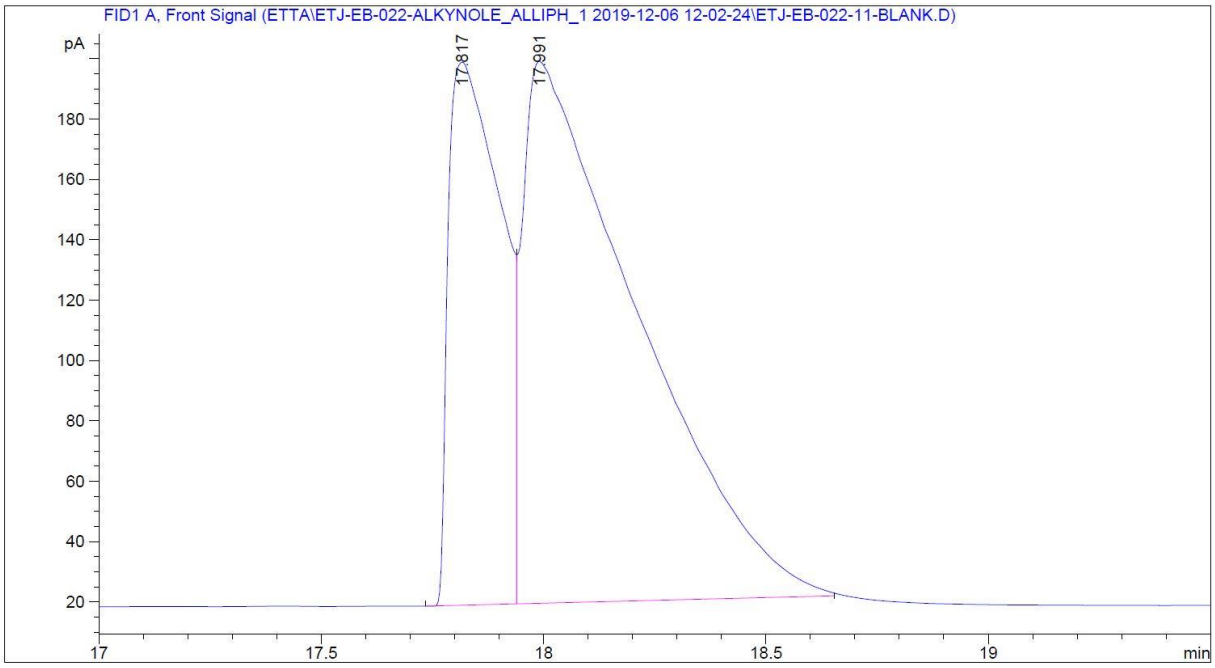

*Figure 84: GC-FID chromatogram of reference compound rac-**10a**.*

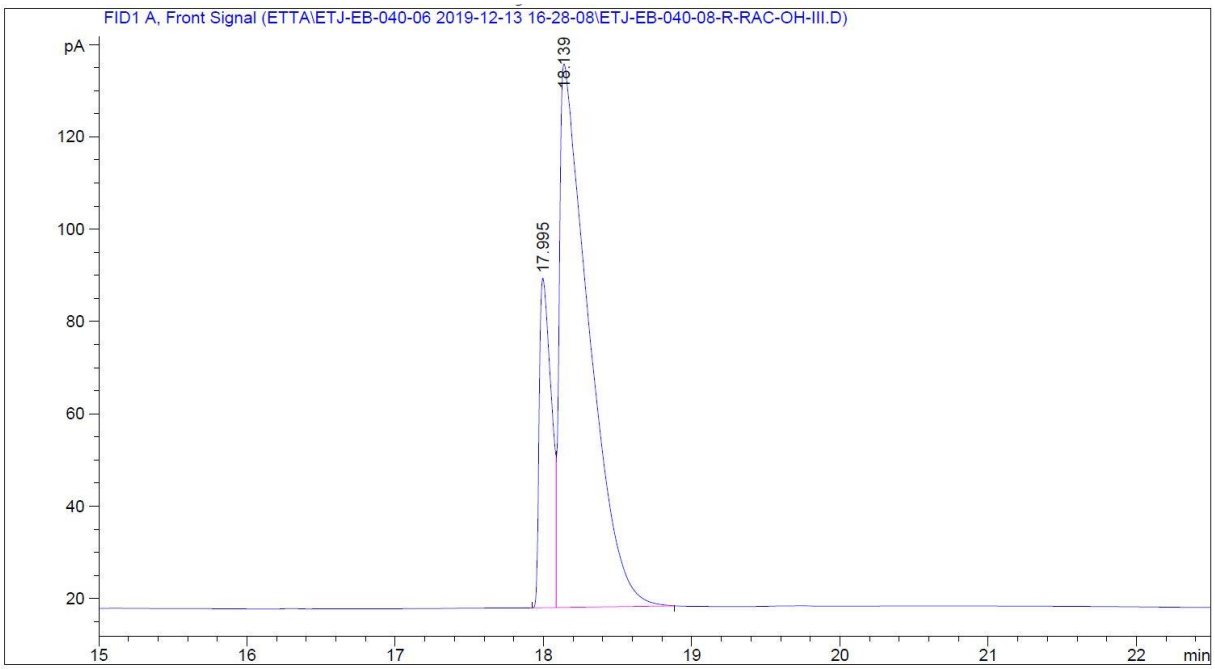

*Figure 85: GC-FID chromatogram of reference compound rac-**10a** with co-injection of (R)-**10a**.*

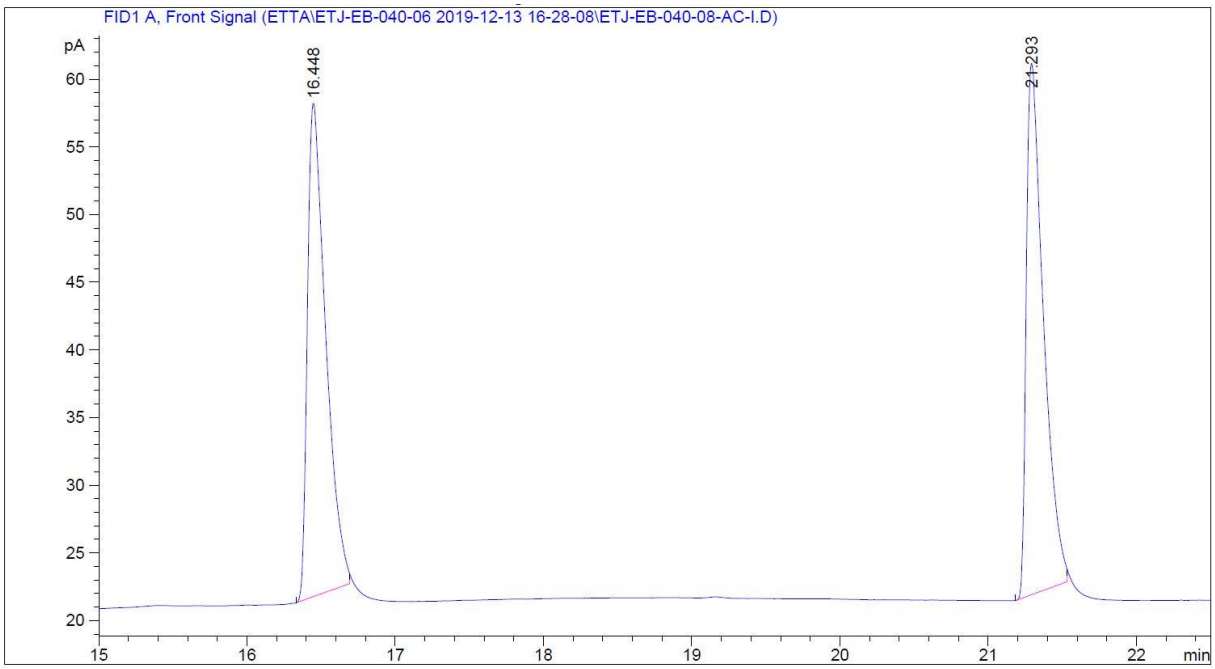

*Figure 86: GC-FID chromatogram of reference compound rac-**10b**.*

8.5.2.6 Substrate **11a/11b**

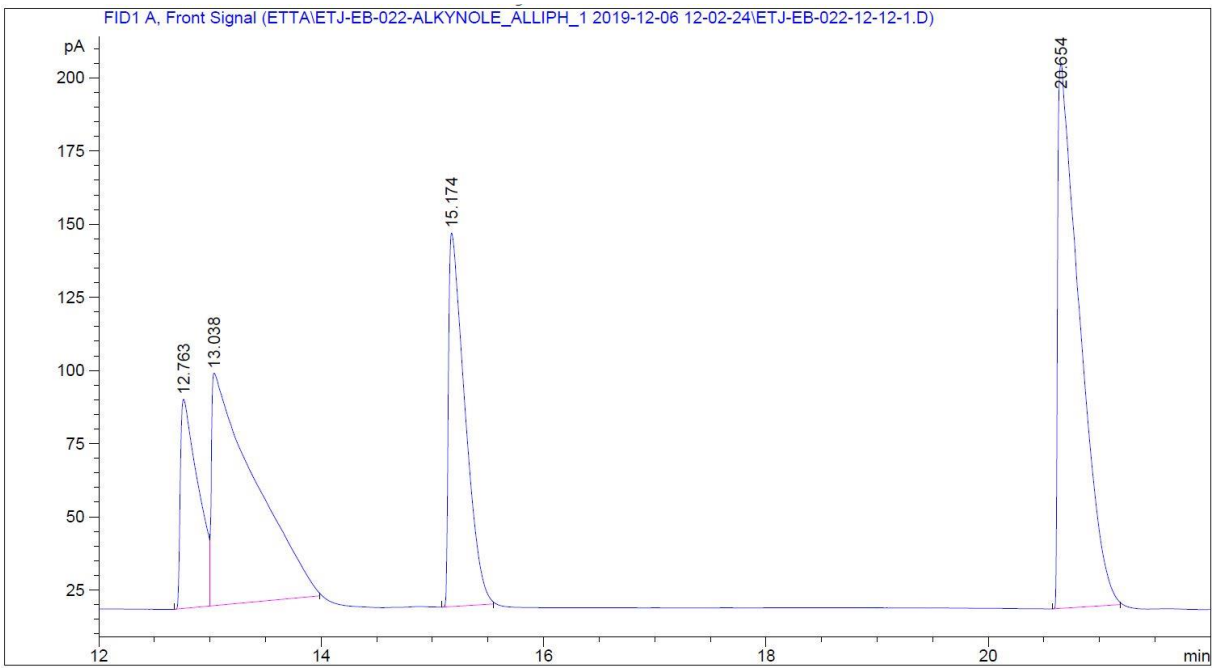

*Figure 87: GC-FID chromatogram of an acyl transfer reaction with substrate **11a**.*

