SYNTHESIS OF ENANTIOPURE BUILDING BLOCKS FOR BIOLOGICALLY ACTIVE COMPOUNDS BY ENZYME CATALYSIS

Optimization of reaction conditions for increased enantioselectivity and activity

by

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Preface

The work presented in this thesis has been carried out under the supervision of Professor Thorleif Anthonsen at the Department of Chemistry, Norwegian University of Science and Technology, NTNU, in the period from 2000-2004.

The thesis consists of an extended summary and 6 papers in an appendix. Chapter 1 gives an introduction to enzymes as catalysts and an overview of several aspects of bicatalysis for synthesis of enantiopure secondary alcohols and monoesters of 3-hydroxy glutaric diesters and 3-hydroxy glutaric amides. The experimental work are summarized in chapter 2 with paragraph 2.1 as a general introduction to the chapter. The synthesis of racemic substrates are discussed in paragraph 2.2 and the synthesis of enantiopure reference compounds are discussed in paragraph 2.3. The results from biocatalytic resolutions are discussed in paragraph 2.4, biocatalytic asymmetrizations are discussed in paragraph 2.5. The papers 1-6 are found in the appendix.

Acknowledgement

First I want to thank my supervisor Professor Thorleif Anthonsen for his enthusiastic, motivating and instructive way of guiding me into the exiting scientific world of organic chemistry and biocatalysis.

Dr. Scient. Bård Hoff and Dr. Scient. Kamilla Lundhaug are thanked for teaching me practical biocatalysis and for being "Oracles" in scientific questions during my Master study. Kamilla is also thanked for valuable comments to the doctor thesis manuscript.

My colleagues at the Department of Chemistry are thanked for cooperation and friendship during the last years.

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At last my family is thanked for encouragement and understanding when I had to stay long days and week-ends at the laboratory.

Trondheim April 2004

Elisabeth Egholm Jacobsen

Summary

Efficient methods for synthesis of enantiomerically pure enantiomers of a series of secondary alcohols and butanoates have been performed by kinetic resolution of the racemic alcohols and esters catalyzed by lipase B from *Candida antarctica* (Novozym 435). The effect of the substrate structure on *E* was different for transesterifications of alcohols in organic media as compared to hydrolysis of esters in buffer. The influence of different acyl donors on the enantioselectivity has also been investigated.

Derivatives of 1-phenoxy-2-alkanols have been kinetically resolved by esterification with irreversible and reversible acyl donors using lipase B from *Candida antarctica* (Novozym 435) as catalyst. Esterifications in eight different solvents with different water activity have been performed. For 3-bromo-1-phenoxy-2-propanol the *E*-values in all of the solvents were higher when the water activity was increased. The water content of the various reaction media at the same water activity was also determined.

In esterifications of secondary alcohols catalyzed by immobilized lipase B from *Candida antarctica* (Novozym 435) the *E*-values decreased during the reaction. Hydrolysis of the corresponding butanoates showed the opposite effect. When an enantiopure *(R)*-alcohol, related but different, was added to the transesterification reaction, the *E*-value was significantly enhanced.

Decreasing enantioselectivity (*E*-value) by conversion has also been observed in transesterification reactions of secondary alcohols catalyzed by a pure protein formulation of lipase B from *Candida antarctica* (Novozym 525 F). It can be concluded that the immobilization of Novozym 435 not was the reason for the decrease in *E*-value which was observed. Addition of a range of enantiopure alcohols caused a temporary increase in enzyme selectivity in the transesterification reaction of 3-chloro-1-phenoxy-2-propanol with vinyl butanoate.

Enantioselective hydrolyses and ammonolyses of diethyl 3-hydroxyglutarate and dimethyl 3-hydroxyglutarate gave a maximum of 91 and 98 % enantiomeric excess, respectively, with use of immobilized lipase B from *Candida antarctica* (Novozym 435). *Ee´*s were determined using chiral GLC of the mono amides and achiral GLC of diastereomeric derivatives of the monoesters. The catalyst was re-used more than ten times with retention of high activity and selectivity.

