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Asymmetrisation of prochiral 1,3-propanediols by enzyme-catalysed acetylation

Master's thesis for the degree Master of Chemistry

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Supervisors:
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Preface

The work presented herein has been conducted at the Department of Chemistry, Norwegian University of Science and Technology (NTNU), Trondheim, Norway.

It has been a long and arduous process to get to this point. The work has been fraught with faulty equipment and ill-timed problems of a kind outside my ability to handle. This also caused the final leg of the work to be short on time. Despite this, the experience from working on this thesis has been immensely educational.

I was also given the opportunity to attend the COST Training School 2009, at the Certosa di Pontignano, Siena, Italy. The training school was a great experience both scientifically and socially.

I want to thank my supervisors for all their help and input during the work with this master's thesis: Bård H. Hoff, Thorbjørn Ljones and Thorleif Anthonsen. In addition, Elisabeth E. Jacobsen and Sina M. Lystvet deserve thanks for their input and guidance.

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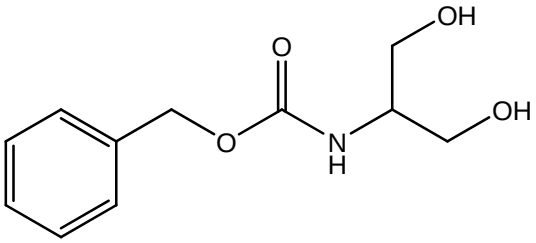
Summary

The enzymatic asymmetric transesterification of the prochiral *N*-benzyloxycarbonyl-2-aminopropane-1,3-diol (**1**) using porcine pancreas lipase (PPL) as catalyst and vinyl acetate as acyl donor was investigated. By monitoring the changes in the composition of the reaction mixture throughout the reaction by chiral HPLC or GC, an unexpected distribution of the enantiomers of *N*-benzyloxycarbonyl-2-amino-3-hydroxypropanyl acetate (**2**) is shown to occur. Initial fast conversion of **1** into ***S*-2** in high enantiomeric excess (*e.e*).is followed by the steady depletion of this species while the relative amount of the other enantiomer, ***R*-2**, slowly increases. At a point in the reaction, the *e.e.* of **2** present in the reaction mixture is 0 %, but as the reaction proceeds, the amount of ***R*-2** continues to accumulate, while ***S*-2** decreases until nearly absent from the mixture, giving an *e.e.* of the *R*-enantiomer. Several reactions with varying conditions were investigated. The selectivity is assumed to be caused by the presence of several enzymes in the crude PPL preparation.

Compounds



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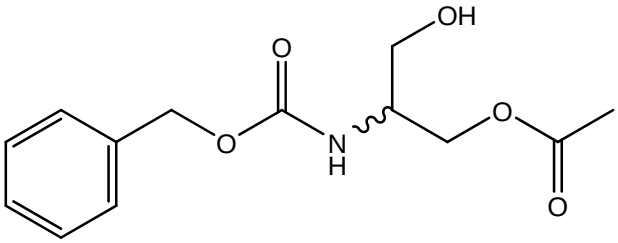

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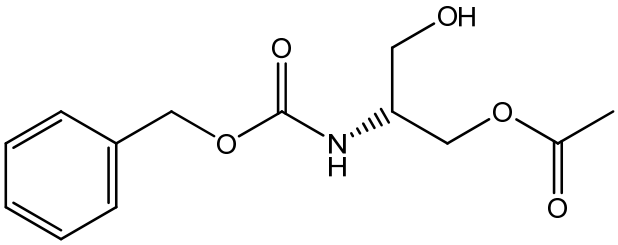
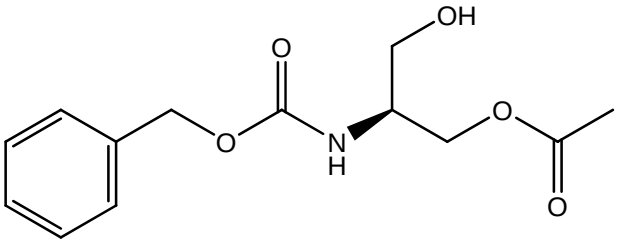
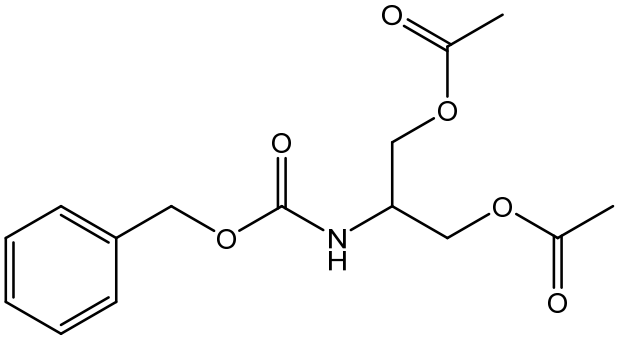


(*R*)-2



(*S*)-2



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Abbrevations

ACN	-	Acetonitrile
DCM	-	Dichloromethane
EC	-	Enzyme Commission
EtOAc	-	Ethyl Acetate
GC	-	Gas Chromatography
HPLC	-	High Performance Liquid Chromatography
PPL	-	Porcine Pancreas Lipase
TFA	-	Trifluoroacetic acid
THF	-	Tetrahydrofuran
TLC	-	Thin Layer Chromatography
TMS	-	Tetramethylsilane

Symbols

$[\alpha]_D^t$	-	Specific rotation
c	-	Concentration in $\frac{g}{100\ mL}$
k	-	Rate constant
l	-	Length in dm of sample cell for polarimeter
n	-	Amount of substance
R_f	-	Retention factor
t_R	-	Retention time

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1 Introduction

The use of biocatalysts in organic chemical processes has increased during the last decades, and is today fairly widespread. Commercial processes using biocatalysts can be found in the food, textile and pharmaceutical industries, and, in addition, biocatalysts are becoming increasingly important for the production of fine chemicals and specialty organic chemicals [1, 2].

Hydrolases – as the name implies, catalysing hydrolytic transformations – see extensive use in industry and research, and lipases, a subclass of hydrolases, are some of the most widely used biocatalysts. About 40 % of research performed in the period 1987-2003 employed lipases [3]. Microbial lipases and porcine pancreas lipase (PPL) are the preferred enzymes [4], based on activity, selectivity and commercial availability. In particular, the advantages of lipases are low cost and high stability, allowing them to be utilised in non-aqueous media [5].

An important area of use for lipases is the synthesis of chiral synthons. Two such processes are the asymmetric esterification of prochiral 1,3-propanediols and the asymmetric hydrolysis of the corresponding diester derivatives, which can give a number of chiral synthons depending on the subsituent in the 2-position.

In Marte Frigstad's master's thesis on synthesising iminocyclitols [6], an important step was the enzymatic asymmetrisation of *N*-benzyloxycarbonyl-2-aminopropane-1,3-diol (**1**) using PPL. When following this reaction by chiral HPLC, a set of interesting results was obtained [7]. The reaction – carried out as a transesterification in organic solvent, catalyzed by immobilised PPL, and using vinyl acetate as the acyl donor – showed an unexpected distribution of the enantiomers of *N*-benzyloxycarbonyl-2-amino-3-hydroxypropanyl acetate (**2**) as the reaction proceeded. During the reaction time studied both enantiomers of **2** appeared in excess at subsequent times. Previous reports of similar catalytic behaviour could not be uncovered and these results were contradictory to the descriptions of the expected selectivity of PPL, as described in the literature [8].

The goal of this master's thesis was to further investigate the selectivity of PPL in the asymmetric transesterification reaction of **1**, with an aim towards determining the underlying causes of the unexpected properties and the extent of their possible practical utilisation.

1.1 Prochiral 1,3-Propanediols

Prochiral 1,3-propanediols are the precursors for chiral synthons. The chirality is introduced in asymmetric processes based on transesterification, or on hydrolysis of the diester derivatives. Often these transformations are performed using lipases as biocatalysts. These processes have been shown to possess high specificity and reproducibility [9]. The chiral 1,3-propanediol-derivative synthons are used for the further synthesis of bioactive compounds such as phospholipids, platelet activating factor and renin inhibitors [10].

The prochirality of these starting materials is of the type most commonly encountered and stems from the 1,3-diol substitution. The prochiral centre is a tetrahedrally substituted carbon atom possessing two identical ligands, while the two remaining substituents are different from each other and the identical ligands. This makes the identical ligands heterotopic, meaning that if either of them is replaced by a unique ligand, a stereogenic centre is created. The two positions are specifically referred to as enantiotopic, and are differentiated by the designations pro-*R* and pro-*S*.

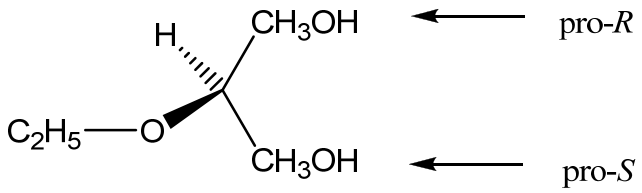

Figure 1-1: Example of prochirality and the differentiation of the enantiotopic groups.

The pro-*R/S* designations are based on the assignment of a higher sequence order priority to one of the enantiotopic groups. The stereochemistry of the resulting stereogenic centre determines the designation, as shown in Figure 1-1. The ligand assigned higher priority giving an *R* centre is designated as pro-*R*. The ligand assigned higher priority giving an *S* centre is designated as pro-*S*. This designation does not necessarily correspond to the stereochemistry obtained in actual reactions involving one of the enantiotopic groups [11].

1.2 Lipases

One of the most used classes of enzymes in organic chemistry is the lipases – triacylglycerol hydrolases. As the classification name indicates, the natural role of lipases is to catalyse hydrolytic reactions, and their natural substrates are triacylglycerols.

In order to readily identify the properties of the several thousand enzymes identified, a systematic classification of enzymes has been provided by the Enzyme Commission (EC) based on reaction type catalysed, substrate types and other properties. The classification is a four number code of the format #.#.#.#, where each number serves to identify properties of the enzyme. Examples of classifications are hydrolases (3.-.-.-), ester hydrolases (3.1.-.-), and lipases (3.1.1.3). More than 3000 enzymes have been classified [12, 13].

1.2.1 Lipases in Organic Synthesis

Since 1936, when it was discovered that the presence of benzene improved the enzymatic ester synthesis catalysed by pancreatic lipase, the application of enzymes in the presence of organic solvents has been of interest to organic chemists. Reasons for addition of organic solvents may be because of insufficient solubility of the substrates in water, or because the equilibrium of the desired reaction is unfavourable in aqueous solution.

Three general approaches are used: 1) Addition of increasing concentrations of solvents miscible with water to the reaction system; 2) Use of a two-phase systems composed of water and an immiscible solvent as the reaction medium; 3) Use of nearly anhydrous

organic solvents as the reaction medium. The presence of water is a necessity for the enzyme to be in the natural configuration and preserve its activity, but the amount of this so-called ‘structural water’ [14], which is tightly bound to the enzyme surface, is very low. In fact, 0.02 % residual water may be sufficient, together with attention to avoiding exhaustive drying of the enzyme preparation [15].

One of the main reasons for using hydrolytic enzymes in non-aqueous media, is the possibility for the alteration of the equilibria and kinetics, even so far as to effectively reversing the hydrolytic reaction and achieving the synthesis of esters. Additional reasons for choosing low-water media are that reactants don’t have to be water soluble, the specificity can be tuned by medium engineering, enzymes can be more stable, and in the case of multi-step processes, the integration of biocatalytic and chemical steps is significantly easier in non-aqueous media. It is now often preferred to carry out enzyme-catalyzed biotransformations in non-aqueous media. These are commonly based on organic solvents, but a variety of low-water media can be useful [16].

A challenge with enzymatic transesterification reactions in organic media is the inherent reversibility of the reaction. For hydrolytic reactions in aqueous media, the concentration of water is so high that the reaction is effectively irreversible. To adopt comparable conditions in organic media it is necessary to add acyl donor in excess, but even more effective is the use of acyl donors that have low reactivity towards re-esterification. Such acyl donors are activated esters (e.g. 2-haloethyl esters, cyanomethyl ester), or better yet, enol esters (vinyl or isopropenyl esters). The enol esters are particularly effective because the released enols tautomerise to the corresponding aldehydes or ketones, ensuring a completely irreversible reaction, as the carbonyl tautomers cannot react in transesterification reactions [17].

Lipases see extensive application in organic chemistry for several reasons. They show high activity in a broad range of non-aqueous solvents and very high stereoselectivity is often evident. They also accept a broad range of esters are as substrates, and they accept other nucleophiles besides water [18].

1.2.2 Selectivity

Enzymes exhibit chiral recognition, preferentially catalysing the reaction of one of a pair of enantiomers. But the selectivity of enzymes isn't limited to enantiomers. Because of the chiral nature of the active sites, prochiral substrates will also be preferentially catalysed, either from one side, or to one of two identical reactive groups.

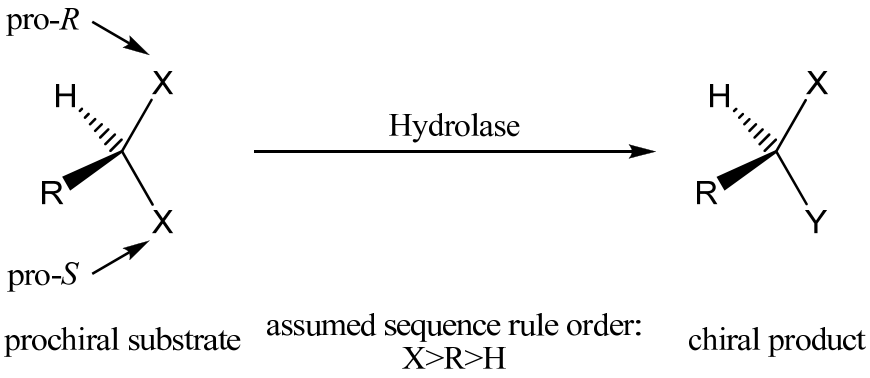

Figure 1-2: Enantiotopos differentiation.

This latter situation is referred to as enantiotopos differentiation and applies to prochiral 1,3-propanediols. The prochiral substrate undergoes a selective transformation of one of the enantiotopic groups, giving a chiral product [19], as shown in Figure 1-2.

Another important aspect of the selectivity is that it is independent of the direction of the reaction. That is, even when reversing the equilibrium of the reaction, such as when using lipases in non-aqueous media to achieve the synthesis of esters, the selectivity remains the same.

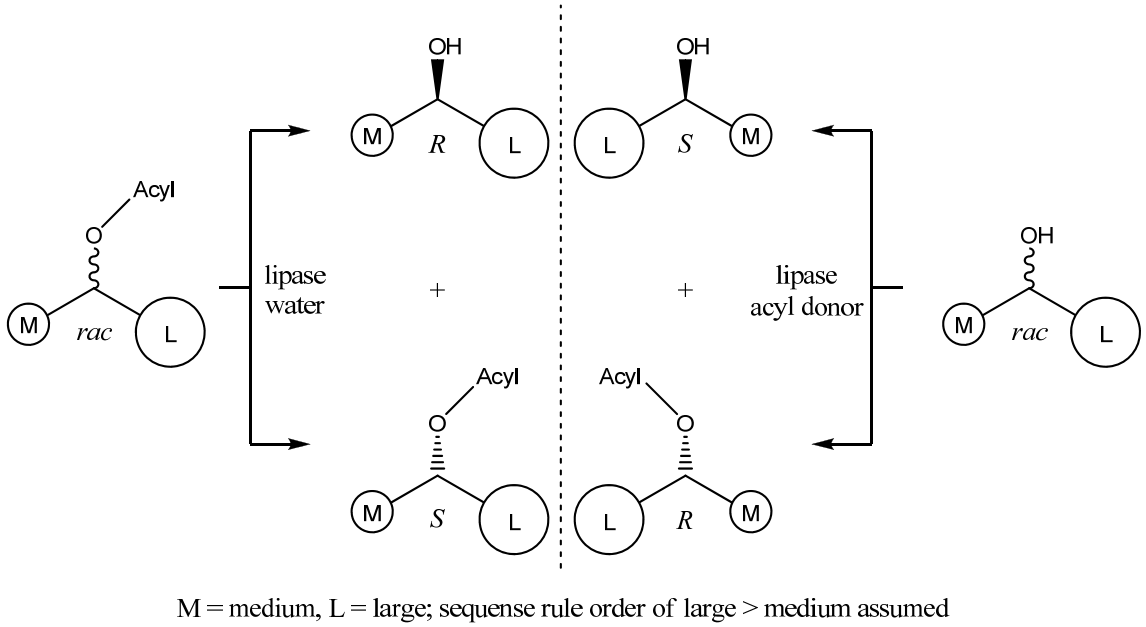

Figure 1-3: Symmetry of selectivity in enzymatic hydrolysis and synthesis of esters [20].