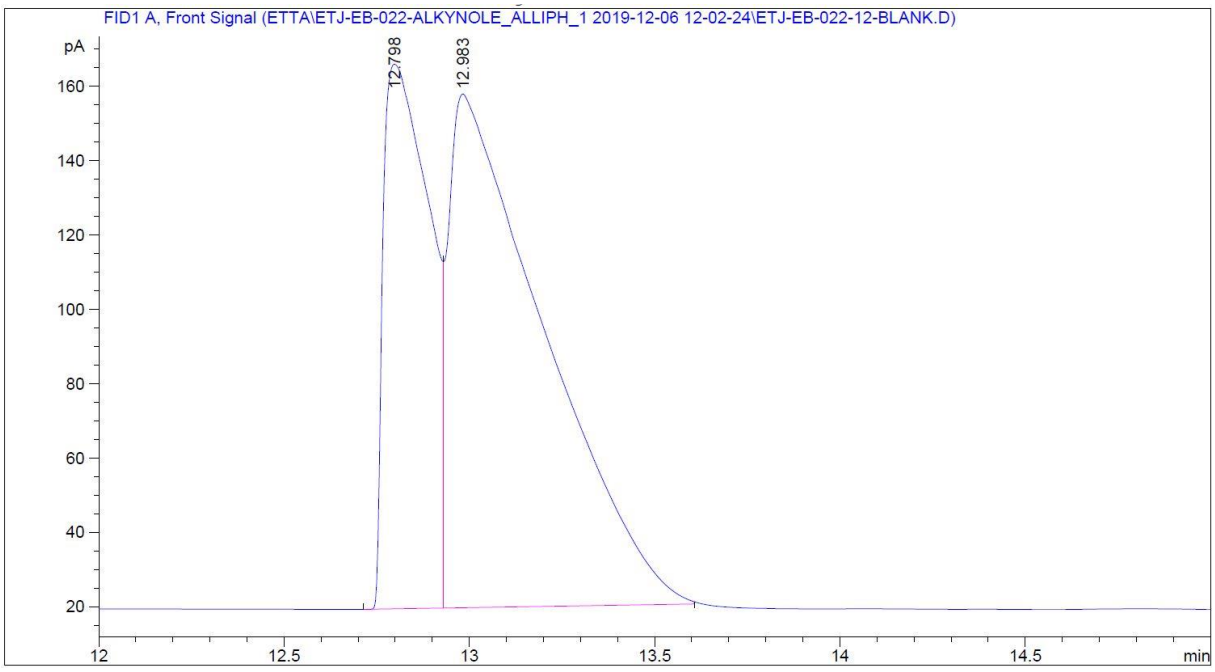

*Figure 88: GC-FID chromatogram of reference compound rac-**11a**.*

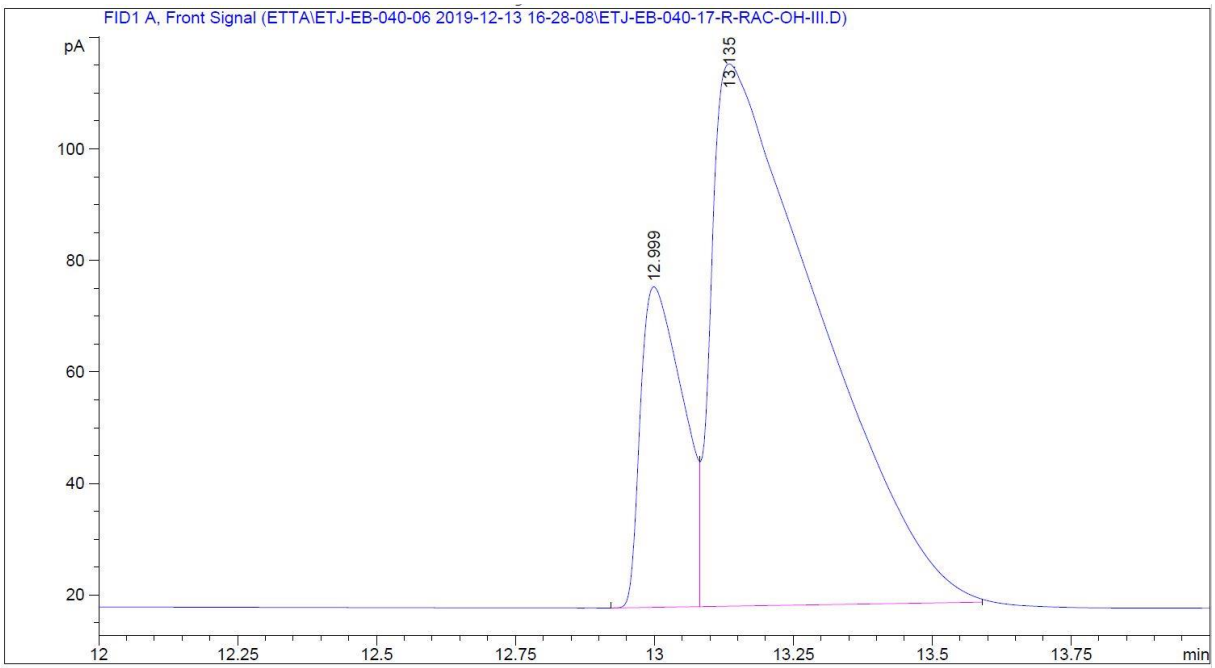

*Figure 89: GC-FID chromatogram of reference compound rac-**11a** with co-injection of (R)-**11a**.*

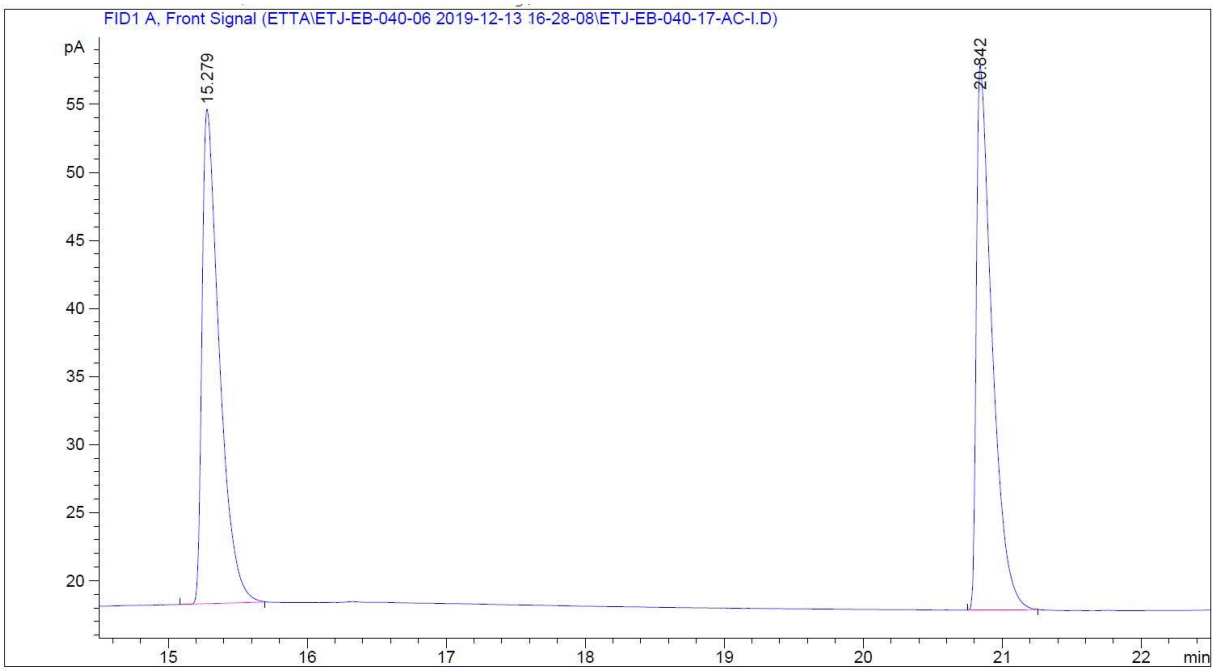

*Figure 90: GC-FID chromatogram of reference compound rac-**11b**.*

8.5.2.7 Substrate ***12a/12b***

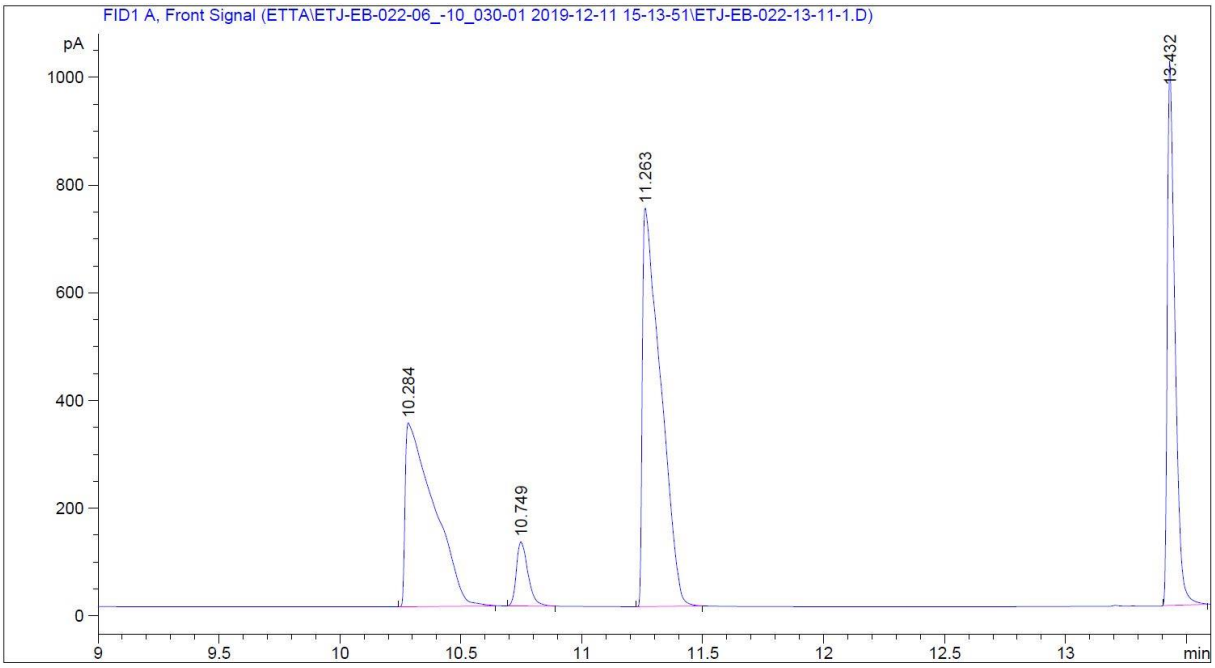

*Figure 91: GC-FID chromatogram of an acyl transfer reaction with substrate **12a** with internal standard 1-decanol (RT 13.43 min).*