Biocatalytic asymmetrizations of diethyl 3-hydroxyglutarate furnish a route to enantiomers of ethyl 4-cyano-3-hydroxybutanoate. The enantiopreference of different enzymes has been established by chiral chromatography. Conclusive evidence for absolute configurations has been provided by X-ray crystallographic structure determination of co-crystals of the predominant monoester *(3S)*-3-hydroxy pentanedioic monoethyl ester with *(R)*-phenylethylamine. The predominant enantiopure monoester produced by ammonolysis of diethyl 3-hydroxyglutarate catalyzed by immobilized lipase B from *Candida antarctica* (Novozym 435) was ethyl *(3S)*-4-carbamoyl-3-hydroxybutanoate. It was converted to ethyl *(3S)*-4-cyano-3-hydroxybutanoate in high yield and enantiomeric excess.

Contents

List of appended papers

1. Jacobsen, E. E., Hoff, B. H., Anthonsen, T. *Enantiopure Derivatives of 1,2-Alkanediols. Substrate Requirements for Lipase B from Candida antarctica*. Chirality **2000**, *12*, 654-659. (Reference 213)

2. Jacobsen, E. E., Anthonsen, T. *Water Content Influences the Selectivity of CALB-catalyzed Kinetic Resolution of Phenoxymethyl-substituted Secondary Alcohols*. Can. J. Chem. **2002**, *80*, 577-581. (Reference 214)

3. Jacobsen, E. E., van Hellemond, E. W., Moen, A. R., Prado, L. C. V, Anthonsen, T. *Enhanced Selectivity in Novozym 435 Catalyzed Kinetic Resolution of Secondary Alcohols and Butanoates caused by the (R)-Alcohols.* Tetrahedron Lett. **2003**, *44*, 8453-8455. (Reference 48)

4. Jacobsen, E. E., Andresen, L. S., Anthonsen, T. *Immobilization does not Influence the Enantioselectivity of Novozym Catalyzed Kinetic Resolution of Secondary Alcohols.* Manuscript.

5. Jacobsen, E. E., Hoff, B. H., Riise Moen, A., Anthonsen, T. *Enantioselective Enzymatic Preparation of Chiral Glutaric Monocarboxylic Acids and Amides*. J. Mol. Catal., B **2003**, *21*, 55-58. (Reference 10)

6. Moen, A. R., Hoff, B. H., Hansen, L. K., Anthonsen, T., Jacobsen, E. E. *Absolute Configurations of Monoesters Produced by Enzyme Catalyzed Hydrolysis of Diethyl 3-hydroxyglutarate*. Tetrahedron:*Asymmetry*. In press.

1 Introduction

1.1 Biocatalysis

The use of enzymes and whole cells in industrial processes have been important for centuries. The production of wine, beer and cheese all involves microorganisms and for many years the fermentation technology industry has been providing a number of amino acids, citric acid etc. The first enzyme to be made commercially active (in 1874!) was the acid protease rennin (chymosine), which was used to hydrolyze κ-casein to paracasein in cheese production.[1]

Organic chemistry processes catalyzed by enzymes and especially enzyme catalysis for the production of fine chemicals in the pharmaceutical industry have increased during the past 30 years.[2,3]

There are two main reasons why biocatalysis has become popular in organic chemistry, i) biocatalysis takes place under mild conditions and is considered to be environmentally friendly and ii) biocatalysis is both chemo-, regio- and stereoselective. Besides acting as chiral catalysts against their natural substrates, a lot of enzymes are also able to catalyze reactions of a wide range of unnatural substrates.[4]

Today, high-throughput screening and modern biotechnology techniques such as directed evolution in combination with genomics and bioinformatics, have led to a substantial increase in the availability of enzymes. Also the stability of biocatalysts applied in industrial processes have been improved over the years by the use of genetic engineering and improvements in their formulation (immobilization).[5] Since 2000, patents describing use of microorganisms or enzymes to produce higher purity specialty chemicals have reached more than 400.[6]