Figure 1-3 shows how this symmetry of selectivity can be utilised to obtain both enantiomers of alcohols and esters starting from racemic mixtures.

Predicting the selectivity of lipases 1,3-propanediol substrates is a difficult matter. An often quoted prediction of the selectivity is known as the ‘Kazlauskas-rule’ [21]. However, this applies to secondary alcohols and their ester derivatives and does not apply to 1,3-propanediols. The closest analogy would be to look at the substrate types of esterases and peptidases, which include prochiral diol esters, as shown in Figure 1-4.

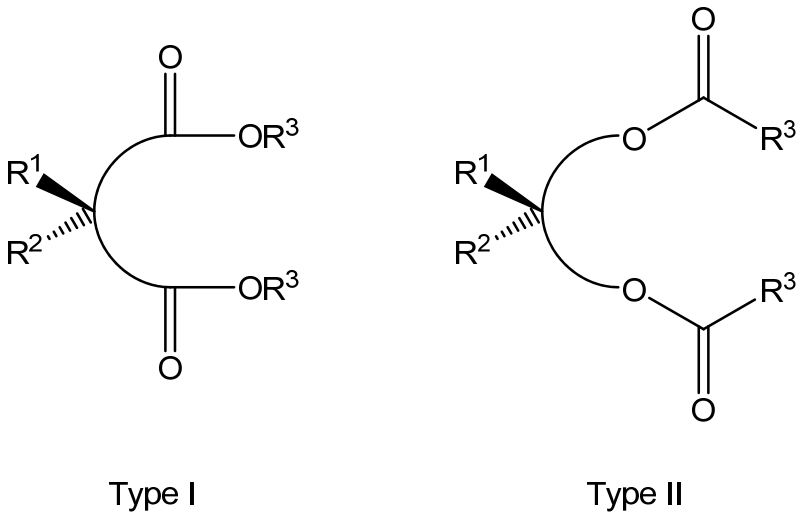

Figure 1-4: Types of prochiral substrates for esterases and peptidases. R^1, R^2 = alkyl, aryl. R^3 = Me, Et.

Increased differences in size and polarity between the substituents R^1 and R^2, connected to the prochiral centre, increases the chiral recognition, and the configuration most closely resembling L-amino acids will be preferred [22]. However, one of the properties that separate lipases from the other hydrolytic enzymes, and in particular from the esterases, is the substrate specificity of the enzymes. Unlike esterases, lipases readily accept water-insoluble substrates, which also has significance concerning the selectivity [23].

1.2.3 Reaction Mechanism and Kinetics

All the hydrolytic enzymes exhibit similar reaction mechanisms, which resemble the mechanism of chemical hydrolysis by a base. The reactive ‘operator’ is a nucleophilic group in the active site of the enzyme, which can be either the carboxyl group of an aspartic acid, the hydroxyl group of a serine or the thiol group of a cysteine [24].

The mechanim is roughly outlined as a nucleophilic group in the active site of the enzyme attacking the carbonyl group of the substrate, leading to the acylation of the enzyme and release of an alcohol or amine. Then another nucleophile, water, can attack the acyl-enzyme intermediate and regenerate the enzyme through release of a carboxylic acid. In low-water-activity media, another nucleophile can compete for the acyl-enzyme intermediate, giving transesterification reactions [25].

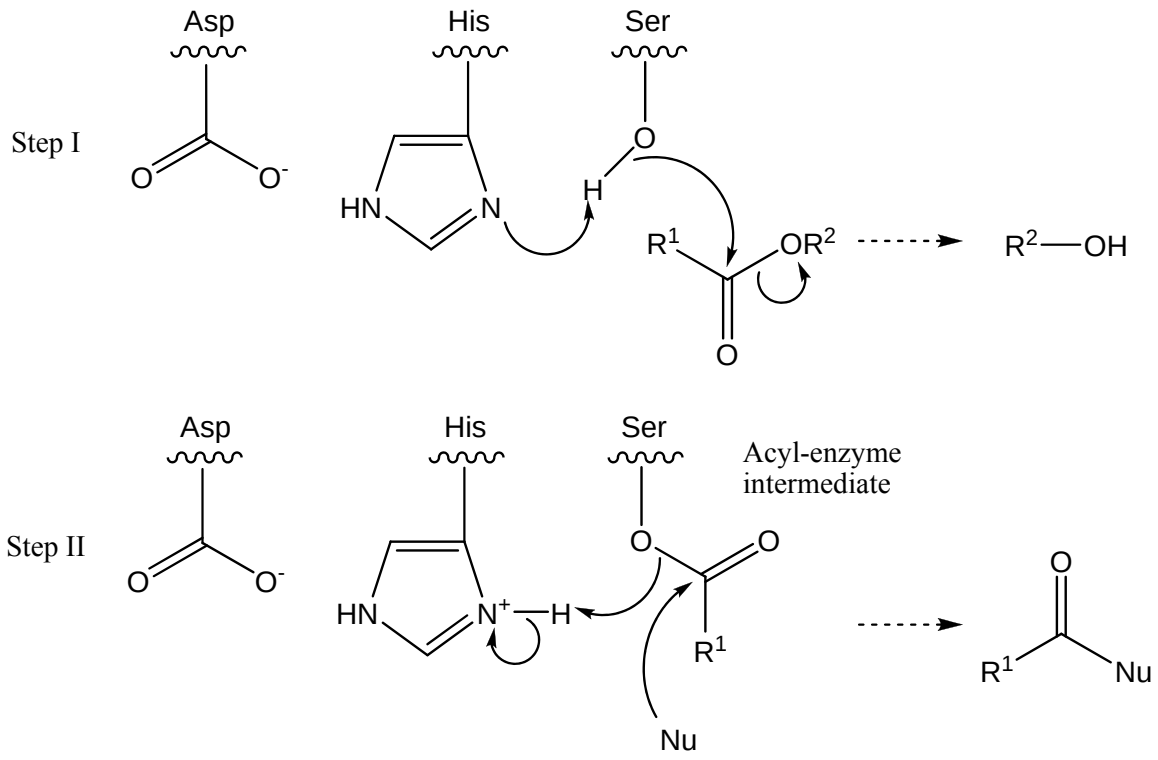

Figure 1-5: The serine hydrolase mechanism [26, 27].

The mechanism of serine hydrolases has been elucidated in detail, and follows the scheme shown in Figure 1-5. Notable aspects of this mechanism, is the activation of the serine hydroxyl group by interaction with the histidin moiety. The positive charge developed on the histidine is stabilised by the negative charge on the aspartate moiety.

A unique property of lipases is their physicochemical interaction with substrates. Most enzymes, including the other hydrolases, show Michaelis-Menten activity, which is dependent on the substrate concentration. Increased substrate concentration leads to increased activity, until saturation of the enzyme occurs, and maximum activity is achieved. In contrast, lipases exhibit almost no activity as long as the substrate is fully dissolved in aqueous solution. Increasinf the substrate concentration to above the solubility limit, when the formation of a second phase takes place, leads to the activity increasing rapidly with further increases in substrate concentration. This property of the so-called critical micellar concentration being necessary for the hydrolytic activity of lipases is referred to as interfacial activation [28].

As indicated, this applies to lipases in aqueous solution. When used in organic solvents, there is always a water-organic solvent interface present, between the structural water bound to the enzyme and the surrounding medium, ensuring activation of the enzyme. Thus Michaelis-Menten activity and dependence of activity on substrate concentration applies to lipases in organic solvents.

1.2.3.1 Double-step Reactions with Prochiral Substrates

In an esterification of a prochiral diol, the reaction will take place over two steps. In the first step the diol is acylated to the enantiomeric monoesters in an enantiopos differentiation reaction. In the second step the monoesters are acylated to give the diester in a kinetic resolution. The second step is generally assumed to be slower than the first, giving accumulation of and enabling the isolation of the enantiomeric first-step products [29].

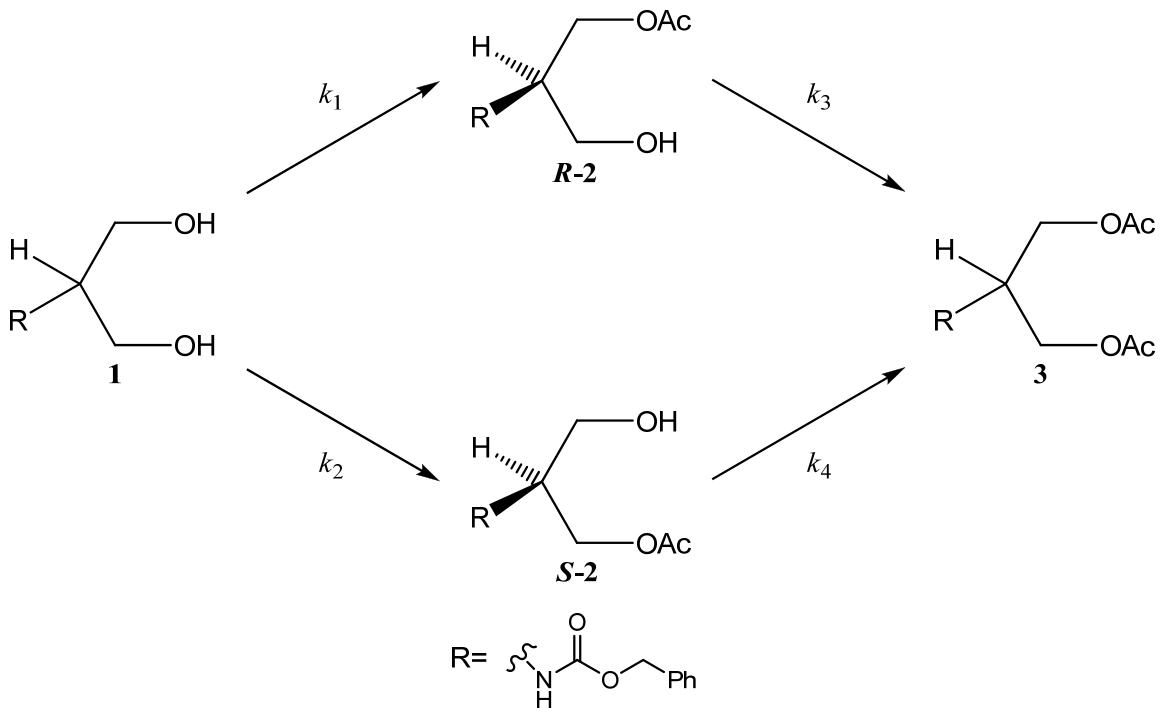

Figure 1-6: Double-step esterification of a prochiral 1,3-propanediol. k_1 - k_4 are rate constants.

The generally assumed reaction scheme for a two-step asymmetric acylation is illustrated in Figure 1-6. The ratio of enantiomers depends on four rate constants, k_1 through k_4. Enzymes usually show a continuous preference for substrates of the same configuration, so if **1** is transformed faster into ***R*-2**, then ***S*-2** should be acylated more swiftly into **3**. In other words, the rate constants governing the selectivity is commonly assumed to be in an order of $k_1 > k_2$ and $k_4 > k_3$. This means that the enantiomeric excess[1] (*e.e.*) and the amount of monoester formed is a function of the conversion, and follows the curves shown in Figure 1-7.

[1] See section 1.4, p. 10

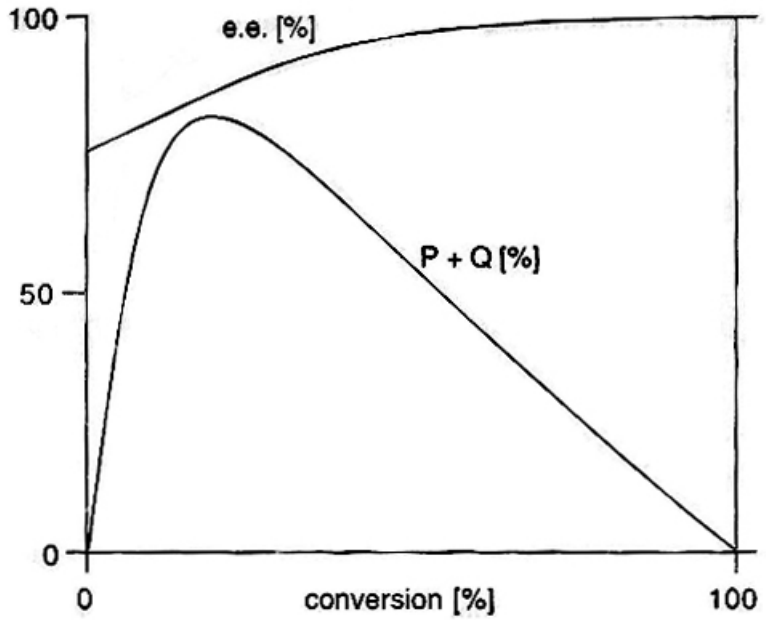

Figure 1-7: Relative amount of product and *e.e.* as a function of conversion in double-step reactions. P and Q indicate enantiomers of monoester. Rate constants for this reaction are k_1 = 100, k_2 = 10, k_3 = 1, k_4 = 10 [30].

A general consideration of these double-step kinetics makes it clear that the ratio of the reaction rates of the first and the second step ($(k_1 + k_2) / (k_3 + k_4)$) affects the chemical yield of **2** to a large extent, whereas the enantiomeric purity is determined by the symmetry of the rate constant inequalities (($k_1 > k_2$, $k_3 > k_4$) or ($k_1 > k_2$, $k_4 > k_3$)). The first step should be considerably faster to ensure accumulation of monoesters, while to achieve enantiomeric purity, the selectivities of both steps should be for the same configuration. This leads to an increased accumulation of only one of the enantiomers [31].

Conversion for a double-step reaction of this kind is defined as going from 100% diol substrate to 100% diester product [32]. The conversion for the reaction in Figure 1-6 would be given as

$$conversion\ (\%) = \frac{\frac{1}{2}(n_2)+n_3}{n_1+n_2+n_3} \cdot 100 \qquad (1\text{-}1)$$

where n_1 through n_3 are the amounts of compounds **1** to **3**.

1.2.3.2 Ping-pong Bi-bi Reactions

A more detailed description of the reaction mechanism for lipases has is classified it as a ping-pong bi-bi reaction [33]. Figure 1-8 gives an overview of this type of reaction.

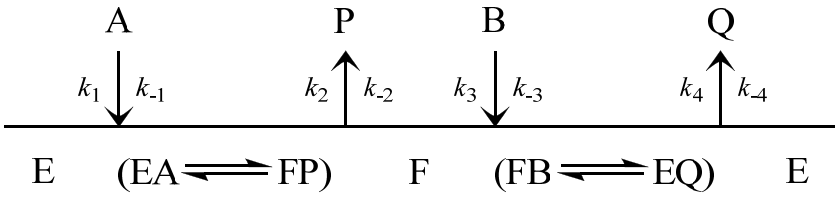

Figure 1-8: Cleland notation of a ping-pong bi-bi reaction. By convention, A is the first substrate to interact with the enzyme, B is the second substrate, P is the first product released, and Q is the second product. E is the enzyme, while F represents a modified enzyme intermediate [33].