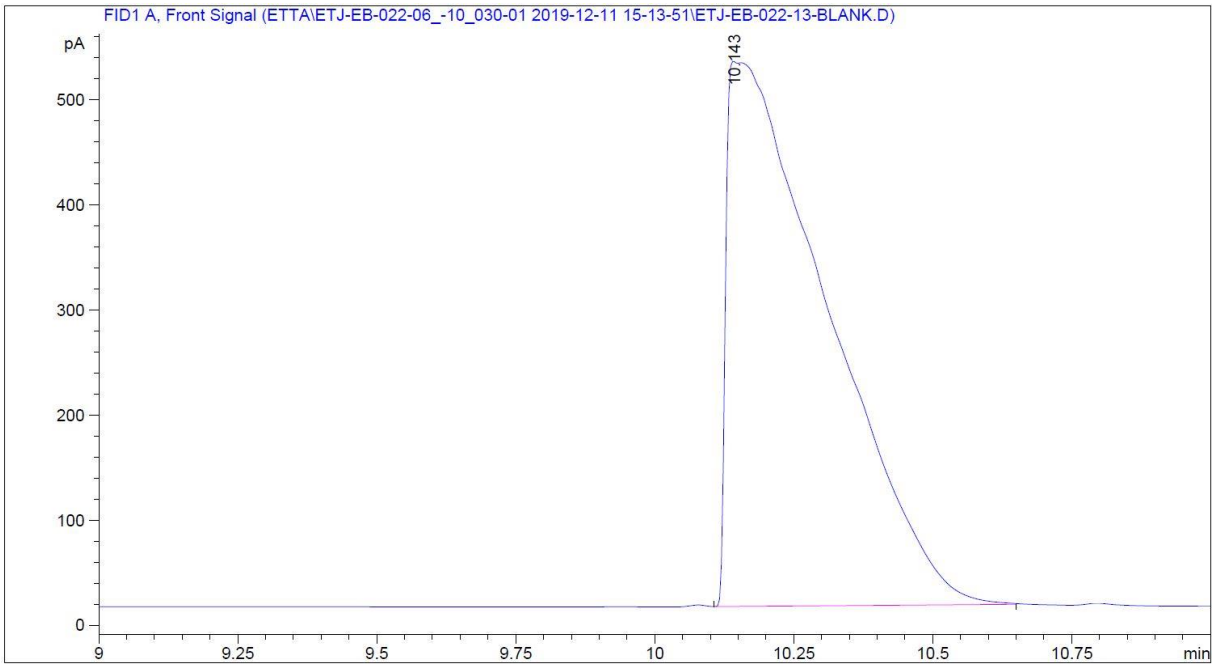

*Figure 92: GC-FID chromatogram of reference compound rac-**12a**.*

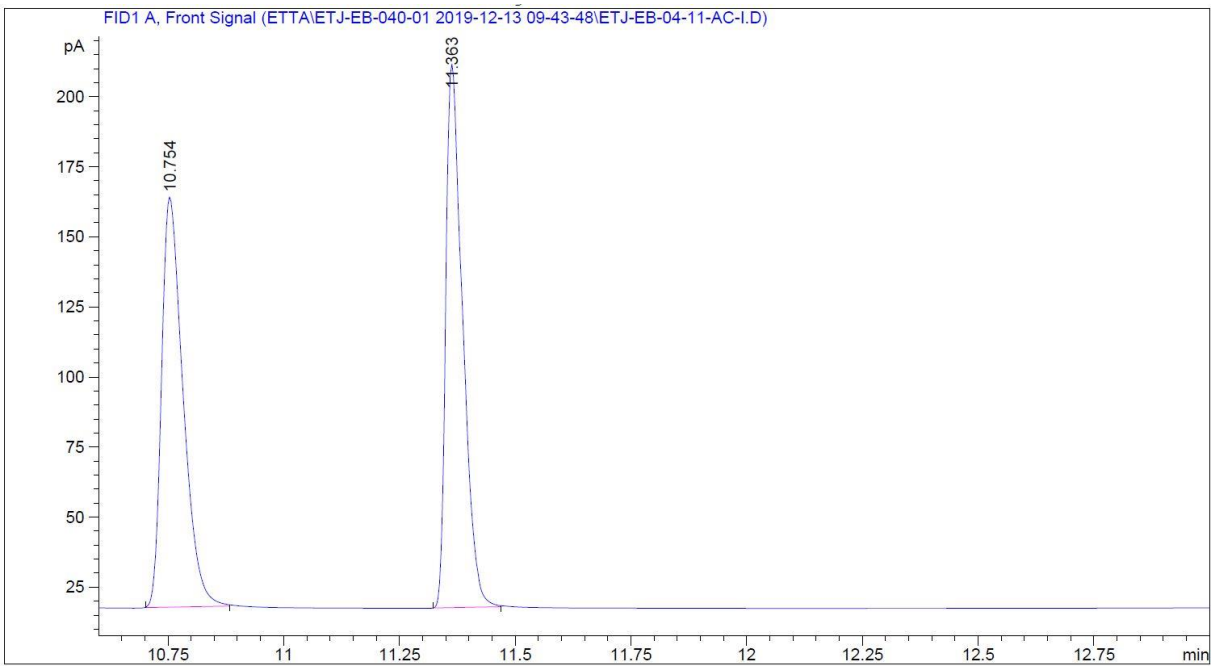

*Figure 93: GC-FID chromatogram of reference compound rac-**12b**.*

8.5.2.8 Substrate **13a/13b**

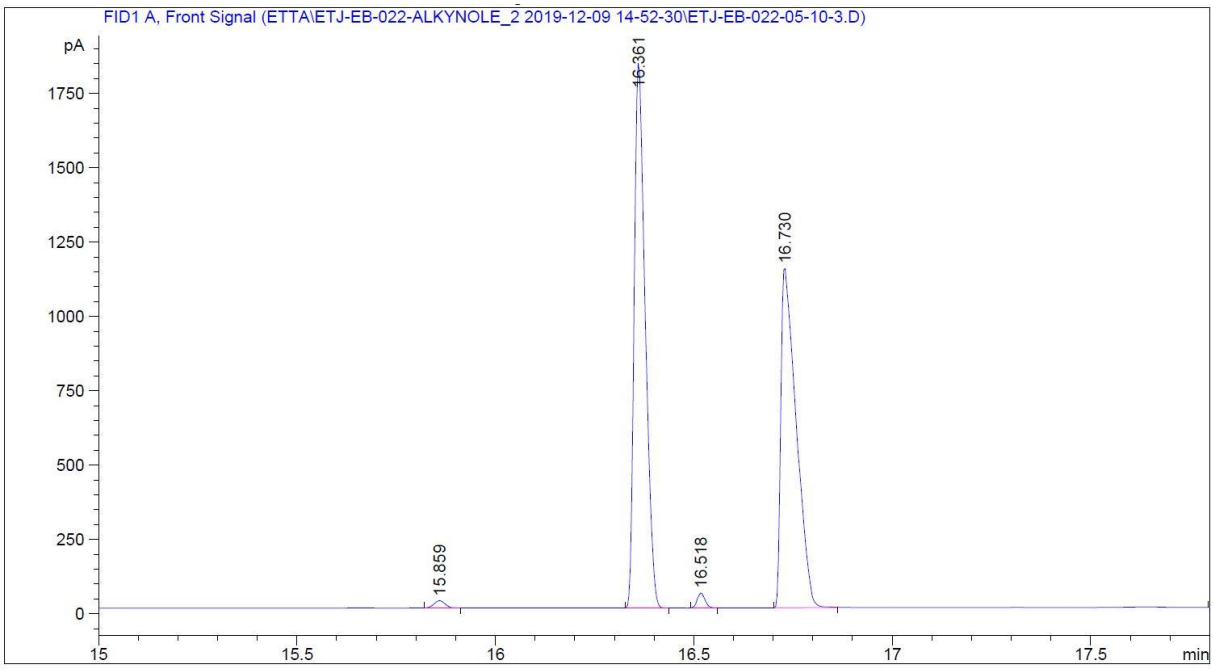

*Figure 94: GC-FID chromatogram of an acyl transfer reaction with substrate **13a**.*