Enzymes are water soluble, active in the pH-range from 5-8 (typically around pH 7) and in the temperature range of 20-40^{o}C. Some enzymes show activity even at 100^{o}C![7] As

catalysts enzymes affect the rate of the reaction but not the equilibrium. Recently it was found enzymes that were enhancing the reaction rates by factors as large as 10^{20}-fold![8] Enzymes are completely degradable to natural compounds unlike f. inst. heavy metal catalysts. Immobilized enzymes can also be re-used many times with retention of activity and selectivity.[9,10]

Green chemistry is a topic that has received more and more attention in recent years. It is defined as the utilization of a set of principles that reduces or eliminates the use or generation of hazardous substances in the design, manufacture and application of chemical products.[11] One goal is to find substitutions for the traditional organic solvents by environmently friendly reaction media which will lead to increased reaction rates, lower temperatures as well as higher selectivities. In view of increasing environmental and economic pressure to use renewable sources for energy and chemical feedstocks in industry, biocatalysts seem to be potentially attractive tools.[12] Figure 1.1 shows a model of the ”green catalyst” lipase B from *Candida antarctica.*

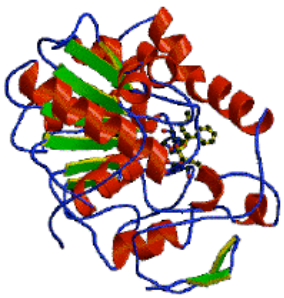


Figure 1.1 Model of lipase B from *Candida antarctica* (CAL-B).

Ionic liquids and supercritical carbon dioxide have also been suggested as interesting reaction media in the area of Green chemistry. Ionic liquids are organic salts with melting points below 100^{o}C which are used as substitutes for traditional organic solvents. The most common ionic liquids are imidazolium and pyridinium derivatives.[13]

Examples of combining ”green chemicals” are lipases providing high selectivity in asymmetrization reactions in supercritical CO_2 after showing low or no selectivity in ordinary organic solvents.[14,15]

1.2 Enzyme classification

Enzymes are divided by The Enzyme Commision into six classes based on the total reaction catalyzed. Each enzyme is assigned a code number consisting of four digits separated by dots. The first digit comprise the main classes: 1. Oxidoreductases, 2. Transferases, 3. Hydrolases, 4. Lyases, 5. Isomerases and 6. Ligases, which are further described in Table 1.1. The second, third and fourth digits in the code further describe the detailed/exact kind of the catalysis reaction.[16,1]

Table 1.1 Enzyme classification and examples of enzymes useful in synthesis.[16]

Enzyme class	Examples of enzymes useful in synthesis
1. Oxidoreductases Interconvert ketones with alcohols, double bonds with single bonds, etc.	Yeast/horse liver alcohol dehydrogenase, oxygenases
2. Transferases Transfer acyl, phosphoryl, sugar, amino groups, etc.	Transaminases, kinases
3. Hydrolases Hydrolysis of esters, peptides, glycerides, anhydrides, etc.	Lipases, esterases, acylases, proteases, phosphatases, glycosidases
4. Lyases Addition to double bonds C=C, C=N, C=O, etc.	(C=O)aldolases, mandelonitrile lyase, (C=C)aspartase, fumarase
5. Isomerases Various isomerizations, C=C bond migration, *cis-trans* isomerizations, etc.	Fructose-glucose isomerase
6. Ligases Formation of C–O, C–S, C–N and phosphoryl bonds	Important in molecular biology

Mainly oxidoreductases, hydrolases and lyases (aldolases) are frequently used in organic synteses. Hydrolytic transformations involving amide and ester bonds are most easy to perform using proteases, esterases and lipases (no need for cofactor systems) and more than 40 % of the biotransformations in synthetic organic chemistry over the past 5-10 years involve hydrolysis and esterification reactions.[2]