The pattern of the reaction is that a first substrate, A, an ester, interacts with the enzyme, leading to the release of the first product, P, an alcohol, and the formation of the acyl-enzyme intermediate. This is followed by the second substrate, B, a nucleophile, interacting with the acyl-enzyme, resulting in the reformation of the enzyme and the release of an acyl derivative. The process corresponds to the mechanism given in Figure 1-5, and applies whether the lipase is used for hydrolysis or ester synthesis. Seen in connection to the double-step kinetics presented above, this mechanism occurs for each of the two steps.

A thorough description of the mechanism for the desymmetrisation of a prochiral compound could not be found, but the description of a single step kinetic resolution can serve as an analogy for each of the steps in the double-step reaction.

The reaction involves the transformation of a chiral substrate B into chiral product Q. Subatrate A and product P are achiral. The reaction is referred to as a ping-pong bi-bi B-Q resolution. The reactions taking place are

$$A + B^R \rightleftarrows P + Q^R \tag{1-2}$$

$$A + B^S \rightleftarrows P + Q^S \tag{1-3}$$

This results in the following mechanistic scheme

$$\begin{array}{ccccccccc} & & & & & \overset{k_3^R[B^R]}{\rightleftarrows} & EQ^R & \overset{k_4^R}{\rightleftarrows} & \\ & \overset{k_1[A]}{\rightleftarrows} & & \overset{k_2}{\rightleftarrows} & & & & & \\ E & & EA & & F & & & & E \\ & \underset{k_{-1}}{} & & \underset{k_{-2}[P]}{} & & \underset{k_3^S[B^S]}{\rightleftarrows} & EQ^S & \underset{k_{-4}^S[Q^S]}{\rightleftarrows} & \\ & & & & & \underset{k_{-3}^S}{} & & & \end{array} \tag{1-4}$$

The enzyme will selectively catalyse one of the reactions given as equation (1-2) and (1-3) based on the relationship between complex relationship between the rate constants given in the mechanistic scheme [34].

1.2.4 Porcine Pancreas Lipase

Together with microbial lipases, PPL is one of the most widely used enzymes. In particular, the crude PPL preparations see wide use, partially because it is one of the cheapest lipases. The preparations usually contain less than 5 % protein, and contain several other hydrolase impurities: α-chymotrypsin, cholesterol esterase, carboxypeptidase B, and other known and unknown hydrolases [35]. For a crude PPL preparation available from Sigma-Aldrich (L3126 – Type II, crude (steapsin)) the manufacturer reports amylase and protease activity [36]. It has also been shown that this PPL preparation from Sigma-Aldrich in fact contains two lipases [37].

Sigma-Aldrich also has available a purified PPL preparation (L0382, Type VI-S, lyophilized powder). For this product there is no other enzyme activity listed and the activity is quoted as vastly increased from the crude PPL preparation. However, there is no information given on what the purification entails [38].

1.3 Immobilisation of Enzymes

Immobilisation of an enzyme is the conversion of the enzyme to a form with artificially restricted mobility and retained catalytic activity. This is commonly achieved by linking the enzyme to insoluble particles or by entrapping the enzyme within a selectively permeable membrane [39].

Immobilisation serves as a means to potentially grant the enzyme several advantageous properties, such as ease of handling, ease of separation from products, the possibility of reuse, and enhancement of catalytic properties, pH-activity profile and enzyme stability. The specific properties of immobilised enzyme preparations are governed by the enzyme and the carrier material, which give the preparation distinct chemical, biochemical, mechanical and kinetic properties [40, 41].

However, there are also some limitations associated with immobilisation. The processes rarely give quantitative yields of protein binding, and the cost of the carrier material can be significant. Together with steric hindrance and mass transfer limitations, this results in a decreased activity per weight of solid. In addition, the activity is often further decreased as a result of chemical modification of the protein [42].

Despite the limitations, the immobilisation of enzymes has seen great success in several industries as well as in research since the first intentionally immobilised enzyme was prepared in the 1950s [43]. Adsorption onto a water-insoluble macroscopic carrier is the easiest and oldest method of immobilisation, but there is a number of methods employed, which can be separated into four broadly defined groups [44]:

- Non-covalent adsorption of the enzyme onto a solid support.
- Covalent attachment of the enzyme to a solid support.
- Entrapment of the enzyme in a polymeric gel, membrane or capsule.
- Cross-linking of the enzyme with a polyfunctional agent.

For use in non-aqueous systems immobilisation by physical adsorption is a common and effective approach. Advantages to this method are its simplicity, that chemical modification of the enzyme is avoided and low cost. Disadvantages are the weak and non-specific binding of the enzyme, the possibility of enzyme leaching, that there is little or no increase in enzyme stability and that the bulk of the carrier material may limit mass transfer.

For lipases it has been shown that desorption from the carrier is avoided because the reaction solvent doesn’t solvate the protein molecule sufficiently to overcome the weak interaction forces with the solid surface or the carrier. In addition, dispersion of lipases over a high-surface hydrophobic polymeric carrier such as polypropylene has been shown to activate the enzyme in organic media relative to particles of the untreated protein prepared by lyophilisation. The hydrophobic binding of lipases is even sufficient to allow their use in purely aqueous media [45].

1.4 Determination of Enantiomer Composition

In any synthesis it is necessary to analyse any isolated intermediate and product to determine identity and purity. This is commonly done using a number of methods based on the physicochemical properties of the compounds. Unique combinations of boiling points, melting points, density, solubility, spectrometric properties, etc. enable the chemist to attain a high degree of certainty of the identity of the obtained compound, and the purity of the sample.

For enantiomeric compounds the analysis can to a large extent be performed in a similar fashion, however at one point the previously mentioned properties does no longer suffice. The mentioned physicochemical properties are the same for both enantiomers, and additional methods are necessary to determine whether one has obtained one or both, and the enantiomeric composition.

The composition of enantiomers is often reported as the *enantiomeric excess* (*e.e.*). This is defined as

$$e.e. = \frac{R-S}{R+S} \cdot 100\ \% \tag{1-5}$$

where *R* and *S* denote relative or absolute amounts of the two enantiomers.

1.4.1 Optical Rotation

Chiral compounds have the property that when plane-polarised light passes through them, the plane is rotated. Such molecules are said to be optically active. Enantiomers rotate the polarized light in opposite directions and, subject to equal conditions, in equal amounts.

The optical rotation, α, is measured with a polarimeter, giving a readout of degrees of rotation. The specific rotation of the sample can be calculated from the formula

$$[\alpha]_{\mathrm{D}}^{t} = \frac{100\alpha}{l \cdot c} \tag{1-6}$$

where $[\alpha]_{\mathrm{D}}^{t}$ is the specific rotation at t °C, α is the number of degrees the polarised light has been rotated, l is the length of the sample cell in dm and c is the concentration of the sample in $\frac{\mathrm{g}}{100\ \mathrm{mL}}$. The D indicates that the measurement has been obtained at a wavelength of λ = 589.3 nm, the so-called D line of sodium. By convention the specific rotation is quoted as a dimensionless figure preceded by a '+' or '-' sign to indicate the direction of rotation, where '+' is clockwise and '-' is counter-clockwise. The specific rotation is dependent on the solvent used, and the relationship between concentration and rotation is dependent on the compound and is not necessarily linear. The sample must be pure to ensure correct rotation [46].

1.4.2 Chiral Chromatography

Chromatographic methods are suitable for the separation and detection of a large number of organic compounds. Especially HPLC and GC provide high sensitivities and broad ranges of application, enabling the direct determination of sample compositions.

The development of chiral stationary phases has made it possible to achieve the separation of chiral compounds. A chiral stationary phase possesses surface groups with one specific absolute configuration. This leads to selective interactions with enantiomers

in the sample analysed, resulting in different retention times for each of a pair of enantiomers. Thus the enantiomers are analysed as individual compounds, enabling direct determination of relative composition and the calculation of *e.e*.

1.5 Previous Research

The use of prochiral 1,3-propanediols as starting material for chiral synthons is fairly common. For an overview of previously conducted work, see the reviews from Schoffers et al.[47] and Garcia-Urdiales et al. [48] and the references cited therein. Work conducted with compounds **1** through **3** in enzymatic hydrolytic or transesterification reactions is significantly rarer.

Choi and Borch report the enzymatic asymmetric transesterification of **1** using PPL and vinyl acetate as a key step in the synthesis of 2-hydroxymethylaziridines [49]. Two sets of reaction conditions are reported, with variation of substrate concentration, relative amount of enzyme and reaction time. Vinyl acetate is the acyl donor and is used as the reaction solvent. For both sets of conditions a yield of >85 % was obtained of ***R*-2** in *e.e* > 98 %. The PPL preparation was from Sigma-Aldrich. Condition specifics:

- **1** (1 mmol, 50 mM) in vinyl acetate (20 mL, acyl donor & solvent) with PPL (300 $\frac{\text{mg}}{\text{mmol substrate}}$) at room temperature for 1.5 h.
- **1** (1 mmol, 100 mM) in vinyl acetate (10 mL, acyl donor & solvent) with PPL (100 $\frac{\text{mg}}{\text{mmol substrate}}$) at room temperature for 3 h.

Wang and Wong report the enzymatic asymmetric transesterification of **1** using PPL and vinyl valerate as a reaction studied in the development of a general method for lipase-catalysed transesterification of *meso*-1,3-diols [50]. From the reaction a yield of 77 % was obtained of the *R*-enantiomer of the monovalerate derivative of **1** in *e.e.* > 97 %. The source of the PPL preparation is not stated. Hydrolysis of the divalerate derivative of **1** is also reported to give the S-monovalerate in *e.e.* > 97 %. Condition specifics:

- **1** (1.1 mmol, 48 mM) and vinyl valerate (4 mmol, 3.6x excess) in tetrahydrofuran (23 mL) incubated with PPL (818$\frac{\text{mg}}{\text{mmol substrate}}$) at 28°C for 11 h.

Analogous reactions with other 1,3-propanediols have also been reported [51; 52; 53]. Common for these reports is that only at the end of the reaction, after a certain reaction time, the product mixture is analysed. The monoacyl product is reported as isolated in high enantiomeric purity or, in a few cases, as a racemic mixture. Specific selectivity may vary with the substituent in the 2-position on the propane backbone.

1.5.1 Unexpected Catalytic Behaviour of PPL

As mentioned above, Marte Frigstad discovered a surprising catalytic behaviour when using PPL to catalyse the acetylation of **1** [54]. Using HPLC she analysed the relative composition of the reaction mixture at various reaction times. She reports the results from three instances of the same reaction, with variations in substrate concentration and

relative amount of enzyme. The reactions vary in reaction activity, as expected, depending on the conditions, but for all the reported reactions, the tendencies of selectivity are the same. An initial fast conversion of substrate into the monoacetate enantiomer reported as ***S*-2** is followed by a steady depletion, while the relative amount of the other enantiomer, ***R*-2** slowly increases. At a point in the reaction, the *e.e.* of the monoacetate present in the reaction mixture is 0 %, but as the reaction proceeds further, the amount of ***R*-2** continues to accumulate, while ***S*-2** decreases until nearly absent from the mixture. The formation of diacetate is detected after the point of *e.e.* = 0 %.

Frigstad compares her results to the expected behaviour of *e.e.* and relative amount of product in double-step reactions, presented above as Figure 1-7. The results do not correspond, but beyond stating that the unequality of the rate constants for the second step of the reaction is the reverse of commonly the expected situation, no explanation for the catalytic behaviour is given.

1.5.2 Optimisation of Reaction Conditions with PPL

Banfi et al. investigated the effects of various reaction conditions on PPL-catalysed monoacetylation of prochiral 1,3-propanediols [55]. Their results are interesting in concern to the reaction conditions employed when using PPL as a catalyst.

Some generally applicable results can be gathered from the report:

- Immobilisation of the enzyme by adsorption on a carrier enhances the activity.
- The enzyme shows a fairly high tolerance for water in the medium (1 % w/v added) without loss of activity.
- The inhibition from acetaldehyde is negligible.

Compound **1** is not included among the 1,3-propanediols used in the studied reactions.

2 Results and Discussion

Presented here are the results obtained and considerations developed during the work towards the goal of this thesis.

The reaction studied was the PPL-catalyzed transesterification of **1**, shown in Figure 2-1. The reaction was conducted in organic solvent with vinyl acetate was acyl donor.

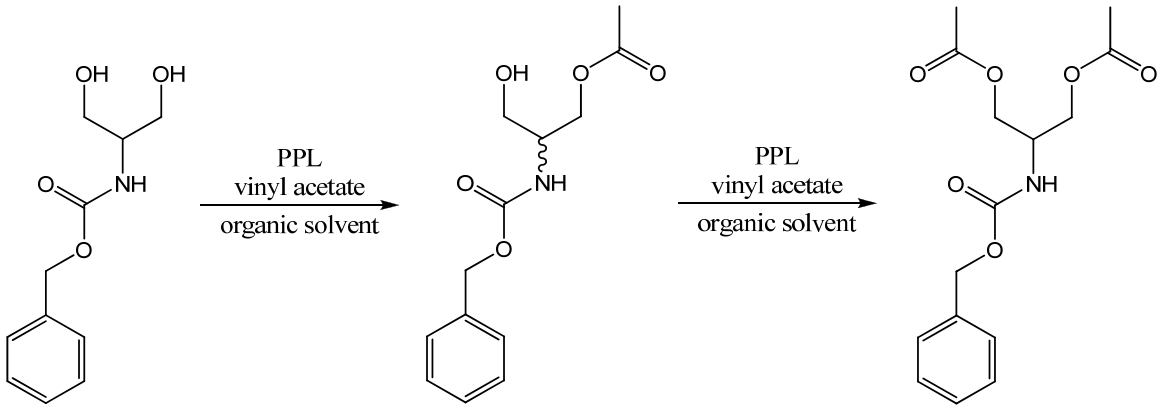

Figure 2-1: Reaction scheme for the enzymatic transesterification of 1.

The reaction is a double-step enantiotopos differentiation acetylation, as discussed in section 1.2.3.

2.1 Kinetics for Lipase-catalyzed Asymmetrisation Reactions

The detailed reaction mechanics for ping-pong bi-bi reactions presented in section 1.2.3 apply to lipase-catalyzed kinetic resolutions of alcohols. The reaction considered here is the asymmetrisation of a prochiral substrate, but the reaction is analogous in the selectivity of the enzyme, and the mechanical scheme (1-4) can be modified to representing the reaction under consideration.

The asymmetric transesterification of **1** is a double-step reaction. In the first step both substrate A, vinyl acetate, and substrate B, **1**, are achiral compounds. The substrates react with the enzyme in turn, to give the achiral product P, vinyl alcohol, and the chiral product Q, **2**.
The first-step reactions are

$$A + B \rightleftarrows P + Q^R \quad (2\text{-}1)$$

$$A + B \rightleftarrows P + Q^S \quad (2\text{-}2)$$

In the second step, substrate A again reacts into product P. But now the chiral Q functions as substrate, being acetylated into achiral product X, **3**.
The second-step reactions are

$$A + Q^R \rightleftarrows P + X \quad (2\text{-}3)$$

$$A + Q^S \rightleftarrows P + X \quad (2\text{-}4)$$

The mechanistic scheme (1-4) can then be modified to give the following schemes for each reaction step:

First-step mechanistic scheme:

$$\mathrm{E} \underset{k_{-1}}{\overset{k_1[\mathrm{A}]}{\rightleftarrows}} \mathrm{EA} \underset{k_{-2}[\mathrm{P}]}{\overset{k_2}{\rightleftarrows}} \mathrm{F} \begin{matrix} \overset{k_3^{\mathrm{R}}[\mathrm{B}]}{\underset{k_{-3}^{\mathrm{R}}}{\rightleftarrows}} \mathrm{EQ^R} \overset{k_4^{\mathrm{R}}}{\underset{k_{-4}^{\mathrm{R}}[\mathrm{Q^R}]}{\rightleftarrows}} \\ \underset{k_{-3}^{\mathrm{S}}}{\overset{k_3^{\mathrm{S}}[\mathrm{B}]}{\rightleftarrows}} \mathrm{EQ^S} \underset{k_{-4}^{\mathrm{S}}[\mathrm{Q^S}]}{\overset{k_4^{\mathrm{S}}}{\rightleftarrows}} \end{matrix} \mathrm{E} \qquad (2\text{-}5)$$

Second-step mechanistic scheme:

$$\mathrm{E} \underset{k_{-1}}{\overset{k_1[\mathrm{A}]}{\rightleftarrows}} \mathrm{EA} \underset{k_{-2}[\mathrm{P}]}{\overset{k_2}{\rightleftarrows}} \mathrm{F} \begin{matrix} \overset{k_5^{\mathrm{R}}[\mathrm{Q^R}]}{\rightleftarrows} \\ \underset{k_{-5}^{\mathrm{R}}}{} \\ \underset{k_{-5}^{\mathrm{S}}}{\overset{k_5^{\mathrm{S}}[\mathrm{Q^S}]}{\rightleftarrows}} \end{matrix} \mathrm{EX} \underset{k_{-6}[\mathrm{X}]}{\overset{k_6}{\rightleftarrows}} \mathrm{E} \qquad (2\text{-}6)$$

Using vinyl acetate as acyl donor makes the conversion of the complex EA into acyl-enzyme intermediate F irreversible, as the concentration [P] ≈ 0 because of the tautomerisation of vinyl alcohol to acetaldehyde.