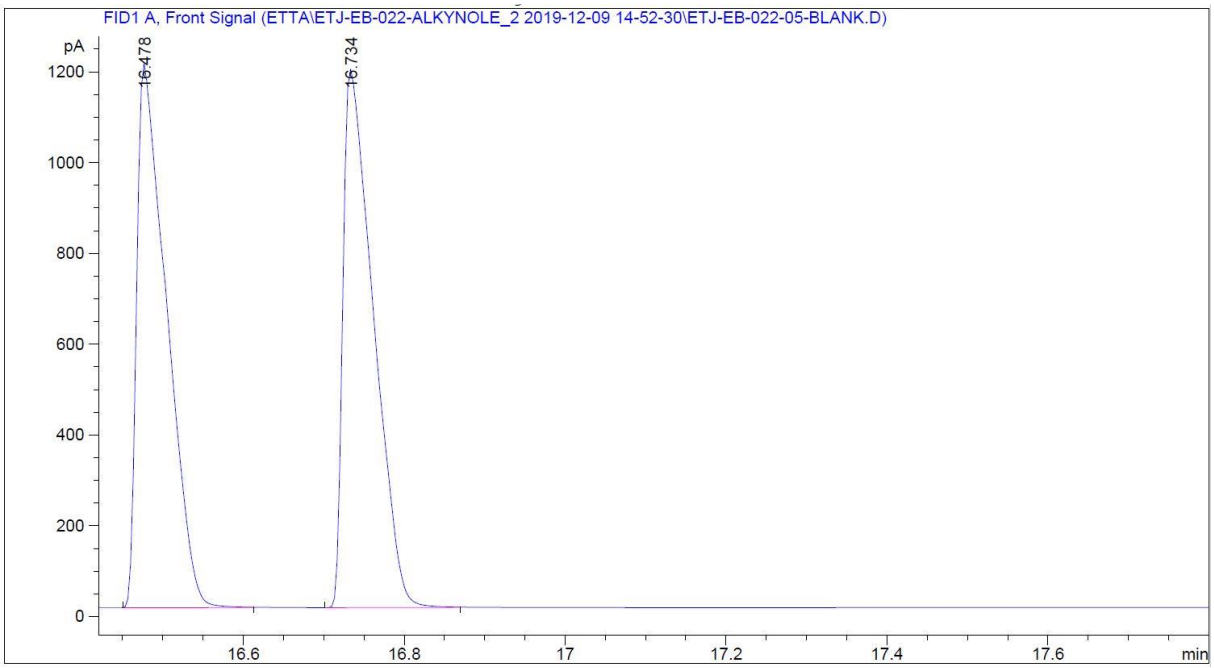

*Figure 95: GC-FID chromatogram of reference compound rac-**13a**.*

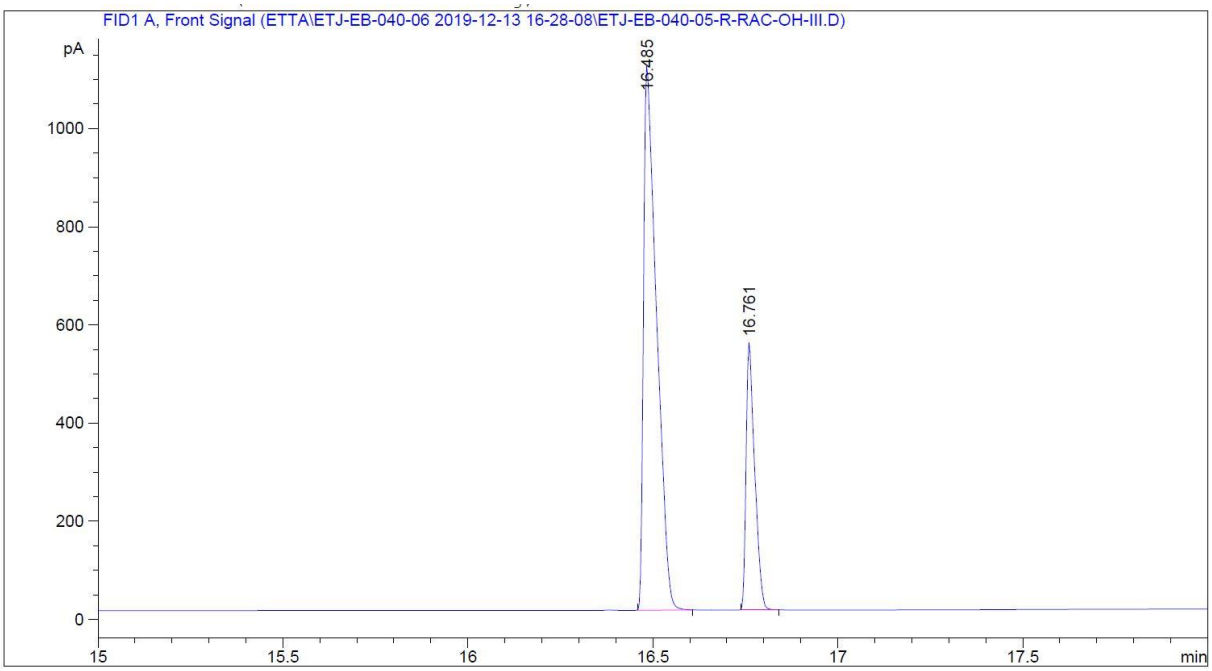

*Figure 96: GC-FID chromatogram of reference compound rac-**13a** with co-injection of (R)-**13a**.*

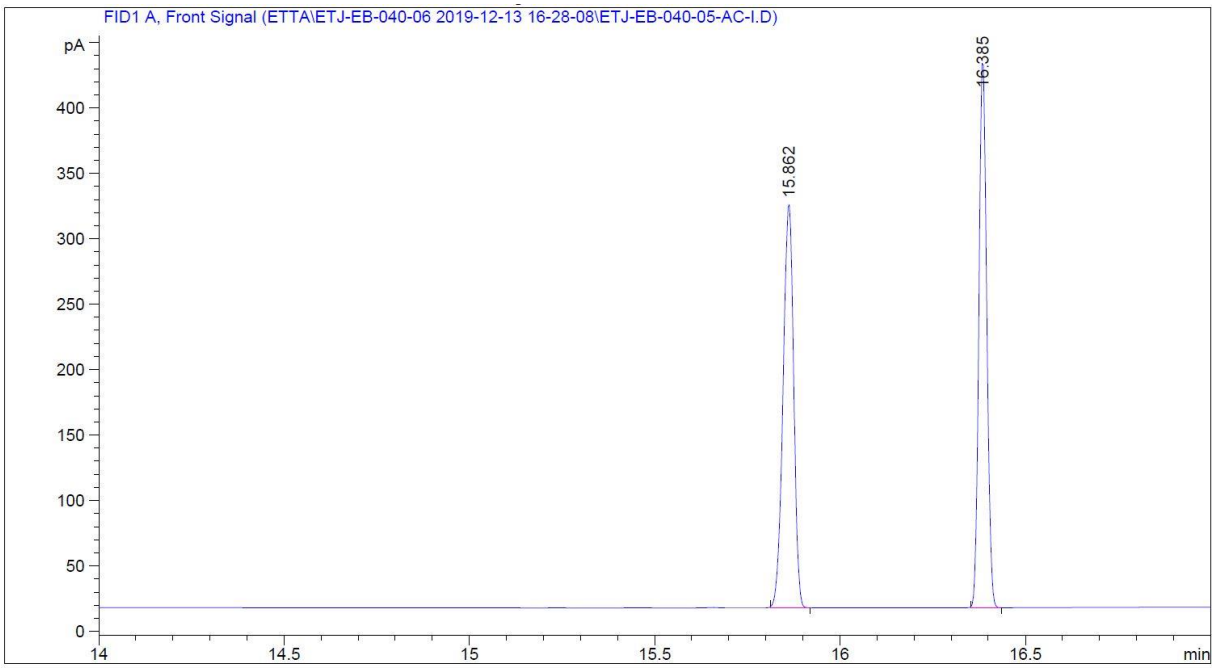

*Figure 97: GC-FID chromatogram of reference compound rac-**13b**.*

8.5.2.9 Substrate **14a/14b**

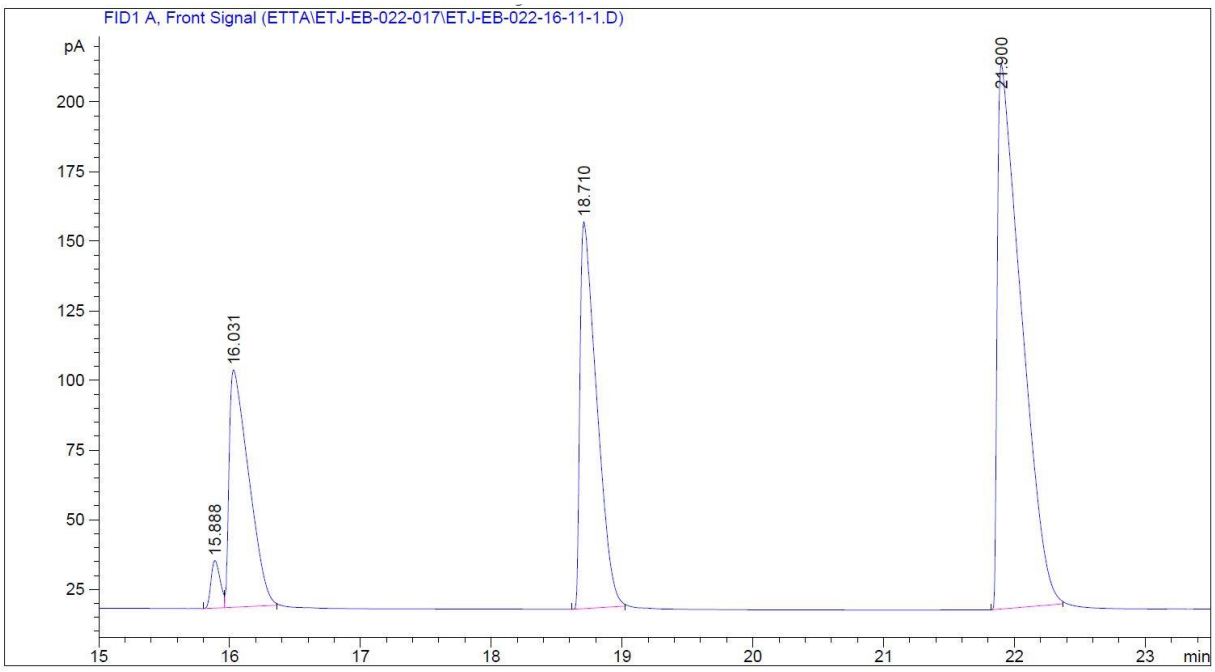

*Figure 98: GC-FID chromatogram of an acyl transfer reaction with substrate **14a**.*