1.3 Chirality and biological activity

The need for enantiopure compounds for treatments of human diseases and combatting microbial attacs on crop etc. can be explained by the fact that all living organisms in Nature are chiral. Protein biosynthesis and most of the metabolic processes are mediated by enzymes which are specific for a particular isomeric form of one substrate, this is essential for ensuring the high degree of three-dimensional organization which is found in structures within cells. It is presumably evolutionary chance which has determined that life is based on L- rather than D-amino acids.[1]

However, chirality is not synonymous to biological activity, but where a stereocenter is present in a bioactive molecule, great differences in biological activity can be observed for the enantiomers.[17]

1.3.1 Pharmaceuticals

The use of drugs with one or more stereocenters is as old as the first natural-product therapeutic agents. Quinine and morphine have always been available as single enantiomers, morphine extracted first time from opium in 1804 by Friedrich W. A. Sertürner. But until recently, as products of synthetic chemistry, chiral drugs have been manufactured and usually used as racemates.

However, it is well known that racemic drugs can cause problems because of the differences in biological effects, and also due to different pharmacokinetics and pharmacodynamics of the two enantiomers. When drug-receptor interactions are considered it is postulated that "the lower the effective dose of a drug, the greater the difference in the pharmacological effect of the optical isomers".[18] The ratio of the more

active enantiomer (eutomer) compared to the less active enantiomer (distomer) is defined as the eudismic ratio, and the higher the eudismic ratio, the higher the effectiveness of the drug.[19]

The authorities who licence pharmaceuticals have begun to demand that when a product is chiral the isomers should be separated and tested separately. It is also undesirable to sell an active chiral drug as the racemate if only one enantiomer is active. The inactive enantiomer is regarded as a 50 % impurity or a so called ”isomeric ballast” and must be avoided in the marketed drug. The need for enantiomeric purity is a driving force for the development of more effective methods of asymmetric synthesis so separation of enantiomers can be avoided. This change of attitude has become known as the ”chiral switch”, and most large pharmaceutical companies consider this topic so important that they have started patenting of the enantiomers of their drugs. Sepracor Inc. and Genencor International Inc. are companies that have specialized in this single-isomer-molecule technology, however, single enantiomer drugs are also marketed by AstraZeneca, Pfizer, Merck and Aventis, among others.[16,20,21]

One example from the 1980´s is Perhexiline, a racemate drug used in the treatment of abnormal hearth rhytms. (Figure 1.2) A number of people have died by accumulated gram quantities of the enantiomer that was more slowly metabolized.[22] The drug is still in use as the racemate, however the dosages are quite decreased since the 1980´s (100-200 mg/day).[23]

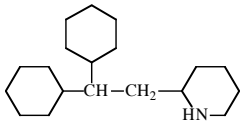


Figure 1.2 Perhexiline is a coronary vasodilator used especially for angina of effort, however it may cause neuropathy and hepatitis. The pharmacological actions are as calcium channel blocker, cardiovascular agent and vasodilator agents.

Statins as cholesterol lowering drugs

An important risk factor in coronary artery disease is hypercholesterolemia. Cholesterol (Figure 1.3) is a steroid molecule that is an essential component of cell membranes. It is also involved in the biosynthesis of steroid hormone and is a building block for very low density lipoproteins (VLDL) synthesized in the liver.

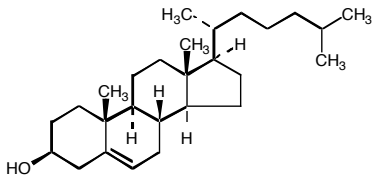


Figure 1.3 Structure of cholesterol.

Low-density lipoprotein (LDL) cholesterol is often called the "bad" cholesterol because it can build up within the walls of the arteries. This plaque buildup (or atherosclerosis) restricts blood flow to the heart and brain, increasing the chances of heart attack or stroke. High-density lipoprotein (HDL) cholesterol is called the "good" cholesterol because it helps the body to remove harmful LDL cholesterol. Higher HDL levels can actually reduce some of the risks of high total cholesterol. Cholesterol and triglycerides circulate in the bloodstream as part of lipoprotein complexes.