If the synthesis is conducted with vinyl ester as solvent, the kinetics will reduce to two steps of uni-uni reactions [56], that is, only the equilibria involving compounds **1** through **3** – denoted as B, Q and X, respectively – need be considered, as the enzyme will be present only as the acyl-enzyme intermediate F.

Comparing the mechanical schemes (2-5) and (2-6) to the double-step kinetics presented in Figure 1-6 reveals that the simplification of only four rate constants applying to the reaction can give a misrepresentation of the complex relationship between the equilibria and rate constants involved.

It also appears that a discussion of Figure 1-7 is appropriate. First of all the curve for *e.e.* should start at 0%, as the substrate for the reaction is prochiral and at 0% conversion there is no chiral compound present. Second, a definition of conversion for a double-step reaction is not provided in connection to the chart [57], but the definition that provides for a maximum of first-step product close to 80 % after only about 20 % conversion does not make sense. Based on the definition of conversion provided in section 1.2.3, the maximum of first-step product is twice the conversion until 50 % conversion, and if the intention behind Figure 1-7 is for the product curve to peak at a value of ca. 80 %, the peak should occur somewhere in the vicinity of 40 % conversion.

These aspects of the reaction mechanics and kinetics should be kept in mind for the presentation of the results below.

2.2 Analysis

Samples acquired from the reaction mixtures were analysed using high performance liquid chromatography (HPLC) and gas chromatography (GC). However, several challenges presented themselves in connection with both the analysis in general and with the methods specifically. The separate methods will be considered below.

The general challenge connected to analysis concerns the fact that although stored at temperatures of between -15 and -20 °C the composition of the samples changed over time, as shown in Figure 2-2.

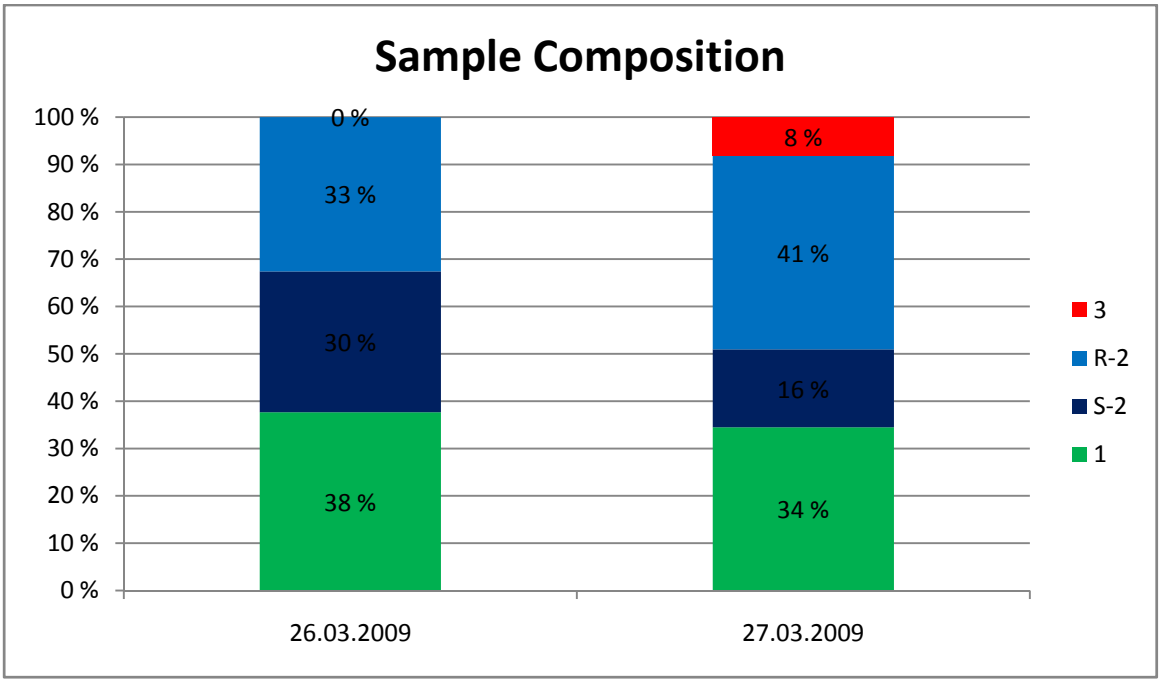

Figure 2-2: Changes in sample composition after overnight storage at between -15 and -20 °C.

The extent of these changes has not been studied, and it is not known how consistent they are with the data indicated in the figure. In connection to the previously presented scheme of reaction rate expressions, it is obvious that the changes would be inherently dependent on the concentrations of the components in the sample, and the effect of temperature on the various specific reaction coefficients.

The most probable cause for these sample instabilities is the presence of enzyme, with reaction taking place even at lowered temperatures. For a number of the reactions conducted with untreated enzyme, it was visually observed that suspended enzyme was present in the samples taken during reaction. However, for reactions conducted with immobilised enzyme, no visual observation indicated the presence of enzyme. Still, leaching of enzyme from the carrier material cannot be ruled out, but it is also significant that in the case of reactions conducted with immobilised enzyme, notable changes took places over months instead of days.

A simple test for the reactivity at lowered temperatures was done using the same conditions as one of the reactions studied[2]. Substrate solution was prepared, enzyme added, and the mixture was stored in the freezer for more than an hour. Acyl donor was added and the reaction glass was immediately put back in the freezer. The next day, after 23 hours, a sample was withdrawn and analysed.

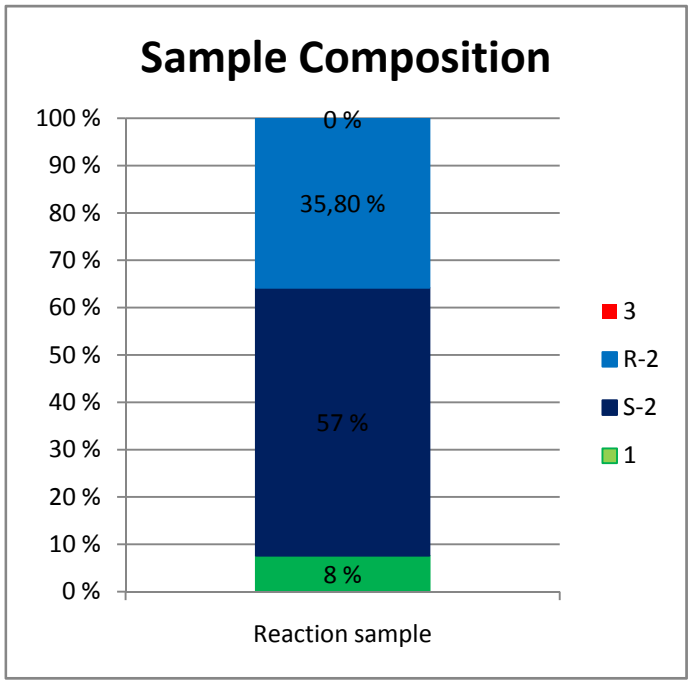

Figure 2-3: Relative composition of reaction mixture for enzymatic acetylation of 1 stored at < -15 °C for 23 hours.

The result is presented in Figure 2-3, and it is clear that the composition of the sample has changed over the intervening time. This does not constitute proof of enzyme activity at < -15 °C, but strongly supports that enzyme activity is not completely suppressed even during storage in a freezer.

Another possible cause for the alteration of sample composition with time is the occurrence of acyl migration. However, this possibility was discounted on the basis of previous work [58, 59]. All the samples were stored in solvents with low water content and medium to high polarity, resulting in conditions that is unfavourable for acyl migration.

From the discussion above, it is obvious that the time passed before analysis is important. Thus, where available, this time is given for the reactions discussed in the following sections.

[2] Reaction D, see p. 26 & p. 34

2.2.1 HPLC Analysis

From previous work parameters for HPLC analysis was available for the reaction studied [60]. However, upon actual analysis, the resulting chromatograms proved unsatisfactory. Baseline separation of the enantiomers was not achieved, and several other problem areas were present in the chromatograms. This made optimisation of HPLC analysis necessary.

First, it was attempted to achieve enantiomeric separation with isocratic elution. One of the eluent components, acetonitrile (ACN), has absorbance at the wavelength of detection, making a gradient elution undesireable because of possible interference. After attempting several eluent mixtures, it was determined that isocratic elution was unsuitable, never achieving baseline separation of the enantiomers, and thus gradient elution was again used.

A large number of gradual steps constituted the process of narrowing in towards the gradient utilised. After optimisation, the separation shown in Figure 2-4 was achieved.

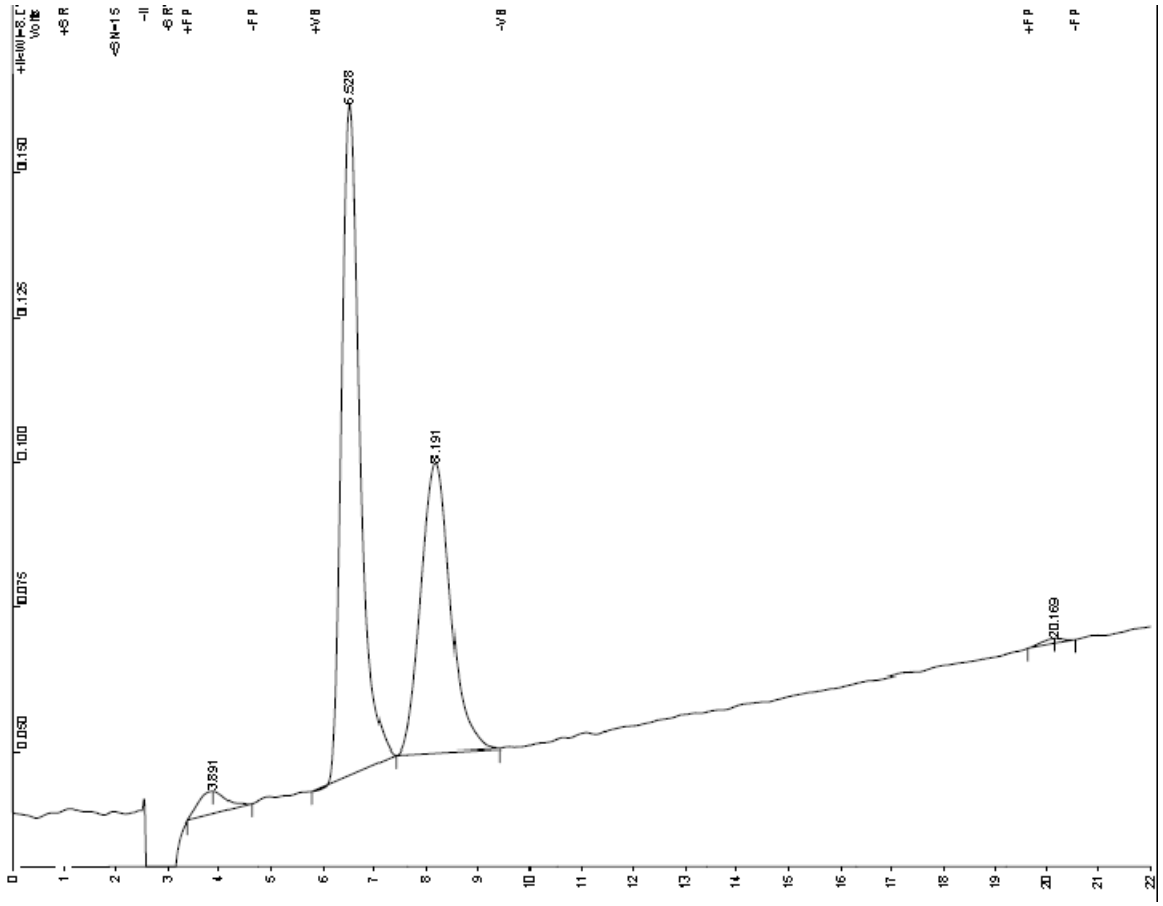

Figure 2-4: HPLC chromatogram of a sample from the enzymatic acetylation of 1**.**

As can be seen, some problems still presented themselves. All attempts to delay the peak of substrate **1** at t_R = 3.9 min by manipulation of the elution parameters were unsuccessful. The peak appears to close to the baseline valley – seemingly connected to injection – making it hard to determine the start of the peak. Also, the increasing amount of ACN in the eluent was the cause for the upward slope. This slope can possibly

interfere with the peak detection and causes doubt as to the accuracy of the relative compositions from the HPLC chromatograms.

The identity of the peaks in the HPLC chromatograms was assigned based on correlation with chromatograms presented in the Frigstad's thesis, as the elution parameters used were comparable [60].

HPLC chromatography analysis was eventually abandoned in favour of GC chromatography.

2.2.2 GC Analysis

The analysis of the samples using GC was initially somewhat problematic because the composition of the samples analysed was uncertain. Considering the enzyme-catalysed nature of the conducted reaction, the primary components of the reaction mixture should be the compounds **1** through **3**, together with solvent and acyl donor derivatives. A pure supply of **1** was available and a pure supply of **3** was synthesised. In addition, vinyl acetate and solvents were of course available.

The analysed pure compounds gave some auxiliary peaks of uncertain origin, making it difficult to assign peak identity unambiguously. Initially it was assumed that the additional peaks were caused by decomposition of the known compounds, however to ascertain the accuracy of this assumption has not been possible. Some of these peaks might of course be caused by contaminants on the column.

Peak assignment of **1** and **3** was possible from analysis of pure samples. In the chromatogram in Figure 2-5, **1** has a retention time of $t_R = 4.3$, while **3** can be seen at $t_R = 24.9$. These analyses also showed that most of the interim peaks between the peaks of **1** and **3** accompanied each of these compounds. However, the intensities and areas of these unidentified peaks did not vary in proportion to variations of concentrations and amounts of the identified peaks.

The peak at $t_R = 25.8$ was identified as an enantiomer of **2** following isolation and characterisation, revealing it to be ***R*-2** [61]. A number of smaller peaks at low retention times and surrounding the peak of **1** were identified as the reaction solvent, following the analysis of a pure sample.

The isolation of ***S*-2** was not achieved, but the peak identity of this compound in the GC chromatogram was assigned partially based on an elimination process, and partially following correlation with spectroscopic analyses of samples of compound mixtures. As mentioned, the interim peaks showed very low variation of intensity and area, while the peak at $t_R = 8.5$ showed variations at an order of magnitude comparable to the already identified peaks.

The optimisation of the parameters for GC analysis was a long process, going hand in hand with the described process of peak assignment. The temperature programs used for analysis ranged from slow increases of temperature (2.5 °min^{-1}) and long isotherms at maximum temperature to shorter programs with varying temperature increases and

several hold intervals of varying durations. A large number of minor changes were incorporated in attempts at tweaking the position of the identified peaks, minimising the influence of the noise. Even after acceptable analyses were achieved, the process was continued in order to cut the analysis time while still achieving fully acceptable results.