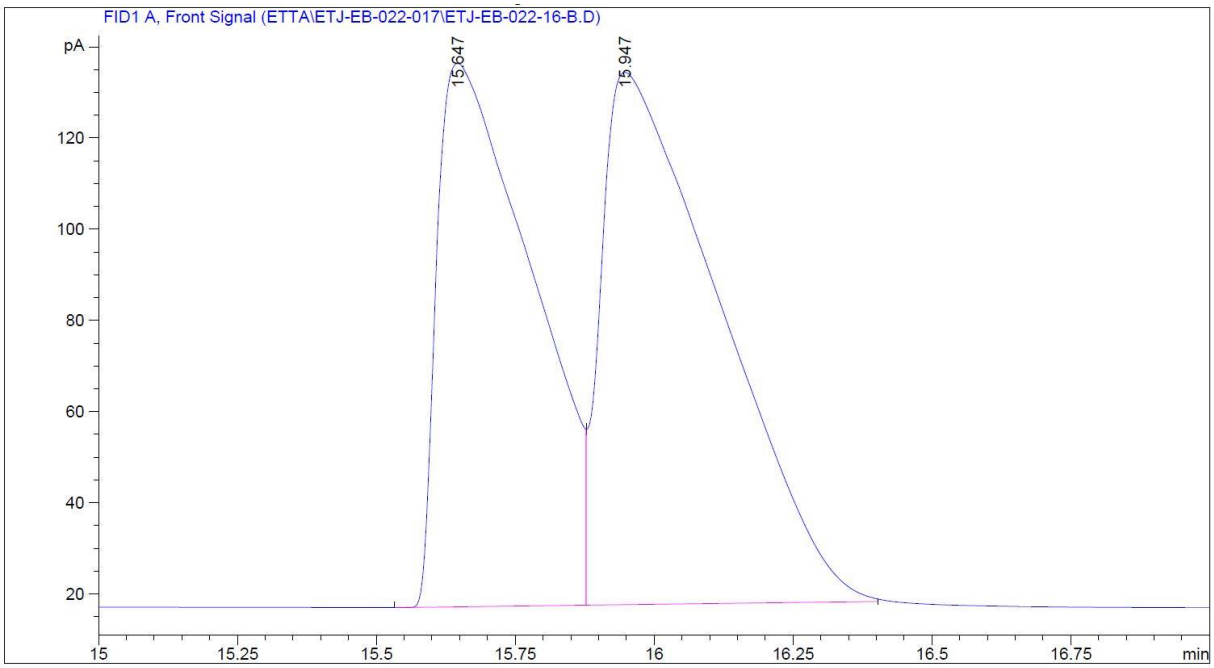

*Figure 99: GC-FID chromatogram of reference compound rac-**14a**.*

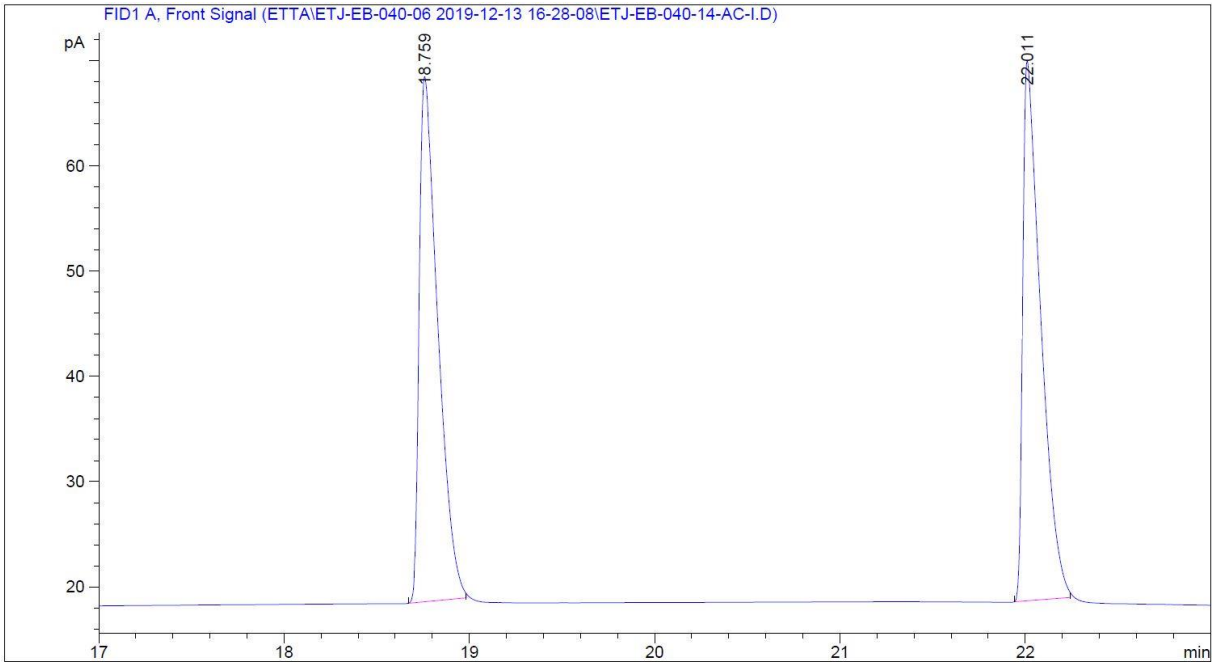

*Figure 100: GC-FID chromatogram of reference compound rac-**14b**.*

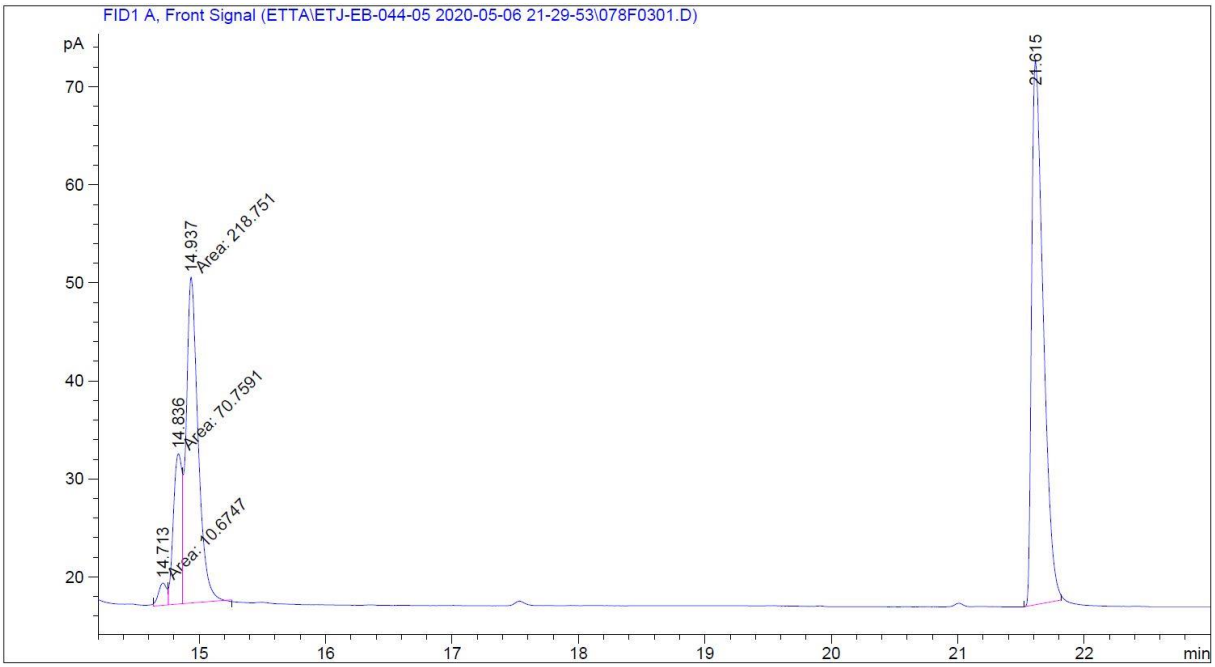

*Figure 101: GC-FID chromatogram of hydrogenated acyl transfer reaction with substrate **14a**.*

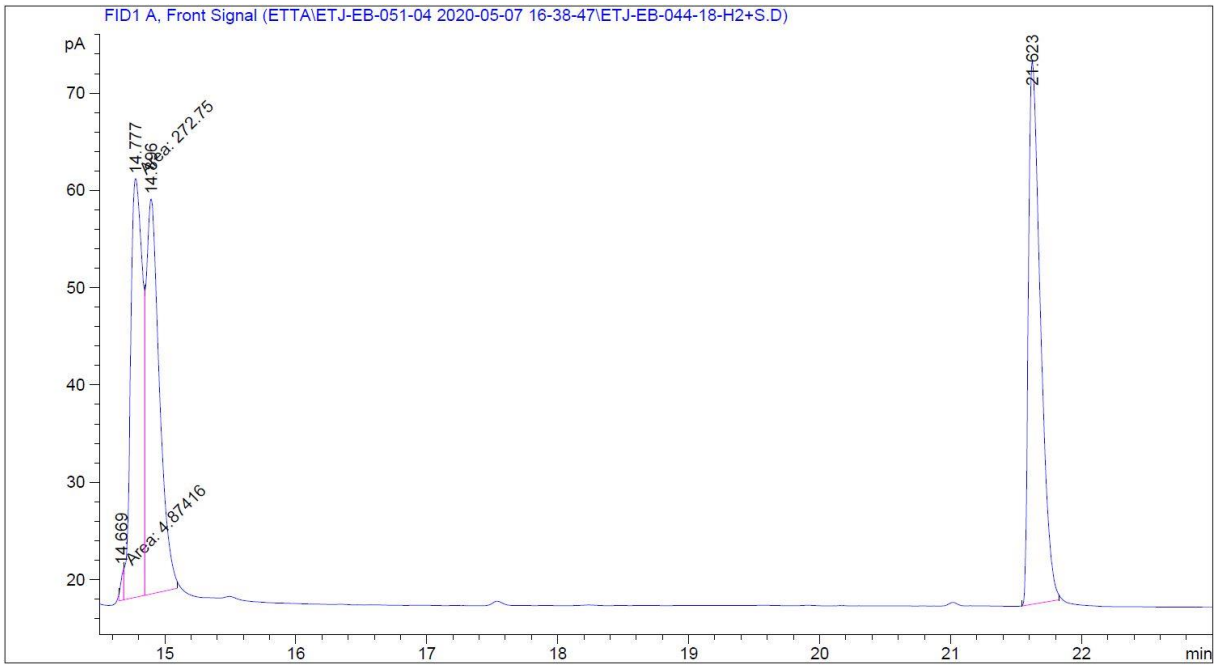

*Figure 102: GC-FID chromatogram of hydrogenated acyl transfer reaction with substrate **14a** with co-injection of (S)-2-pentanol.*

8.5.2.10 Substrate **15a/15b**

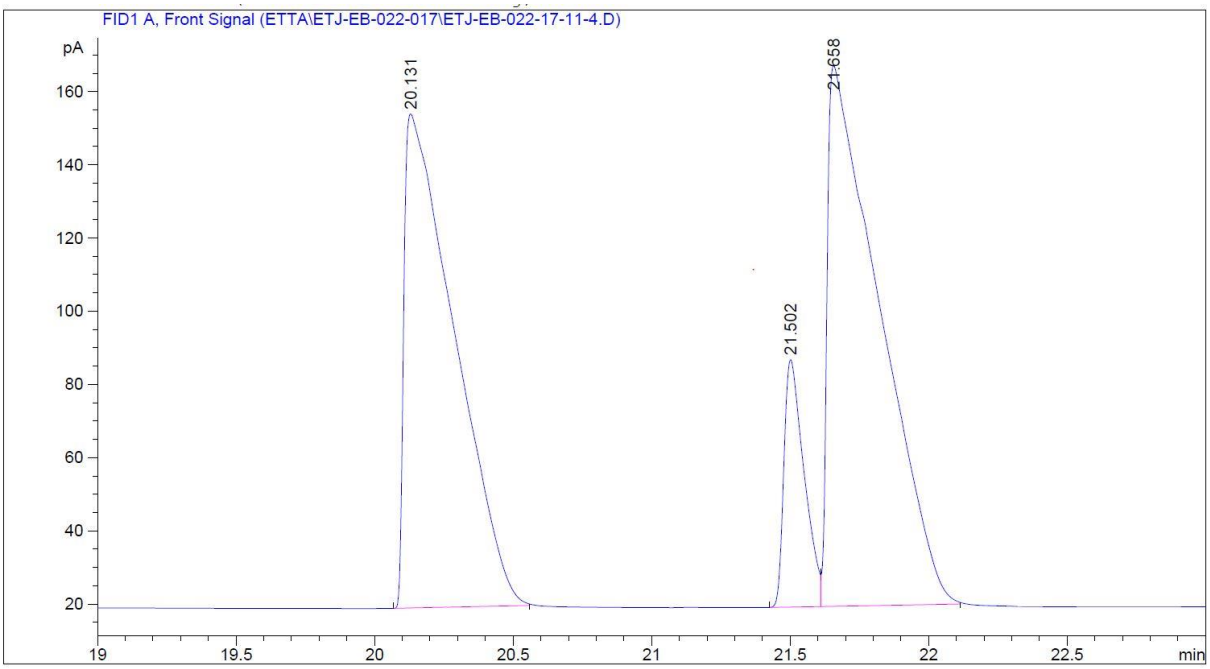

*Figure 103: GC-FID chromatogram of an acyl transfer reaction with substrate **15a**.*