Approximately two-third of the cholesterol is synthesized in the body, the rest comes from the food. Therefore, it is not effective enough to control hypercholesterolemia by dietary restrictions. One effective control of plasma cholesterol levels is to inhibit the hydroxymethyl glutaryl-coenzyme A (HMG-CoA) reductase that is the first enzyme in the cholesterol biosynthesis producing mevalonate. The structure of HMG-CoA is shown in Figure 1.4. Fungal secondary metabolites called statins are found to selectively inhibit (by competitive inhibition) this step in the synthesis as they resemble hydroxymethyl glutaryl-coenzyme A. Applied to patients suffering from hypercholesterolemia this leads to reduced concentrations of cholesterol in the blood.[24,25]

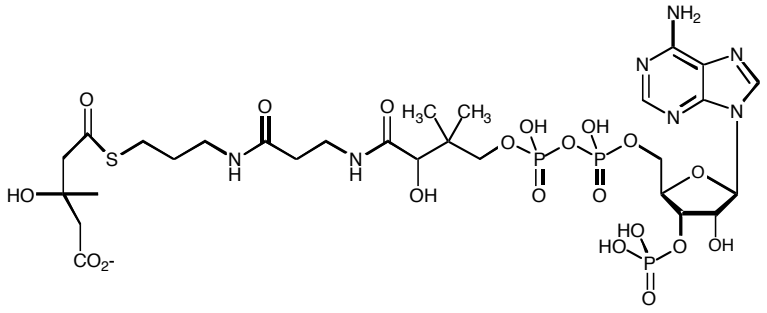
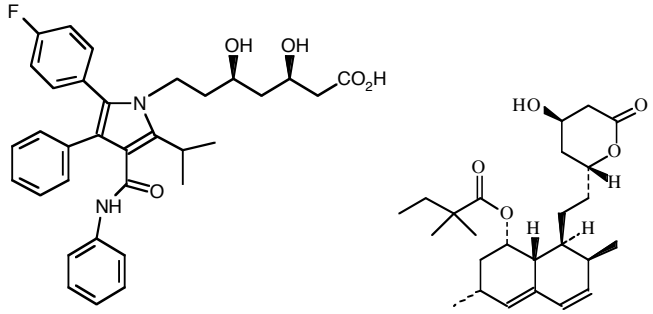


Figure 1.4 Structure of 3-hydroxy-3-methylglutaryl Coenzyme A.

Lipitor (Pfizer) and Zocor (Merck) (Figure 1.5) are single-enantiomer drugs used in the treatment of high blood cholesterol and were on top of the list of worldwide sales in 2002 with almost $ 14 billion together.[26] These drugs both have active ingredients named statins, with similar direction of action although different in structure.[27,24]



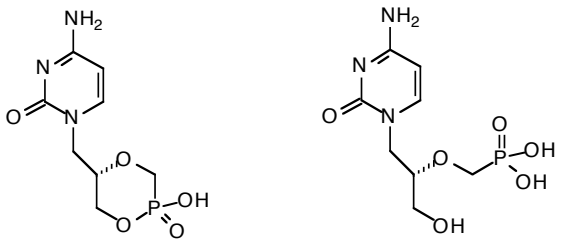
a) b)

Figure 1.5 a) Atorvastatin sold as Lipitor by Pfizer, b) Simvastatin sold as Zocor by Merck**.**

Lipitor (atorvastatin) is the number one sold drug in Norway with sales of 301 mill. NOK in 2003 and Zocor (simvastatin) is the second most sold drug with sales of 284 mill. NOK the same period. The feburary sales of Lipitor this year (2004) was 13.5 mill NOK![28]