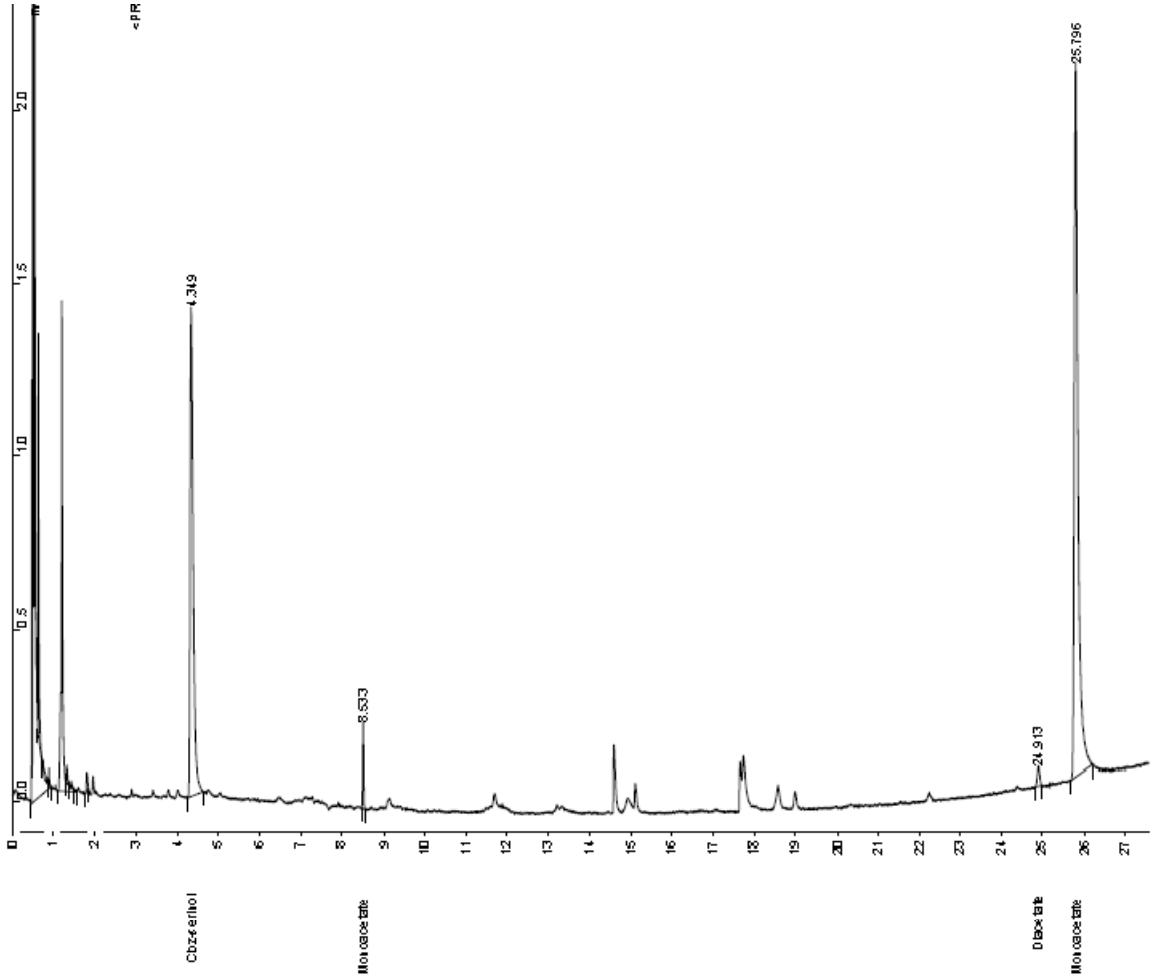

Figure 2-5: GC chromatogram of a sample from the enzymatic acetylation of 1**. Temperature program: 80 - 92 °C, 30 °min^{-1}; 92 - 98 °C, 3 °min^{-1} (2 min); 98 - 104 °C, 3 °min^{-1}; 104 - 140 °C, 30 °min^{-1}; 140 - 200 °C, 3 °min^{-1}; total time 28 min.**

2.3 Enzymatic Acetylation of *N*-Benzyloxycarbonyl-2-aminopropane-1,3-diol

The reaction in Figure 2-1 was conducted with varying reaction conditions. The results obtained are presented below. Inaccuracies in these results are connected to the problem of sample instability discussed above, and unfortunately the time passed before analysis varied for the performed reactions. Poor planning was also an influencing factor. Because of limited supply of substrate **1**, it was attempted to limit the amounts used in each reaction. Based on this, the scale of the performed reactions was slightly reduced, causing the proportion of total sample volume to total volume of reaction solution to vary between 10 % and 35 %. How much this affects any reaction is unclear, but that it will have some effect is certain, as it decreases the volume of solvent in which the enzyme is suspended. Further, considering that some solvent evaporates off with each unstoppering of the reaction flask, the concentrations of reactants was also potentially altered during the reaction. In addition, this exposure to the surrounding atmosphere can lead to introduction of moisture into the reaction solution, potentially affecting the equilibria of the reaction, despite the apparent water tolerance of the enzyme [62]. The time intervals of sample acquisition most likely varied as well, both between individual samplings from the same reaction and across the different reactions.

The charts of relative composition of the reaction solution in the following sections show a lot of fluctuations with time. The smaller fluctuations will be ascribed to the above mentioned causes, and focus will be on the apparent trends exhibited during the reaction time studied.

2.3.1 Results as a Function of Reaction Time

Reaction A was conducted with 180 mM solution of substrate in tetrahydrofuran (THF) with 140 mg immobilised enzyme pr. mmol of substrate and 5 equivalents of vinyl acetate. The samples from the reaction were analysed on HPLC.

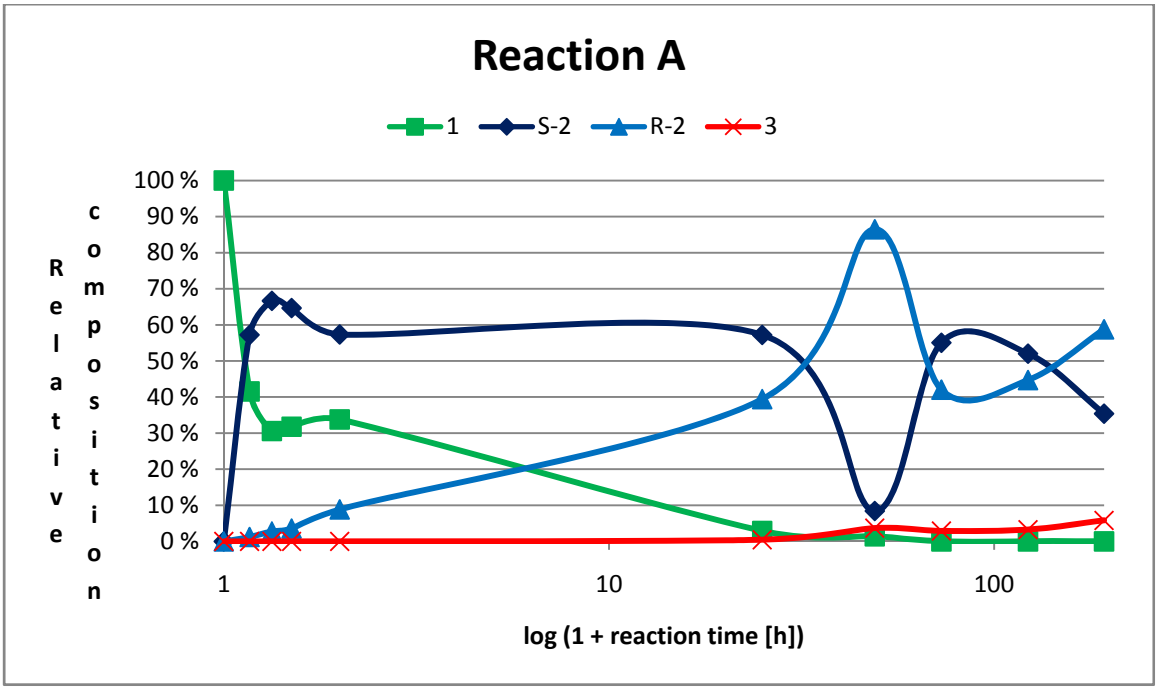

Figure 2-6: Reaction A – enzymatic acetylation of 1 in THF using immobilised enzyme. Reaction conditions: substrate 180 mM; enzyme 140 mg/mmol substrate; 5 equivalents acyl donor .

The results for reaction A show that a substantial conversion of **1** into ***S*-2** took place during the first 20 minutes of the reaction, as can be seen from Figure 2-6. From 20 minutes there was a slight decline in the amount of this enantiomer, while the continued conversion of **1** steadily increased the amount of ***R*-2**, until sometime after 24 hours, where there appeared to be equal amounts of the enantiomers of **2**. Subsequently, the relative amounts of the enantiomers of **2** fluctuated, in what seems to be an interconversion between the enantiomers, as the remaining amount of **1** was minimal. Meanwhile the amount of **3** increased slowly from approximately 24 hours.

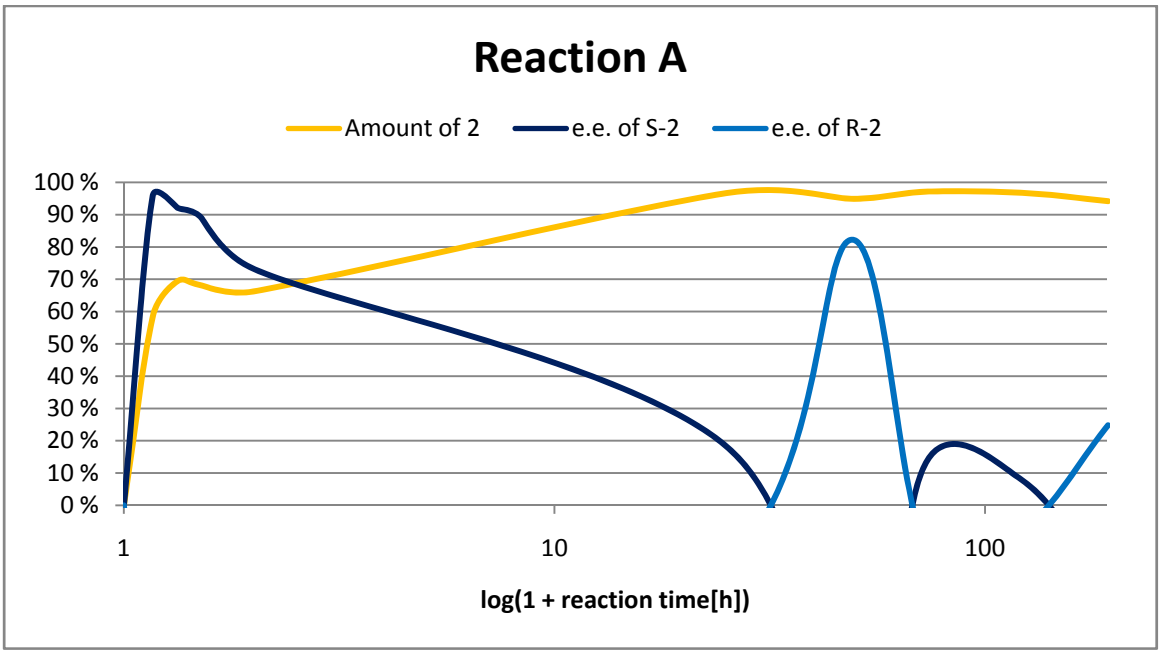

Figure 2-7: Amount of monoacetates and e.e. as a function of reaction time for reaction A.

Figure 2-7 shows the total amount of **2** and the *e.e.* as a function of reaction time for reaction A. This plot makes it apparent how the *e.e.* of **S-2** swiftly increased to > 95 % together with a fairly substantial initial formation of **2**. Though the total amount of **2** continued to increase at a slower pace until leveling off after 24 hours, the *e.e.* of **S-2** declined to below 20 % at 24 hours, and during the time up to 48 hours, the excess is completely inverted, becoming an *e.e.* ≈ 75 % of ***R*-2**. This chart lends further support to the thought that the later fluctuations between the excesses of both ***R*-2** and ***S*-2** were interconversions between the enantiomers, because of the fairly constant total amount of **2** towards the end of the reaction time studied.

The use of immobilised enzyme was abandoned after reaction A, because of the loss of activity for the immobilised preparation.

Reaction B was conducted with 50 mM solution of substrate in vinyl acetate with 300 mg untreated enzyme pr. mmol substrate. The samples from the reaction were analysed by GC. The time from sampling to analysis was 12 days and above, introducing a significant uncertainty to the results.

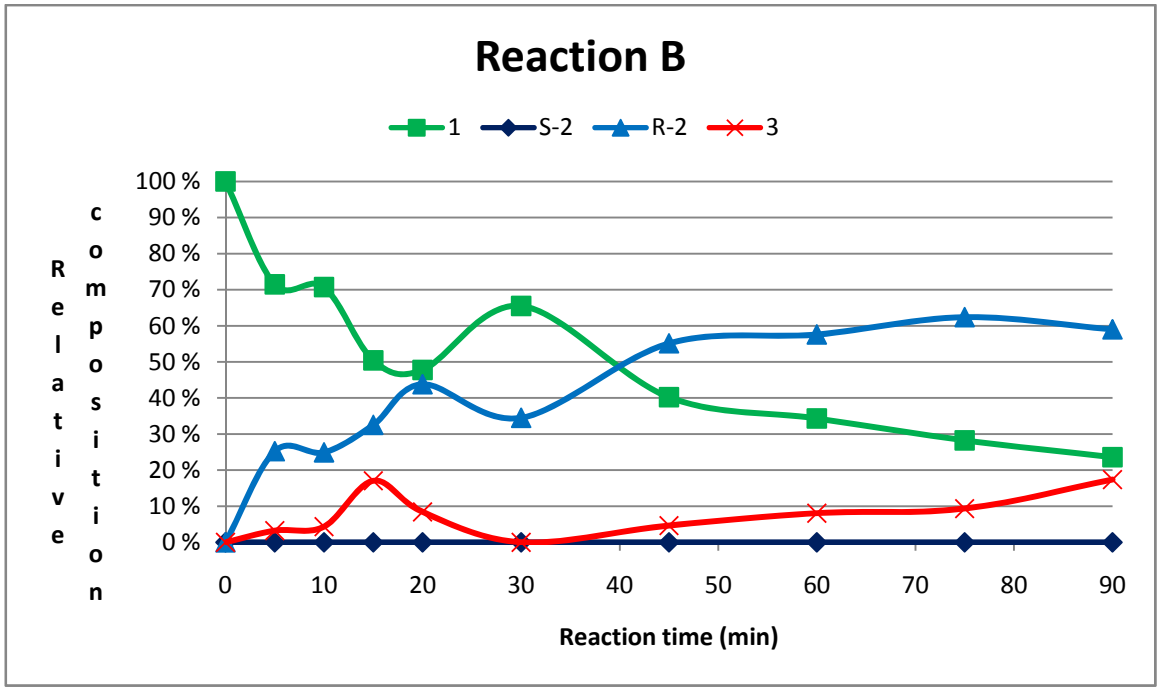

Figure 2-8: Reaction B – enzymatic acetylation of 1 **in acyl donor using untreated enzyme. Reaction conditions: substrate 50 mM; enzyme 300 mg/mmol substrate; excess acyl donor.**

The results for reaction B show that during the first 5 minutes, a small amount of ***S*-2** was formed from **1**, as seen in Figure 2-8. From 5 minutes the increase in the amount of ***S*-2** is somewhat slower, until around 1 hour, where the amount levels off. The amount of **1** decreases steadily throughout the reaction time studied, as **3** is also steadily formed already from the start of the reaction.

The results for reaction B differ notably from reaction A, although both reactions have a similar reaction rate in terms of substrate conversion, which passes 70 % shortly after 1

hour. However, the main difference applies to the enantiomers of **2**, where it is ***R*-2** which is in excess throughout the studied reaction time of reaction B. Also the presence of **3** is apparent from the first sample, acquired after 5 minutes, while for reaction A, it took 24 hours before **3** was detected.