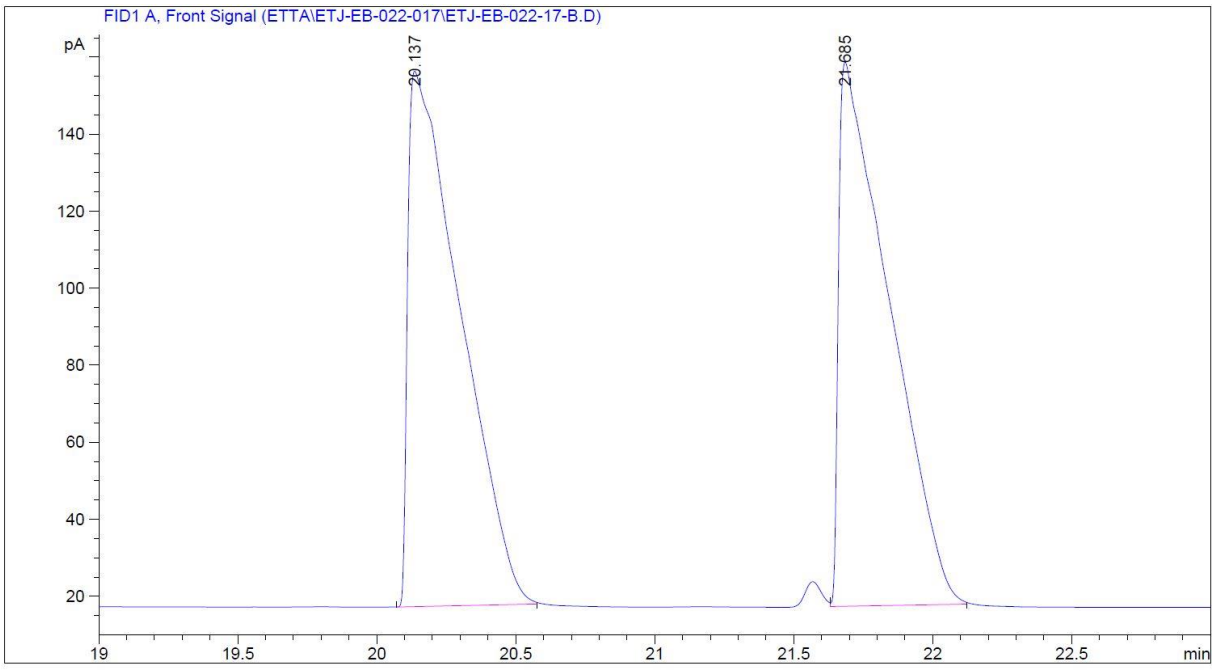

*Figure 104: GC-FID chromatogram of reference compound rac-**15a**.*

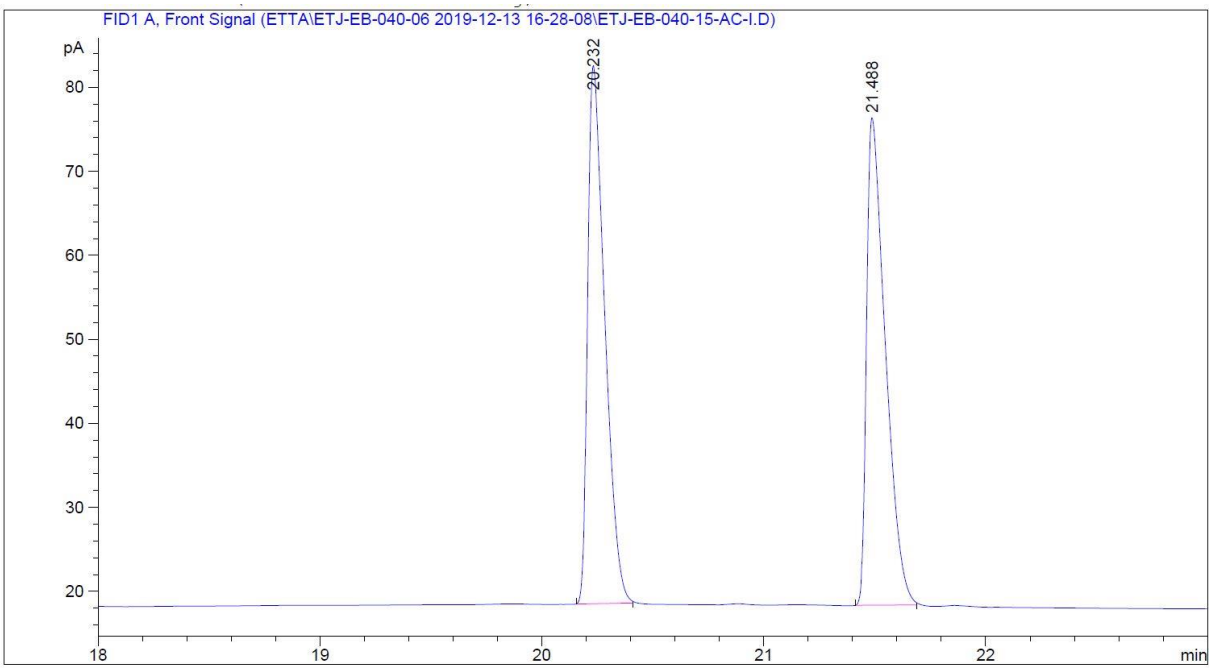

*Figure 105: GC-FID chromatogram of reference compound rac-**15b**.*

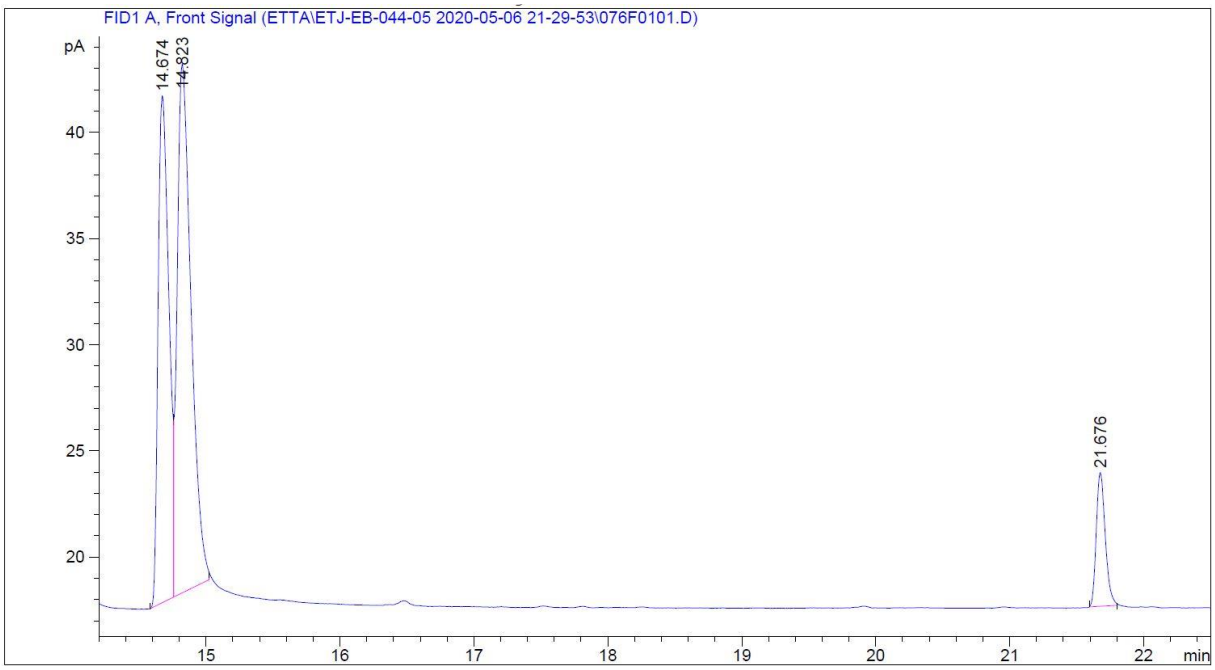

*Figure 106: GC-FID chromatogram of hydrogenated acyl transfer reaction with substrate **15a**.*

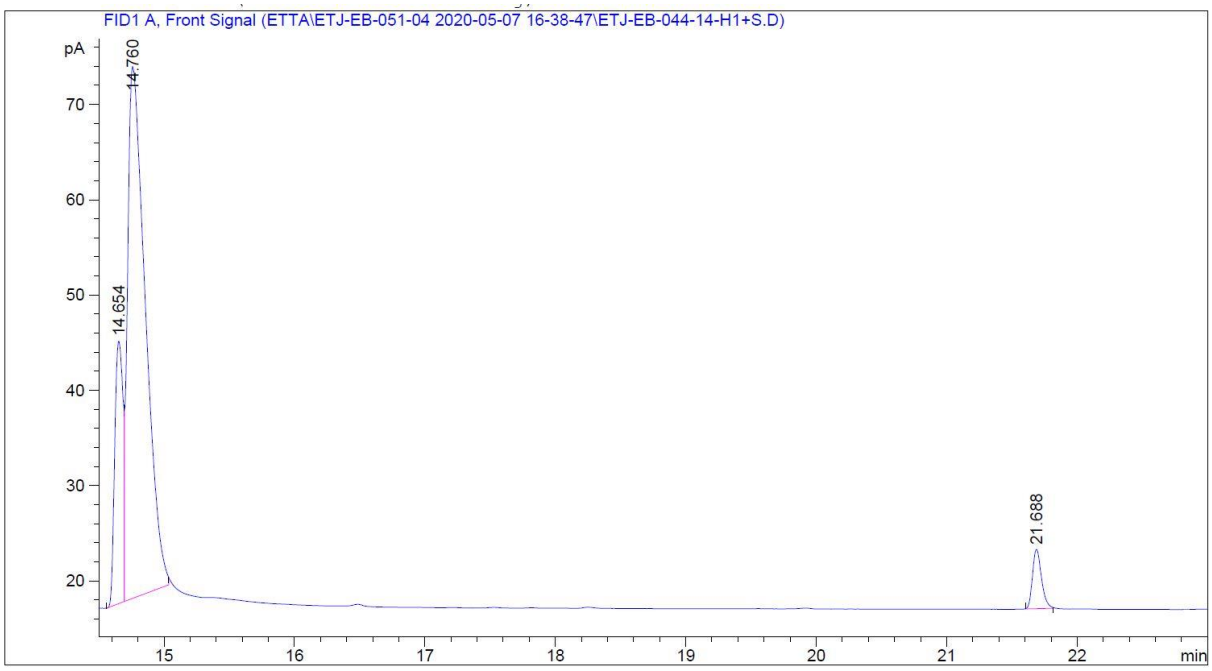

*Figure 107: GC-FID chromatogram of hydrogenated acyl transfer reaction with substrate **15a** with co-injection of (S)-2-pentanol.*