Antiviral drugs

The life cycle of HIV has many steps, and it is theoretically possible to design a drug that will stop the virus growth at each step. Designing drugs to interfere with specific steps in the viral life cycle is called rational drug design. Drugs to fight the infections in patiens with developed AIDS are mostly active against DNA viruses. Cyclic HPMPC (1-[(*(S)*-2-hydroxy-2-oxo-1,4,2-dioxaphosphorinan-5-yl)methyl]cytosine) is a derivative of cidofovir (Figure 1.6) which in 2000 was approved for the systemic treatment of cytalomegavirus (CMV) retinis and human papilloma virus (HPV) in AIDS patients. Currently there are 19 distinct anti-viral drugs on the market which are used in many different combinations to fight HIV infection. In Norway anti-HIV drugs make up 2.5 % of the total pharmaceuticals sales.[28]



Cyclic HPMPC Cidofovir

Figure 1.6 Molecular structures of cyclic HPMPC and cidofovir.

Studies of pharmacological effects of cyclic HPMPC compared to cidofovir suggests that intravenous cyclic HPMPC has a lower potential for nephrotoxicity in humans compared to that of intravenous cidofovir.[29]

1.3.2 Insect pheromones

The use of pheromones in the control of insect populations is an area of increasing commercial importance. The advantage of using pheromones compared to traditional insecticides is that only target insects are affected. In addition low concentrations of the active compounds are sufficient to destroy the pests. Often the enantiomeric purity of the pheromone is of cruicial importance for its effectivity.[30,31]

The accessible amounts of natural pheromones are often less than one milligram, and of that reason it has been difficult to determine the optical activity of these compounds. However, several insect pheromones have recently been syntesized by different methods including the synthesis of sordidin, the banana weevil pheromone, starting from *(S)*-propylene oxide.[32] Last year the main component of this pheromone, the (1*S*, 3*R*, 5*R*, 7*S*)-enantiomer, was synthesized by a method involving enzymatic kinetic resolution of 2,2-(1,3-propylenedisulfanyl)pentan-4-ol catalyzed by lipase B from *Candida antarctica*.[33]

1.3.3 Routes to single enantiomers

Single enantiomers of small molecules can be obtained in the following ways:

- From enantiopure natural compounds
- Resolution of racemates: Non-biological (crystallization) or biocatalytic
- Asymmetric synthesis: Non-biological or biocatalytic

Natural products will still be an important source of novel active agents which may serve as leads for elaboration into efficacious drugs for a multitude of diseases.[34] However, biocatalysis technology with enzymes capable of accepting a wide range of complex molecules as substrates in chemo-, regio-, and stereoselective ways with transformations without tedious blocking and deblocking steps makes it possible for the pharmaceutical industry to use biocatalysis to produce single-enantiomer drugs.[35]

1.4 Kinetics and thermodynamics in enzyme catalysis

The rate constant of a chemical reaction is connected to the free energy of activation, $\Delta G^{\#}$ (= the barrier height), by the Eyring relation:

$$k = \kappa K_B T/h \, e^{-\Delta G\#/RT}$$

where K_B is the Boltzmann constant and κ (kappa) is the transmission coefficient. The latter is most often neglected by giving it the value 1, meaning that all molecules which

pass the transition state will arrive at the product. Multiplying K_B with T/h results in a frequency factor equal to about 6 ps^{-1} at 300 K for crossing the transition state. This is valid in solution as well as in the gas phase.[36,37]

Enzymes as catalysts speed up the rate of equilibrium to be established in a chemical system. The fitting of the substrate in the active site of the enzyme is providing a stabilization of the transition state of the reaction leading to a decrease in the activation energy ($\Delta G^{\#}$), resulting in a significant acceleration of the reaction rate. Calculations show that a 4 kcal/mol decrease in $\Delta G^{\#}$ from a given value onset a 1000 times increase in reaction rate. The substrate is bound in the active site in a manner that the functional groups involved in conversion of the substrate to product come close to each other. When two substrates are meant to react, the enzyme bring them in close contact which allow functional groups to react.[38,39]