The fact that only one enantiomer was detected means that *e.e.* > 99 % at any reaction time analysed, negating the value of plotting *e.e.* as a function of reaction time.

The reaction conditions for reaction B is similar to a reaction conducted by Choi and Borch, and although the obtained yield of ***R*-2** was lower, the results correspond to those reported [63].

Reaction C was conducted with 180 mM solution of substrate in THF with 180 mg untreated enzyme pr. mmol substrate and 1.04 equivalents of vinyl acetate. The samples from the reaction were analysed by GC, and the time from sampling to analysis was ca. 18 hours for the first 7 samples, and approximately 2,5 days for the remaining samples.

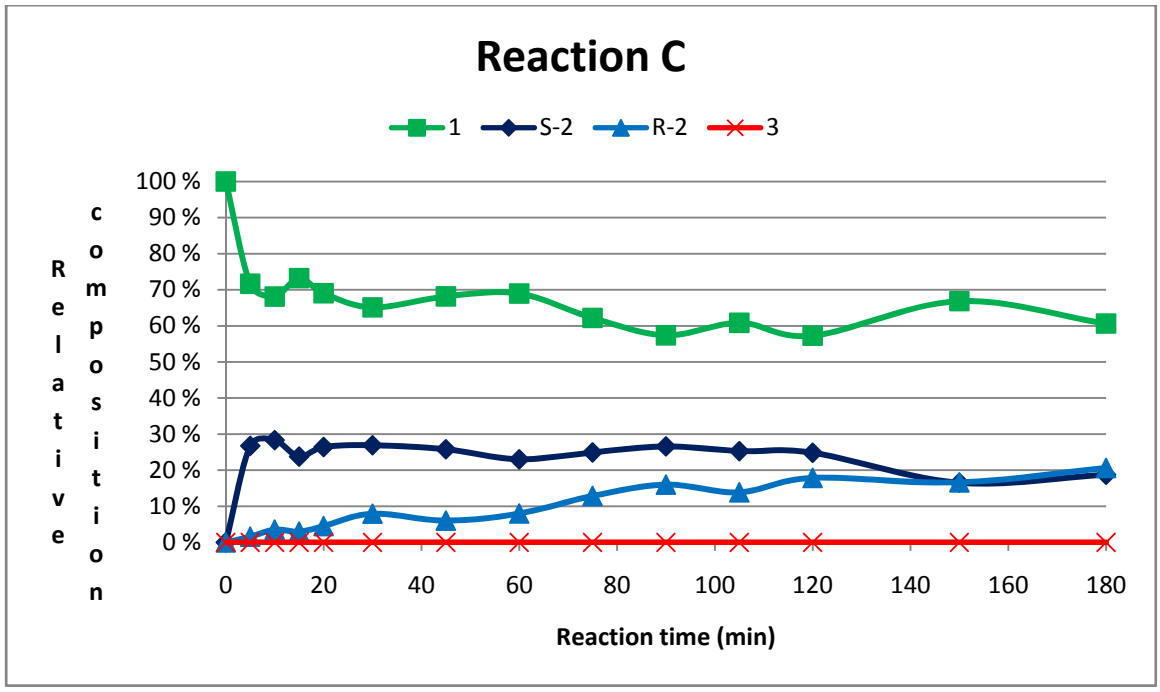

Figure 2-9: Reaction C – enzymatic acetylation of 1 in THF using untreated enzyme. Reaction conditions: substrate 180 mM; enzyme 180 mg/mmol substrate; 1.04 equivalents acyl donor.

The results for reaction C show that an initial esterification of a moderate amount of **1** into ***S*-2** was followed by a very slow decrease in the amount of this enantiomer, seen in Figure 2-9. After the initial conversion, the substrate was slowly converted to ***R*-2**. **3** was not formed during the reaction time studied.

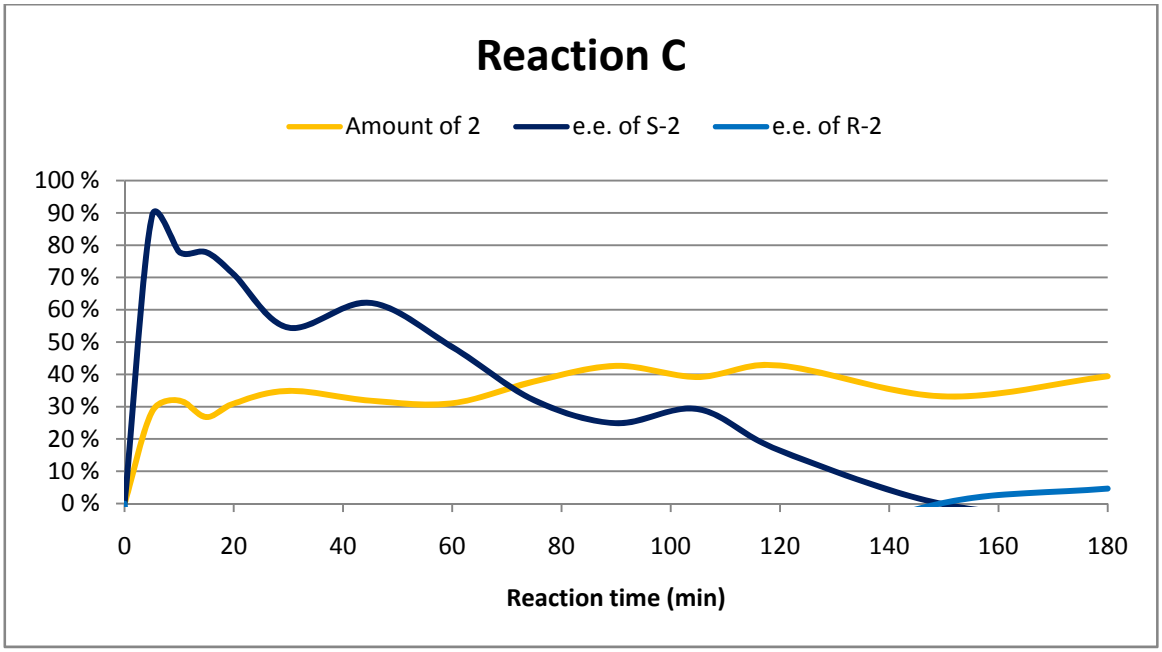

Figure 2-10: Amount of monoacetates and e.e. as a function of reaction time for reaction C.

Figure 2-10 reflects the initial high *e.e.* ≈ 90 % for ***S*-2** together with the rapid formation of about 30 % total of **2**. From this point the *e.e.* of ***S*-2** decreased steadily, while the amount of **2** only increased slightly, indicating that the enantiomers most likely interconverted. Towards the end of the reaction, ***R*-2** was in slight excess.

The overall trend for reaction C resembles reaction A, as ***S*-2** is formed in excess within a short time, followed by a steady decrease coinciding with the steady build-up of the opposite enantiomer, reaching *e.e.* ≈ 0 % after about 2.5 hours, and the following slight excess of *R*-2 at 3 hours, when the reaction was terminated. However, the time frame of the mentioned trends was shorter for reaction C than for reaction A, and the amounts converted were much larger for reaction A. The similarities are supported by comparison of Figure 2-7 with Figure 2-10. Consideration of the specific causes for these differences are difficult, seeing as reaction A was conducted with immobilised enzyme and a larger amount of acyl donor than reaction C, although these factors certainly influence the reaction pattern.

Reaction D was conducted using 180 mM solution of substrate in THF with 360 mg untreated enzyme pr. mmol of substrate and 1.19 equivalents of vinyl acetate. The samples from the reaction were analysed by GC in two batches. The first 5 samples were analysed within 0.5 - 2 hours after sampling, and the remaining the next day. This makes the time interval approximately 18 hours for the samples from 150 and 180 minutes reaction time, while the last sample from 17 hours reaction time was analysed within 4 hours after sampling.

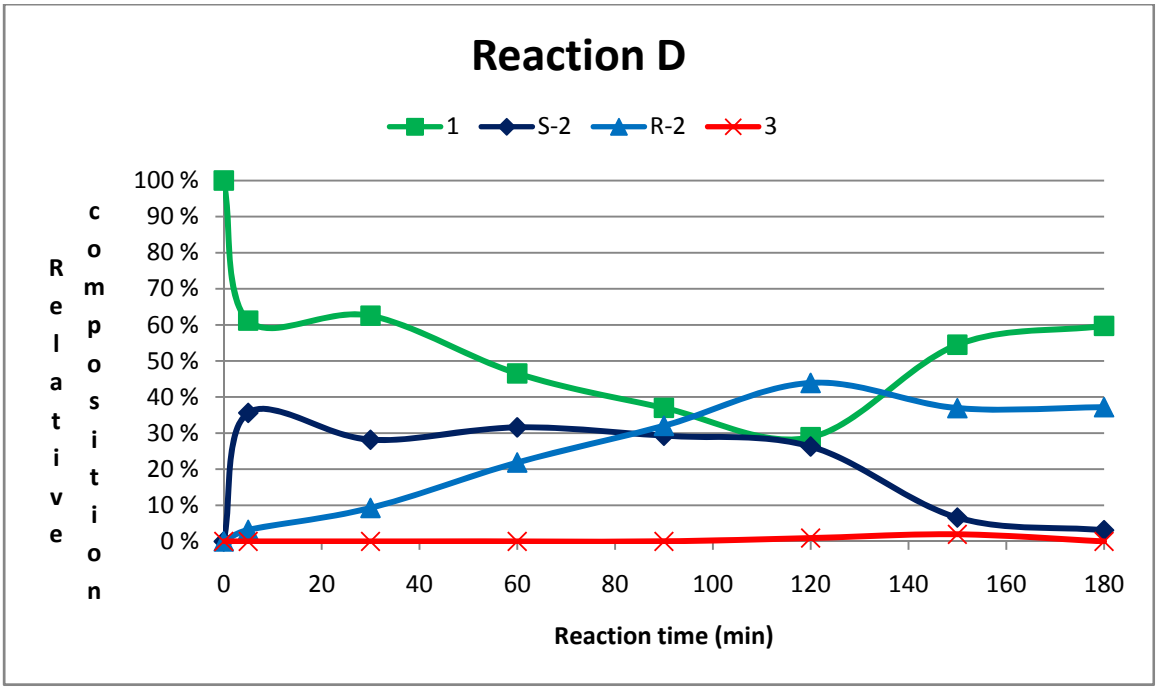

Figure 2-11: Reaction D – enzymatic acetylation of 1 in THF using untreated enzyme. Reaction conditions: substrate 180 mM; enzyme 360 mg/mmol substrate; acyl donor 1.2x excess

The results for reaction D is given in Figure 2-11, which shows that the initial swift esterification of **1** gave a moderate amount of ***S*-2** and a slight amount of ***R*-2** after 5 minutes. Then, the amount of ***S*-2** began decreasing slowly, while the amount of ***R*-2** increased steadily. The formation of **3** appears to have begun around 120 minutes reaction time, but was very slow.

The somewhat strange increase in the amount of substrate **1** at the expense of both enantiomers of **2** shown for the last two reaction times in this figure is probably an effect of the overnight storage which these to samples was subjected to before analysis.

This theory is further supported by the analysis results for the sample taken after 17 hours reaction time, where it is clear that after this extended reaction time, the amount of ***R*-2** has increased further.

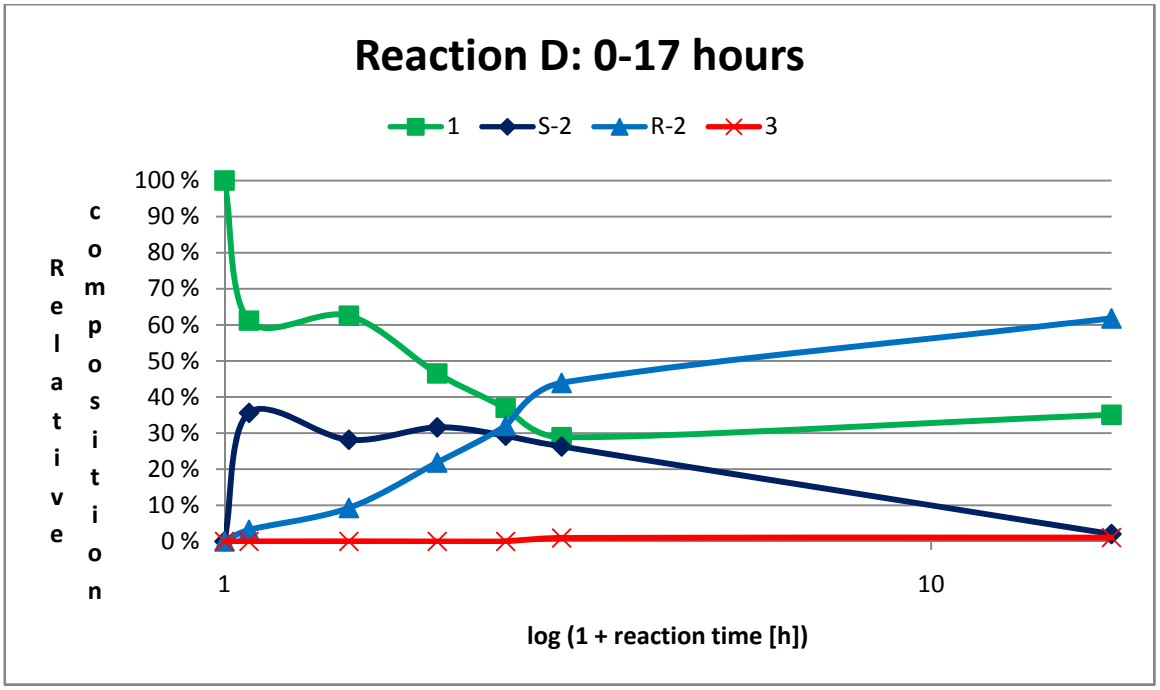

Figure 2-12: Reaction D at complete reaction time. Only results from samples analysed within comparable time after sampling is included.

Removing the data points from the delayed analyses and including the final sample from a reaction time of 17 hours gives Figure 2-12. Here the changes in the relative composition conform to a general trend. The amount of **S-2** continues to decrease while the amount of ***R*-2** increases. The slight increase in amount of **1** from 2 hours to 17 hours is somewhat surprising, but no explanation beyond the possible causes already mentioned is apparent.

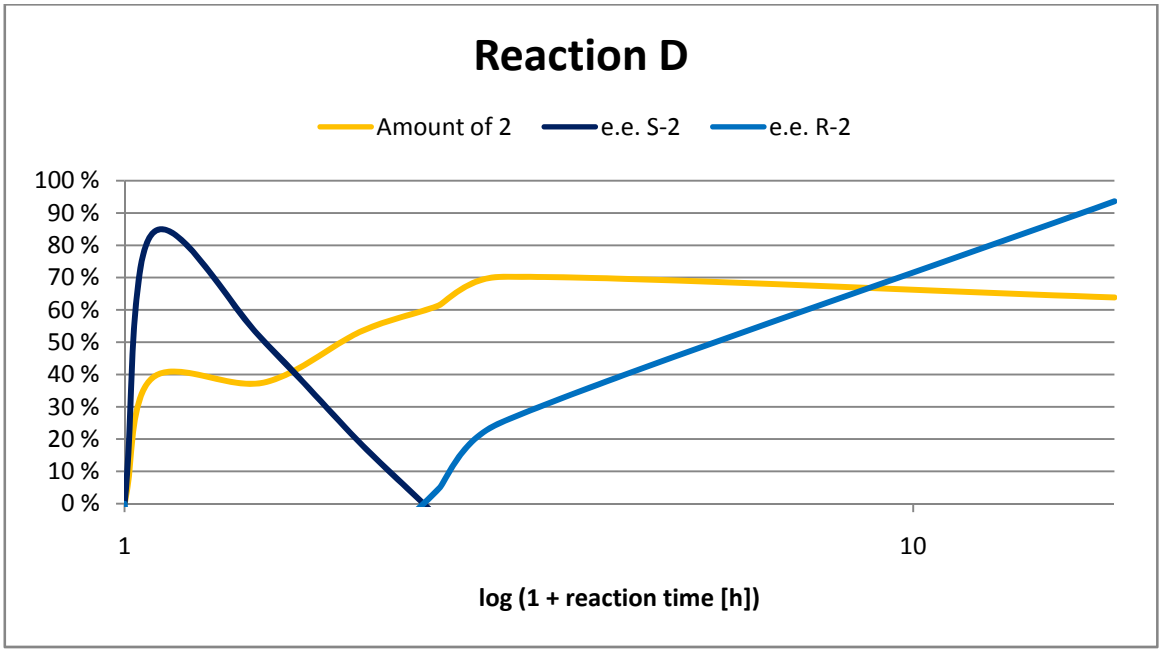

Figure 2-13: Amount of monoacetates and e.e. as a function of reaction time for reaction D.