8.5.2.11 Compounds 2-pentanol and 2-pentanyl acetate

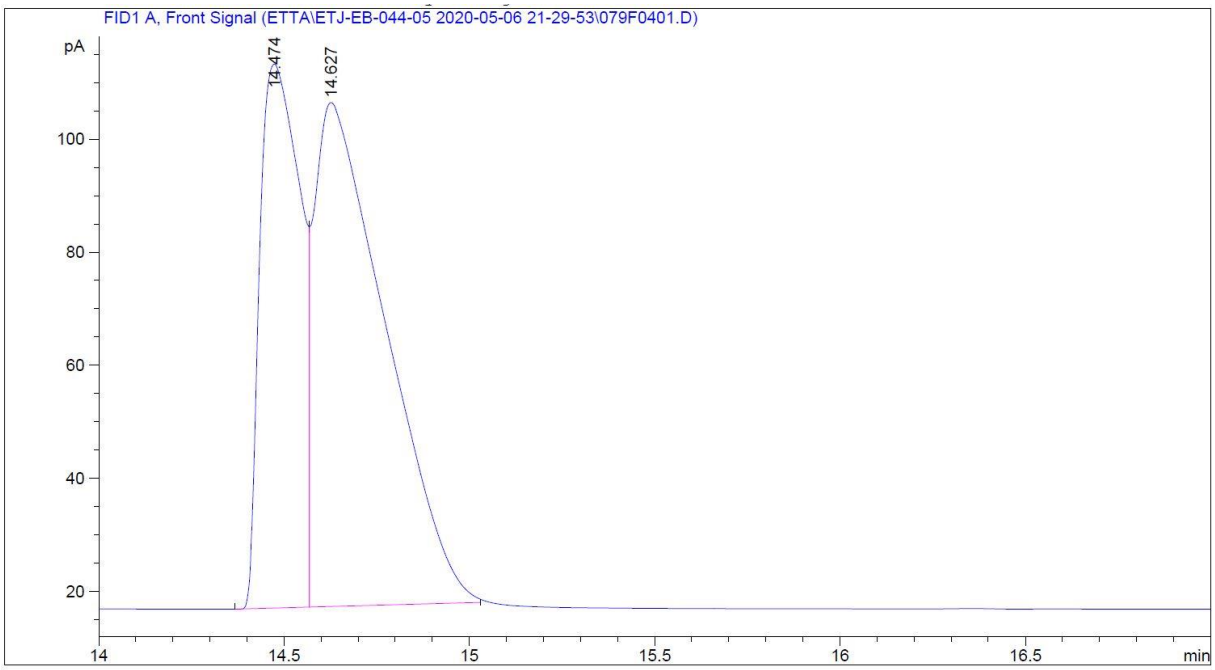

Figure 108: GC-FID chromatogram of reference compound rac-2-pentanol.

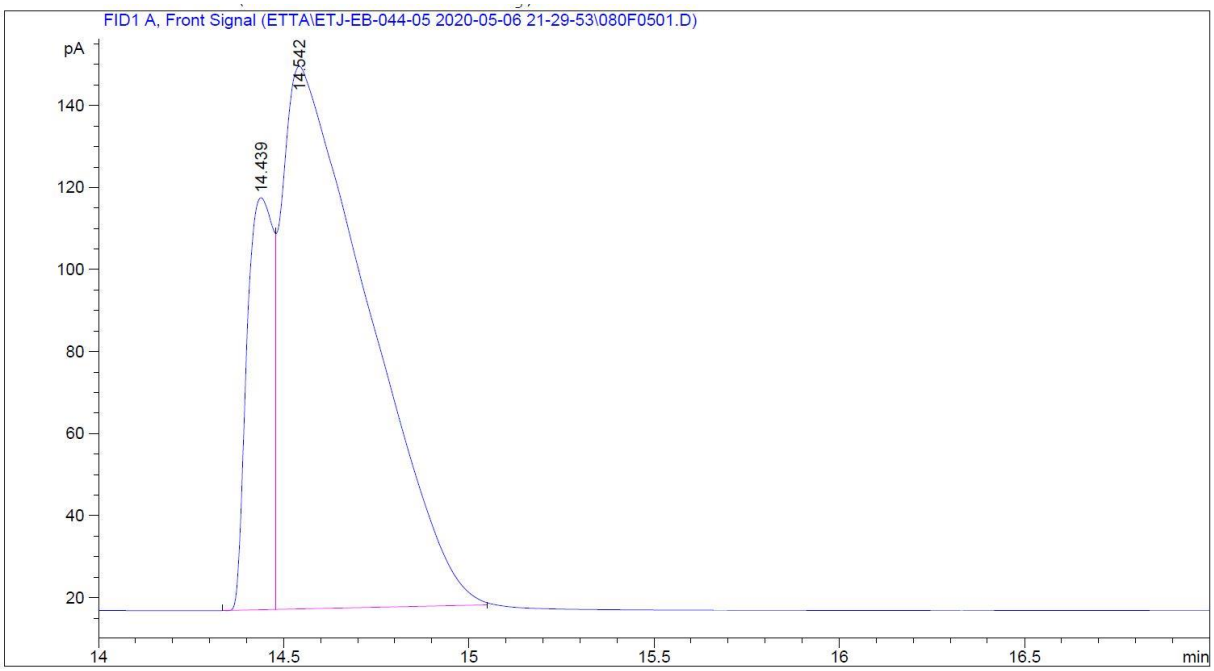

Figure 109: GC-FID chromatogram of reference compound rac-2-pentanol with co-injection of (S)-2-pentanol.

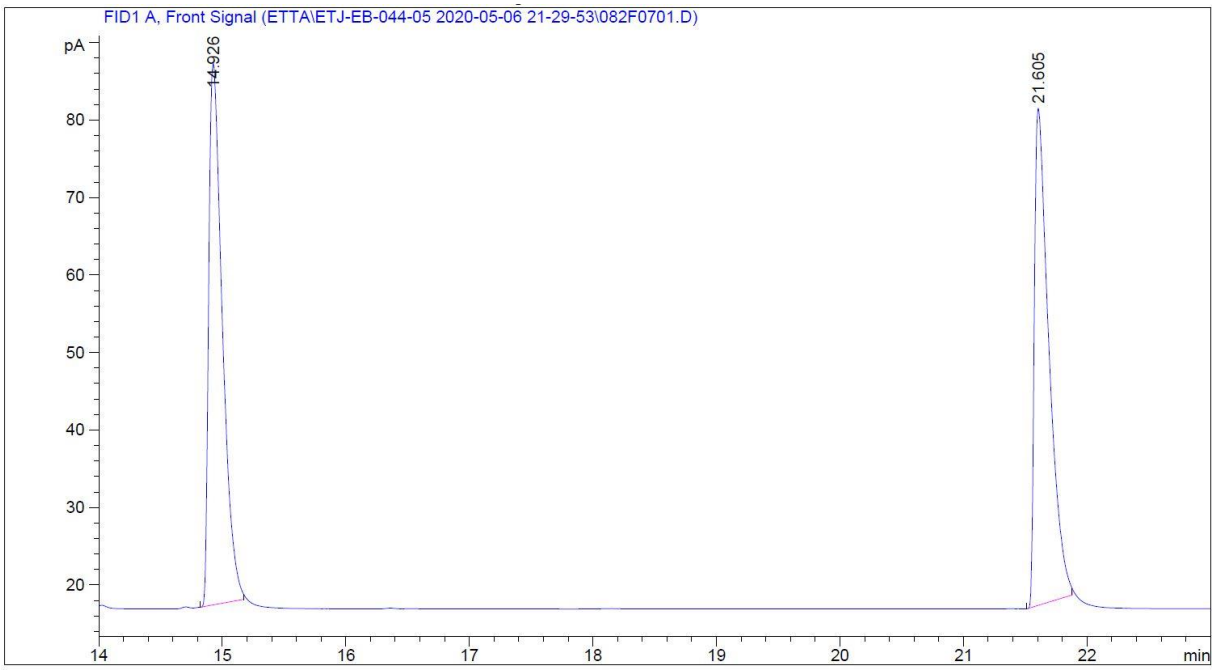

Figure 110: GC-FID chromatogram of reference compound rac-2-pentanyl acetate.