Figure 2-13 shows the initial high *e.e.* ≈ 85 % for ***S*-2** together with the rapid formation of about 40 % total of **2**. From this point the *e.e.* of ***S*-2** decreased steadily to 0 % shortly after 80 minutes, while the amount of **2** increased at a slower rate until leveling off. Towards the end of the reaction the excess of ***R*-2** increased to *e.e.* ≈ 95 % at the time of termination.

The trends of reaction D have similarities to those of both reaction A and C, with the same pattern of enantiomeric excess of both enantiomers of **2**, and the pattern of formation of total amount of **2**. Reaction D differs from reaction C mainly in the relative amount of enzyme used. However, an unfortunate increase in the amount of acyl donor complicates the comparison, but the difference is so slight that it will be assumed to have a negligible effect on the general trends. The main differences to note is then that the total amount of **2** formed in reaction D lies between the amounts for reactions A and C, and the decrease of *e.e.* for ***S*-2** is close to twice as rapid for reaction D compared to reaction C.

A reaction with a purified PPL preparation, reaction E, was also conducted, using 180 mM substrate concentration in THF with 2.8 mg purified PPL pr. mmol of substrate and 1.2 equivalents of vinyl acetate. The activity reported by Sigma-Aldrich for the purified PPL preparation gives an activity ratio of 300:1 compared to crude PPL, making the corresponding relative amount of crude enzyme approximately 800 mg pr. mmol of substrate.

Problems with the analysis, in the form of an increased amount of noise and alien peaks in the GC chromatograms, makes it unfeasible to plot a chart like those shown for the other reactions. What could be ascertained from the analysis results was that throughout the reaction only one enantiomer, ***S*-2**, appeared to be present. Neither the other enantiomer, nor diacetate could be detected within the 6 hour reaction time studied. In other words, the situation for this reaction is the opposite of that for reaction B, with an apparent *e.e.* > 99 % for ***S*-2** throughout the reaction time of reaction E. In addition, the initial conversion of **2** within the first 5 minutes, gave > 80 % of ***S*-2**. These results are, however, very tentative, because of the mentioned instrument problems.

2.3.2 Amount of 2 and *e.e.* as a Function of Conversion

Plotting the amount of **2** and *e.e.* as a function of conversion for the above reactions presents some problems. Because the relative amounts of the compounds **1** through **3** fluctuate over the course of the reaction, the conversion is not proportional to the reaction time. This makes comparisons between the reactions difficult the interference factors vary a lot between the individual reactions.

The charts presented below give the amount of **2** and *e.e.* as a function of conversion for the recorded conversions that are proportional to reaction time. Note that the conversion axis ends at 60 % in the charts below.

Comparison of Figure 2-14, Figure 2-15 and Figure 2-16 makes the similarities between reactions A, C and D quite apparent. For all the reactions the slope for the amount of **2** is close to the maximum provided by the definition of conversion for this kind of reaction.

The *e.e.* initially shows a steady increase for ***S*-2**, before peaking somewhere above 80 % and decreasing again. For reactions A and D *e.e.* reaches 0 %, followed by an increase in the *e.e.* of ***R*-2**. From these results, it seems justified to assume that an increased reaction time for reaction C would have resulted in a similar development as for the other two reactions.

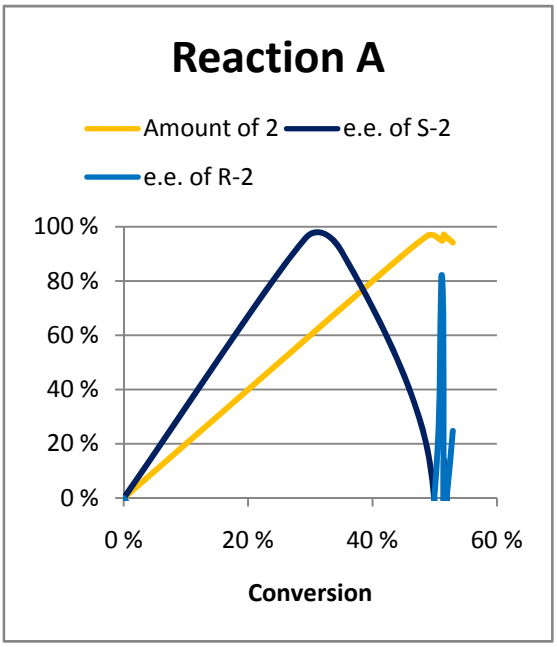

Figure 2-14: Amount of monoacetates and e.e. as a function of conversion for reaction A.

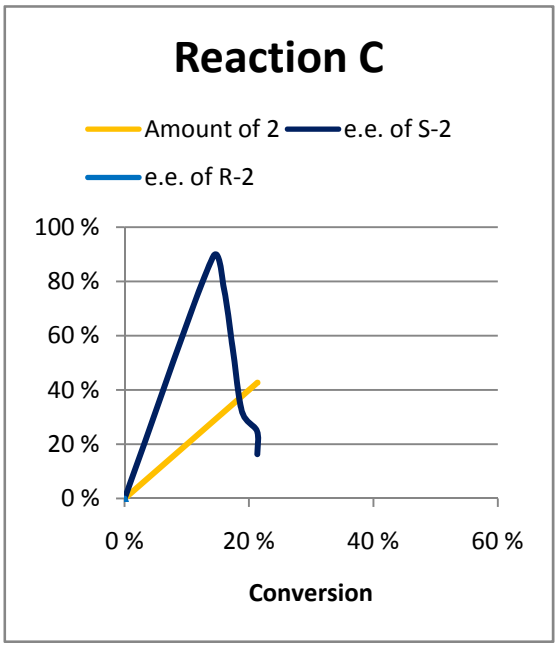

Figure 2-15: Amount of monoacetates and e.e. as a function of conversion for reaction C.

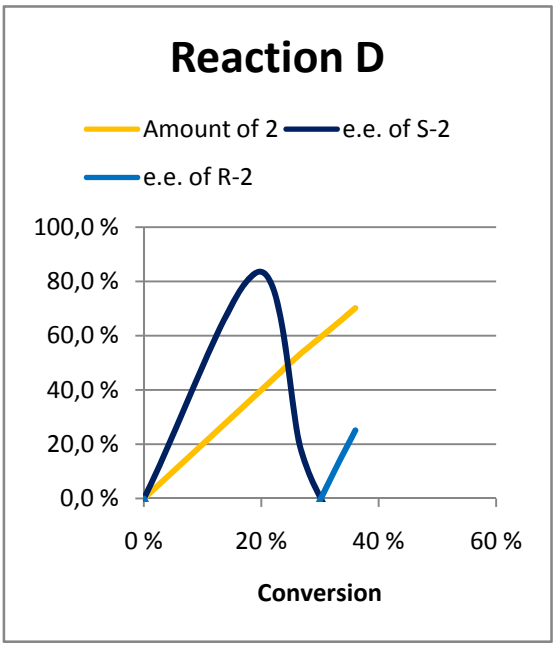

Figure 2-16: Amount of monoacetates and e.e. as a function of conversion for reaction D.

2.3.3 Selectivity of PPL in the transesterification of *N*-Benzyloxycarbonyl-2-aminopropane-1,3-diol

Concluding anything definite from these results is difficult, since the reactions carried out are not satisfyingly uniform in conditions, and because the time before sample analysis varied. However, the trends and tendencies outlined are too prevalent to be only a result of these inconsistencies.

Lacking a thorough investigation of crude PPL preparations, the only assumptions that can be made concerning the causes of the selectivity demonstrated by the results above concerns the composition of the enzyme preparation. As already mentioned in section 1.2.4, the presence of several different enzymes with hydrolytic activity has been established in crude PPL, and the selectivities and activities of these individual catalysts and the interaction between them would constitute a significant body of research in itself.

What the above presented results show is that despite variations in reaction conditions, which obviously affect the selectivity, activity and overall reaction rates, te same trends in selectivity is present for substrate **1** across the reactions.

The two reactions discussed which don't conform to either each other or the other reactions, are reactions B and E. For reaction E the use of a purified PPL preparation makes it highly plausible that only one enzyme is present and providing a consistent selectivity. For reaction B it is difficult to correlated the results with those from reactions A, C and D. However, considering the conditions for the reaction incorporated a significantly lower concentration of substrate and a vastly increased amount of acyl donor compared to the other reactions, it is not too farfetched to postulate that the conditions are less suitable for source of the selectivity at short reaction times for the other reactions, or that the reaction kinetics are altered to such a degree that 5 minutes reaction time for reaction B represents a reaction time above 17 hours for reaction D. There are of course other possibilities and only a more thorough mapping of the catalytic behaviour of PPL in this reaction will provide answers.

2.4 Further Research

The extent of this work was limited by the unforeseen complications with analysis and the availability of substrate **1** within the set time constraints. Thus, it seems justified to include a short section devoted to the basis this work can form for further research, and to present some advice based on the experience acquired.

The main issues with the presented results are the inaccuracies assumed to be connected to the presence of enzyme in the samples, the atmospheric exposure of the reaction mixture leading to solvent evaporation and introduction of moisture, and the additional reduction of solvent volume from sample withdrawal. If these factors could be avoided, the conditions should be favourable for accurate analysis results.

If a satisfactory supply of substrate can be assumed, the most certain way to ensure complete removal of enzyme before analysis would be to run batches of reactions with equal conditions in separate vessels, at a scale of about 1 mL, and, by filtration, halting

the reaction in a separate vessel for each reaction time to be analysed. Important aspects to focus on are to maintain the chosen reaction scale and be diligent with the reaction conditions within the batches of reactions to ensure comparability and conformity of the results.

Utilising this experimental procedure, a first step would be to repeat one of the reactions presented above. A recommendation would be reaction D. The chosen reaction should be repeated twice, to ensure the reproducibility of the conditions and the results. Following acquisition of this base set of results, the chosen reaction can form a centre point in separate reaction series investigating the effects of incremental variations in substrate concentration, relative enzyme amount and amount of acyl donor.

In addition to, and in connection to variations in reaction conditions, both untreated PPL and immobilised PPL should be investigated, and as the solvent can affect the selectivity, so should also the effect of different solvents [64; 65].

3 Experimental Procedures

This section describes the methods employed and the equipment used in the work presented.

3.1 General Experimental

3.1.1 Reactants and Solvents

All reactants and solvents used were, unless indicated, commercially available and of analytical grade.

Except for reaction A, the THF used as solvent for enzymatic reactions was briefly dried over molecular sieves before use. The dry THF used for reaction A had been dried by refluxing with sodium in a solvent still.

Two preparations of PPL were used, both from Sigma: L3126 Lipase from porcine pancreas Type II, crude (steapsin); and L0382, Type VI-S, lyophilized powder.

The preparation of *Candida Antarctica* lipase B (CAL B) used was Novozym 435 from Novozymes.

3.1.2 Chromatographic Methods

Equipment and parameters for the chromatographic methods utilised are given below.

3.1.2.1 HP Liquid Chromatography

HPLC was performed using a Varian Star LC System, consisting of a Varian 9010 Solvent Delivery System, a Varian ProStar 400 Autosampler, and a Varian 2550 UV Detector. The detector was set to $\lambda = 254$ nm. The column was a Daicel Chiralcel OD-R: 0.46 cm x 25 cm (10 μm). Chromatograms were recorded and analysed using Varian Star Chromatography Workstation 6.0 software.

Eluents consisted of acetonitrile (ACN), water and trifluoroacetic acid (TFA).

3.1.2.2 Thin Layer Chromatography

Thin layer chromatography was performed using Merck Silica Gel 60 F_{254} TLC sheets.

3.1.2.3 Flash Column Chromatography

Purification by flash chromatography was performed using a VersaFlash system connected to a Scilog Accu eluent pump. Columns were pre-produced VersaPak: Silica (Spherical); 23 X 110 mm (23 g), from Supelco.

3.1.2.4 Gas Chromatography

Analysis by GC was performed using a Varian 3380 Gas Chromatograph with a Varian CP-8410 Autoinjector. The column was a CP-Chirasil DEX (25 m x 0.32 mm, DF = 0.25), and the system used compressed air, and H_2 (5.0) as carrier and detector gases. All analyses were performed using an injection temperature of 250 °C. Chromatograms were recorded and analysed using Varian Star Chromatography Workstation 6.0 software.

3.1.3 Other Characterisation Methods

Equipment and parameters for the other characterisation methods utilised are given below.

3.1.3.1 NMR Spectroscopy

All ^{1}H-NMR spectra were obtained on a Bruker Avance DPX 300 QNP NMR system using deuterochloroform ($CDCl_3$) with tetramethylsilane (TMS) as internal reference. The spectra were processed using the software XWIN-NMR 3.5 from Bruker Biospin.

3.1.3.2 Optical Rotation

Optical rotation was measured using a Perkin-Elmer 243 B polarimeter and a 1 mL jacketed cell with length $l = 1$ dm. All measurements were obtained at wavelength $\lambda = 589.3$ nm.

3.1.3.3 Melting Points

Melting points were recorded using a Sanyo Gallenkamp capillary tube melting point apparatus. At least two parallels were recorded for each sample, and when sufficient material was available, three parallels were recorded.

3.2 Reactions

Procedures for the reactions conducted are given below.

3.2.1 Immobilisation of PPL

Powdered polypropylene (Accurel MP1000, 1.5 g) was added to ethanol (96%, 3 mL). PPL (Type II, 1.5 g) was suspended in phosphate buffer (20 mM, 30 mL). The two solutions were mixed and stirred for 22.5 hours at room temperature. The immobilised enzyme was filtered, washed with water and lyophilised for at least 16 hours.

3.2.2 General Procedure for Enzymatic Acetylation of *N*-Benzyloxycarbonyl-2-aminopropane-1,3-diol

Substrate was added to a suitably sized round-bottomed flask. The chosen relative amount of enzyme was added. If additional solvent was used, THF was added to obtain the correct concentration of substrate solution. Vinyl acetate was then added in a number of equivalents, or, if used as solvent, to obtain correct concentration of the substrate solution. Stirring by magnet was activated. The reaction proceeded at room temperature in a closed flask. At varying intervals, samples of the reaction mixture were obtained by halting the stirring and extracting 50 µL samples using an auto pipette. The samples were stored at -20 °C. After a set time, the reaction was halted by filtering with etyl acetate (EtOAc) or dichloromethane (DCM). The reaction mixture was concentrated under reduced pressure and stored at < -15 °C.

3.2.2.1 Reaction A

To substrate **1** (0.4086 g, 1.814 mmol, 180 mM) in dry THF (10 mL), was added immobilised PPL (Type II, 140 $\frac{\text{mg}}{\text{mmol substrate}}$, 0.252 g) and vinyl acetate (0.720 mL, 7.73 mmol, 5 equiv.). The reaction mixture was stirred under nitrogen, at room temperature, withdrawing samples (50 µL) at various intervals. After 8 days, 21 hours and 45 minutes the reaction was halted, as all the solvent had evaporated. Ethyl acetate was added to the dry residue, and immobilised enzyme filtered off.

The samples were diluted to $\frac{1}{2250}$ concentration with 1:1 H_2O/ACN and analysed by HPLC:
Eluent A: 40 % H_2O + 60 % ACN + 0.1 % TFA; Eluent B: H_2O + 0.1 % TFA;
Gradient elution: 65 % A + 35 % B to 95 % A + 5 % B over 25.72 min (flow 1.0 $\frac{\text{mL}}{\text{min}}$)

3.2.2.2 Reaction B

To substrate **1** (0.0563 g, 0.250 mmol, 50 mM) in vinyl acetate (5 mL, 53.7 mmol) was added untreated PPL (Type II, 300 $\frac{\text{mg}}{\text{mmol substrate}}$, 0.0755 g). The mixture was stirred in a stoppered flask at room temperature, withdrawing samples (50 µL) at various intervals. After 1.5 hours the reaction was stopped by filtration with ethyl acetate [66].

The samples were diluted to $\frac{1}{3}$ concentration with DCM and analysed by GC:
Temperature program: 80 - 92 °C, 30 °min^{-1}; 92 - 98 °C, 3 °min^{-1} (2 min);
98 - 104 °C, 3 °min^{-1}; 104 - 140 °C, 30 °min^{-1}; 140 - 200 °C, 3 °min^{-1} (5 min);
total time 33 min.

3.2.2.3 Reaction C

To substrate **1** (0.0811 g, 0.360 mmol, 180 mM) in THF (2 mL) was added untreated PPL (Type II, 180 $\frac{\text{mg}}{\text{mmol substrate}}$, 0.0654 g) and vinyl acetate (35 µL, 0.376 mmol, 1.04 equiv.). The mixture was stirred in a stoppered flask at room temperature, withdrawing samples (50 µL) at various intervals. After 3 hours the reaction was stopped by filtration with DCM.

The samples were diluted to $\frac{1}{3}$ concentration with DCM and analysed by GC:
Temperature program: 80 - 92 °C, 30 °min^{-1}; 92 - 98 °C, 3 °min^{-1} (2 min);
98 - 104 °C, 3 °min^{-1}; 104 - 140 °C, 30 °min^{-1}; 140 - 200 °C, 3 °min^{-1} (5 min);
total time 33 min.

3.2.2.4 Reaction D

To substrate **1** (0.0407 g, 0.181 mmol, 180 mM) in THF (1 mL) was added untreated PPL (Type II, 360 $\frac{\text{mg}}{\text{mmol substrate}}$, 0.0651 g) and vinyl acetate (20 µL, 0.215 mmol, 1.2 equiv.). The mixture was stirred in a stoppered flask at room temperature, withdrawing samples (50 µL) at various intervals. After 17 hours the reaction was stopped by filtration with DCM.

The samples were diluted to $\frac{1}{3}$ concentration with DCM and analysed by GC:
Temperature program: 80 - 92 °C, 30 °min^{-1}; 92 - 98 °C, 3 °min^{-1} (2 min);

98 - 104 °C, 3 °min^{-1}; 104 - 140 °C, 30 °min^{-1}; 140 - 200 °C, 3 °min^{-1};
total time 28 min.

3.2.2.5 Reaction E

To substrate **1** (0.0816 g, 0.362 mmol, 180 mM) in THF (2 mL) was added PPL (Type VI-S, 2.8 $\frac{\text{mg}}{\text{mmol substrate}}$, 0.0012 g) and vinyl acetate (40 µL, 0.432 mmol, 1.2 equiv.). The mixture was stirred in a stoppered flask at room temperature, withdrawing samples (50 µL) at various intervals using a syringe (25 µL). After 6 hours the reaction was stopped by filtration with DCM.

The samples were diluted to $\frac{1}{3}$ concentration with DCM and analysed by GC:
Temperature program: 80 - 100 °C, 20 °min^{-1} (3 min); 100 - 150 °C, 20 °min^{-1} (1 min); 150 - 200 °C, 20 °min^{-1} (5 min); total time 15 min.

3.3 Characterisation of Compounds

All the compounds listed in this section have been previously characterised [67; 68; 69]. For NMR data, all chemical shifts are reported in ppm (δ). Multiplicities are indicated as s (singlet), d (doublet), t (triplet), q (quartet) or m (multiplet). Broad peaks are indicated by br.

3.3.1 *N*-Benzyloxycarbonyl-2-aminopropane-1,3-diol

The compound was available from previous research [5].

Melting Point: 110.3-112.2 °C

^{1}H-NMR (300 MHz, $CDCl_3$): δ 7.32-7.55 (5H, m), 5.50 (1H, br s), 5.14 (2H, s), 3.74-3.95 (5H, m), 2.33 (2H, br s)

TLC: R_f (EtoAc/Hexane 2:1) = 0.8

3.3.2 *N*-Benzyloxycarbonyl-2-amino-3-hydroxypropyl Acetate

Isolated from the reaction mixture of reaction B. After filtration the solvent was removed under reduced pressure, and **2** was isolated and purified by flash chromatography, using 2:1 EtOAc/Hexane as eluent.

^{1}H-NMR (300 MHz, $CDCl_3$): δ 7.32-7.43 (5H, m), 5.24 (1H, br), 5.13 (2H, s), 4.23 (2H, d), 3.98 (1H, m), 3.69 (2H, m), 2.39 (1H, br s), 2.08 (3H, s)

TLC: R_f (EtoAc/Hexane 2:1) = 0.4

Optical Rotation: $[\alpha]_D^{20}$ (EtOAc, c = 0.797) = -7.4

3.3.3 *N*-Benzyloxycarbonyl-2-aminopropane-1,3-diyl Diacetate

Synthesised by reacting **1** (0.9013 g, 4.00 mmol) in vinyl acetat (5 mL) with CAL B (0.0401 g, 10 $\frac{\text{mg}}{\text{mmol substrate}}$) for 23 hours. Enzyme was removed by filtration and solvent removed under reduced pressure. The product was purified by flash chromatography, using 2:1 EtOAc/Hexane as eluent, giving a yield of 90%.

Melting Point: 47.1-49.1 °C

^{1}H-NMR (300 MHz, $CDCl_3$): δ 7.31-7.43 (5H, m), 5.12 (2H, s), 5.11 (1H, d), 4.07-4.28 (5H, m), 2.07 (6H, s)

TLC: R_f (EtoAc/Hexane 2:1) = 0.07

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Appendix A

A.1 ^{1}H-NMR Spectra

Below are given the ^{1}H-NMR spectra for the compounds **1**, **2** and **3**. All the spectra were obtained at 300 MHz with $CDCl_3$ as solvent and TMS as internal reference.

A.1.1 *N*-Benzyloxycarbonyl-2-aminopropane-1,3-diol

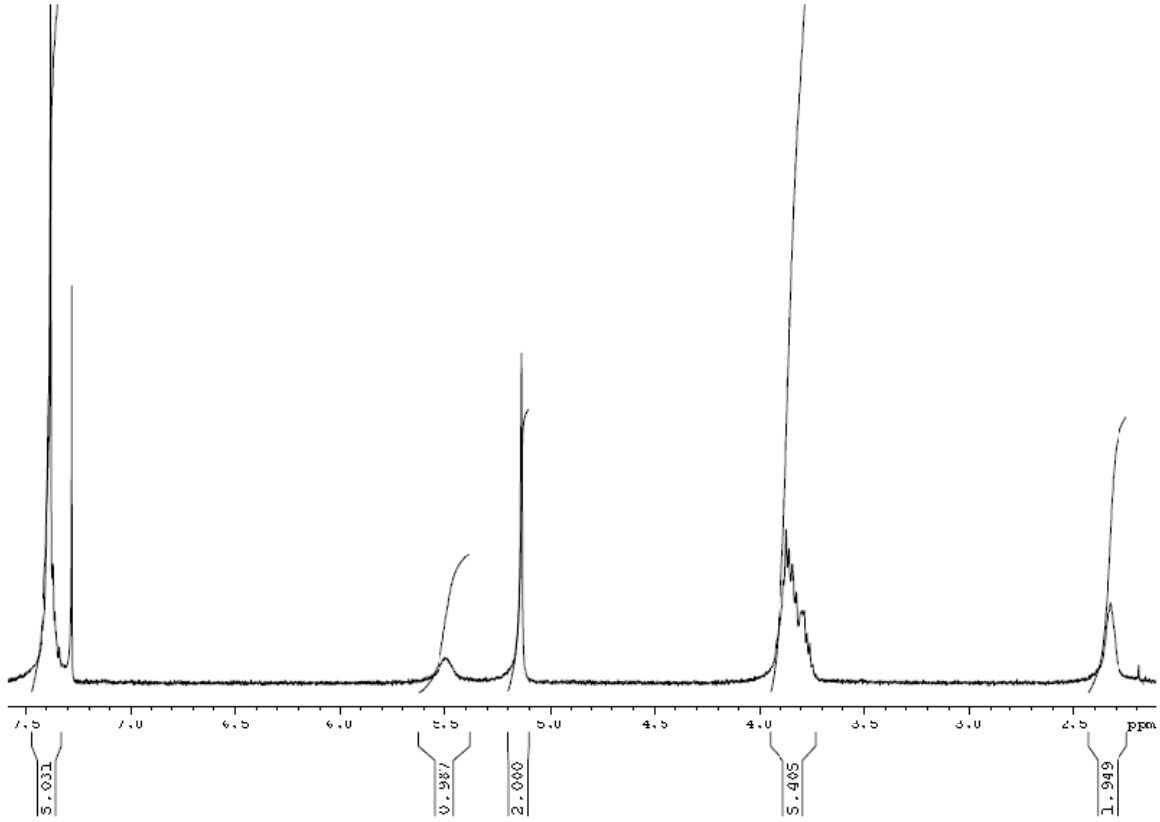

Figure A-1: ^{1}H-NMR spectrum for compound 1.

A.1.2 *N*-Benzyloxycarbonyl-2-amino-3-hydroxypropyl Acetate

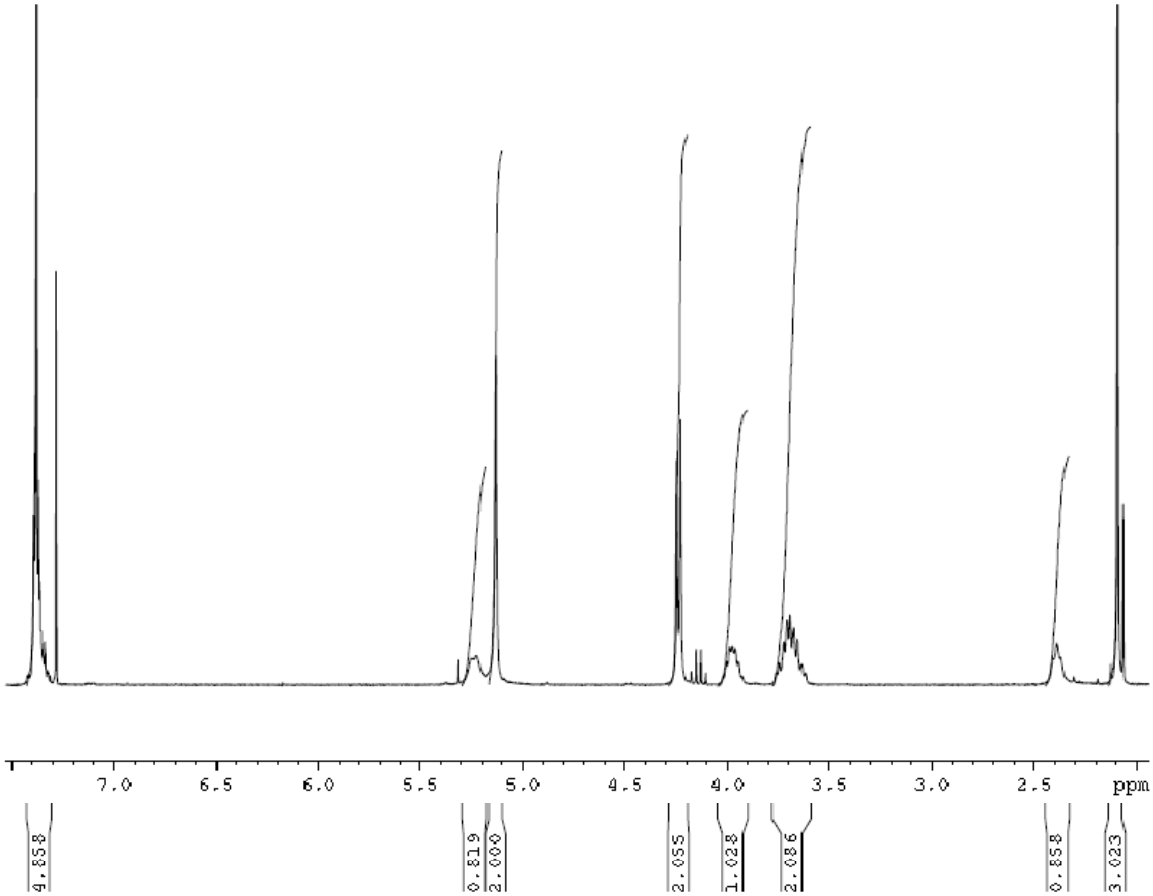

Figure A-2: ^{1}H-NMR spectrum for compound 2**.**

A.1.3 *N*-Benzyloxycarbonyl-2-aminopropane-1,3-diyl Diacetate

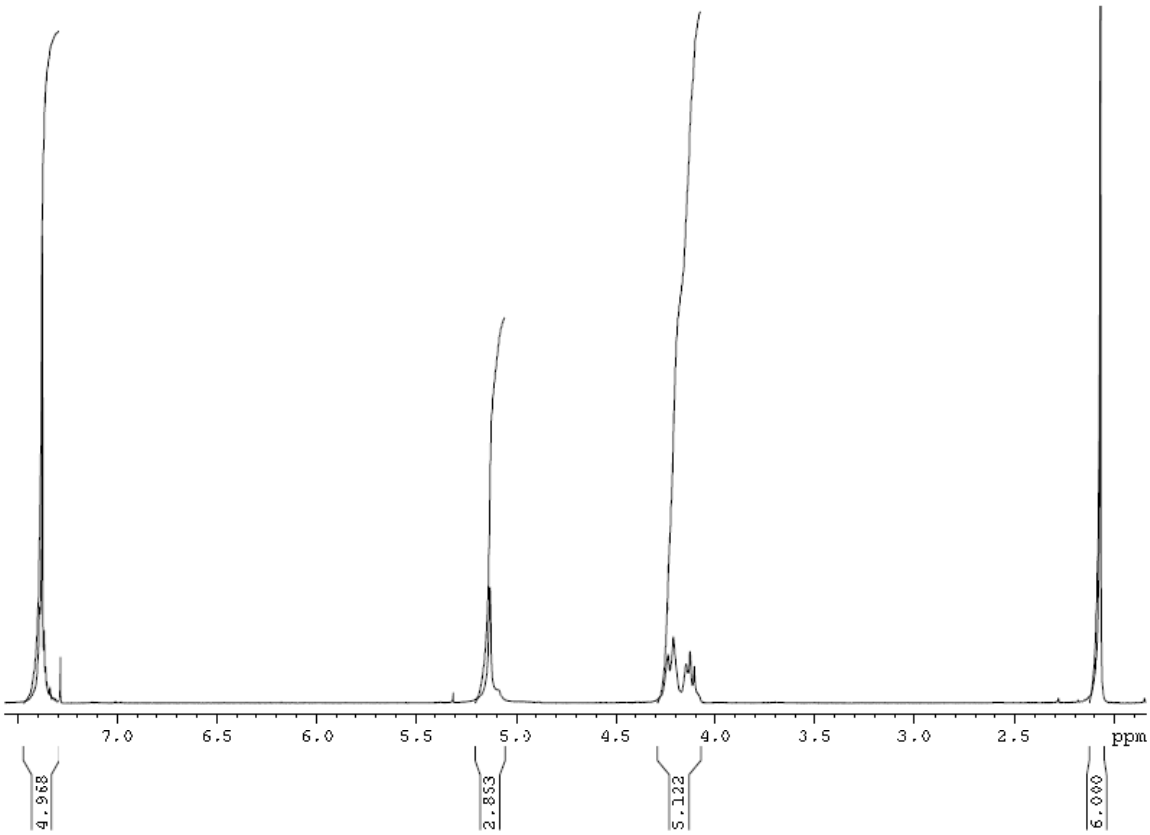

Figure A-3: ^{1}H-NMR spectrum for compound 3**.**