8.5.2.12 Substrate **16a/16b**

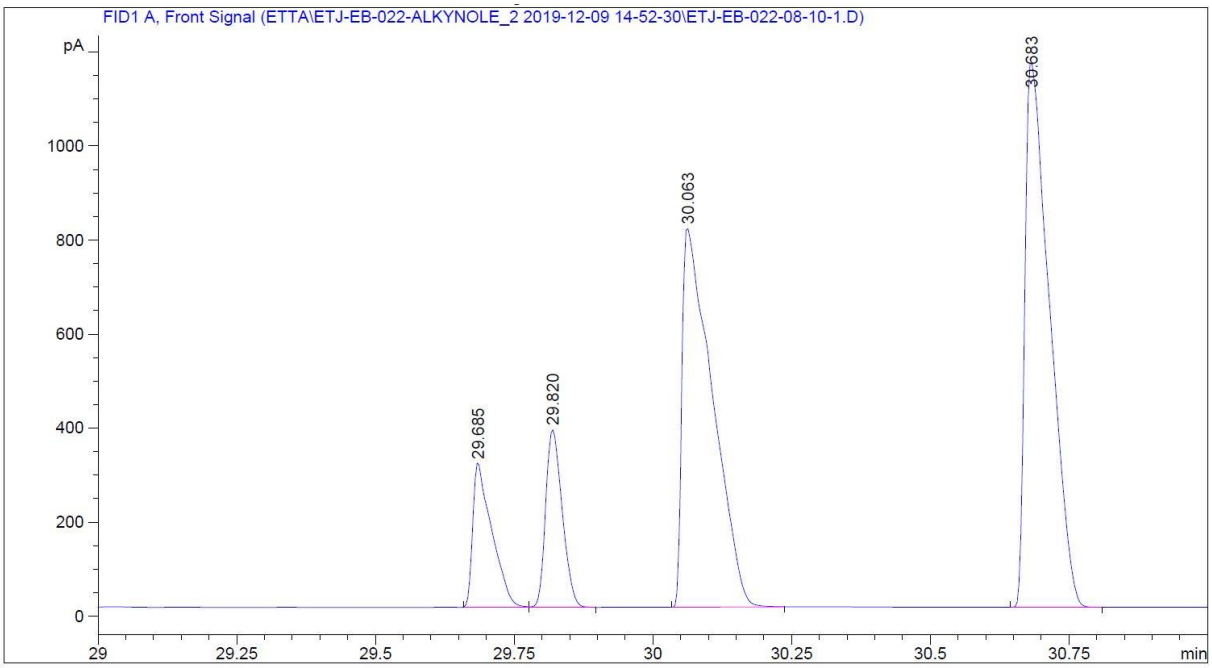

*Figure 111: GC-FID chromatogram of an acyl transfer reaction with substrate **16a**.*

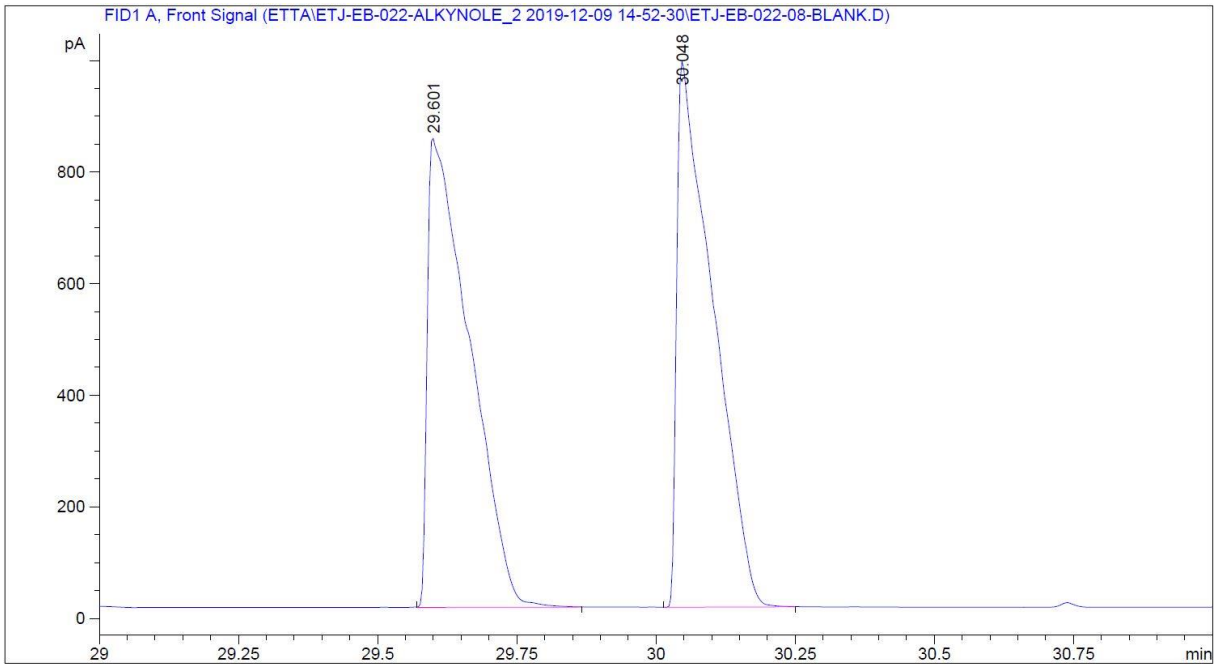

*Figure 112: GC-FID chromatogram of reference compound rac-**16a**.*

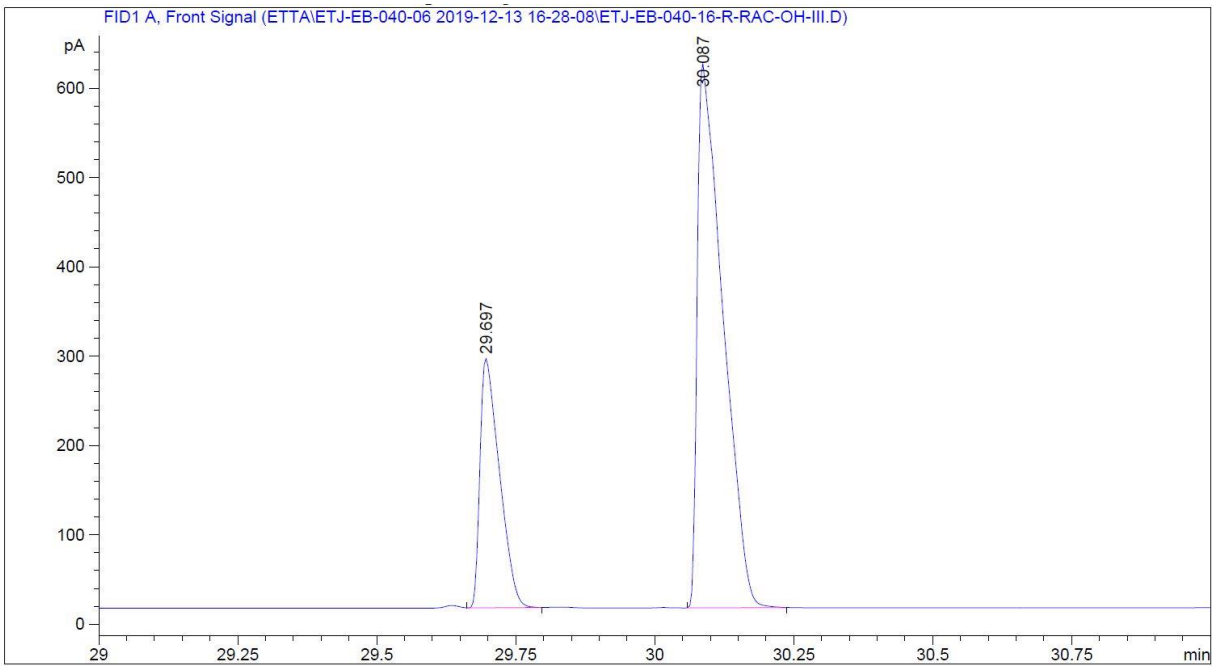

*Figure 113: GC-FID chromatogram of reference compound rac-**16a** with co-injection of (R)-**16a**.*

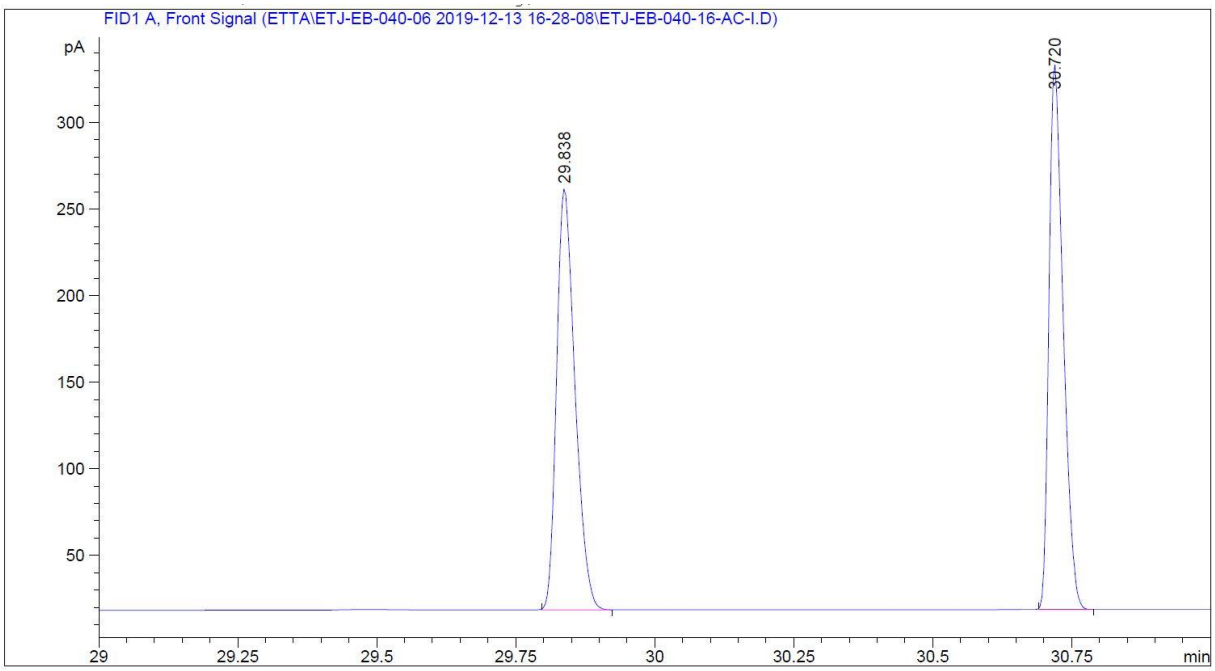

*Figure 114: GC-FID chromatogram of reference compound rac-**16b**.*

Curriculum Vitae - Etta Jost, BSc

Personal Details

Address: Petersgasse 27, 8010 Graz

Telephone: +43 680 12 81 594

E-Mail: etta.jost@student.tugraz.at

Date of Birth: 22.12.1995

Nationality: Austrian

Language Skills

Native Tongue German

Foreign Language English (fluent both orally and in writing)

Education

- Since 2018 Master's degree in Chemistry at the University of Graz and the Technical University of Graz (NAWI Graz)
- 2014-2018 Bachelor's degree in Chemistry at the University of Graz and the Technical University of Graz (NAWI Graz)
- 2006-2014 Secondary school at the BG/BRG Leibnitz, Graduation on the 18th June 2014 with honors
- 2002-2006 Primary school at the VS Lichendorf

Teaching Experiences

- Student laboratory assistant in the Organic Chemistry laboratory course at the University of Graz (SS 2017, SS 2018, SS 2019)
- Student laboratory assistant in the General Chemistry laboratory course for biology students at the Technical University of Graz (WS 2018/2019, WS 2019/2020)
- Student laboratory assistant in the Technical Chemistry laboratory course 1 (Biocatalysis Unit) at the University of Graz (WS 2019/2020)