A reaction where the enzyme E catalyzes the conversion of substrate S to product P, with ES as the enzyme-substrate complex and ES^* as the transition state, is expressed as follows (k_1-k_4 are the rate constants of the different steps):

$$E+S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} ES^{\#} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} EP \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} E+P$$

In some reactions the substrate is covalently bound to the enzyme. However, the enzyme-substrate complex binding can also be caused by non covalent forces as hydrogen bonds, electronic and hydrophobic interactions, as well as van der Waals forces.[40] Enzyme catalysis follows general organic reaction mechanisms, nucleophilic and electrophilic attacks as well as acid-base chemistry.[40,41,1]

1.4.1 Kinetic resolutions

In enzyme catalyzed kinetic resolutions of racemic substrates one enantiomer has a higher specificity constant (k_{cat}/K_M) than the other due to favorized orientation in the

active site. This results in a lowering of the activation energy ($\Delta G^{\#}$) for the transition state of this enantiomer. (Figure 1.7 and 1.8)[4]

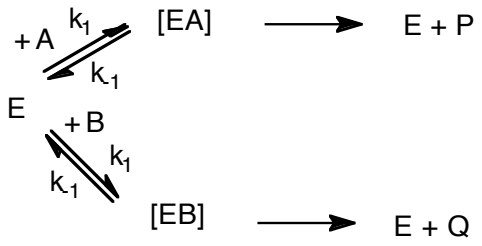


Figure 1.7 Reaction scheme for an enzyme catalyzed enantioselective reaction. E = enzyme, A and B = enantiomers of a racemic substrate, EA and EB = enzyme-substrate complexes, P and Q = enantiomeric products.

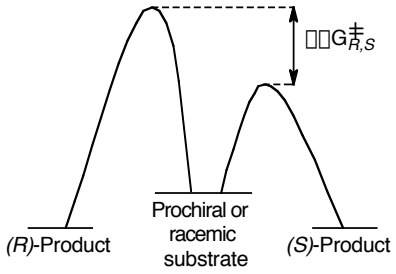


Figure 1.8 Free energy profile illustrating a biocatalytic kinetic resolution or asymmetric synthesis. $\Delta\Delta G^{\#}$ is the difference in activation energy of the two diastereomeric transition states.

1.4.2 The enantiomeric ratio, E

The enantioselectivity of an enzymatic kinetic resolution is described by the enantiomeric ratio, *E*, which is the relative rate constant of reaction of the two enantiomers. It is a kinetic parameter which will vary with different catalysts and it is sensitive to environmental changes as solvent, water content of the medium, acyl donor and acyl acceptor, pH, temperature etc. It is related to the difference in free energy of activation, $\Delta\Delta G^{\#}$, and to the ratio of the specificity constants k_{cat}/K_M for the enantiomers as expressed in the following equations.[42-45]

$$\Delta\Delta G^{\#} = -RT \ln E \qquad E = \frac{k_{sp}^{R}}{k_{sp}^{S}} = \frac{\left(k_{cat} / K_M\right)^{R}}{\left(k_{cat} / K_M\right)^{S}}$$

When $\Delta\Delta G^{\#}$, defined as $\Delta\Delta G^{\#} = -RT \ln k_R/k_S$ or $\Delta\Delta G^{\#} = -RT \ln E$, is f. inst. 1.74 kcal/mole (7.3 kJ/mole) the enantioselectivity, *E*, is determined to 19.

As long as the $\Delta\Delta G^{\#}$ is not affected *E* should be constant throughout a reaction.[42,44] However, examples of changing enantioselectivity in kinetic resolutions have been observed. Different reasons for this deviation from theory were discussed; changing medium by conversion, complex interactions between reaction components, enzyme inhibition and substrate sorption into enzyme beads.[46-50] The relation of $\Delta\Delta G^{\#}$ to *E* with the example of *E* = 180 at 15 % conversion and *E* = 60 at 55 % conversion gives a difference in $\Delta\Delta G^{\#}$ of 0.66 kcal/mole. (Figure 1.9)

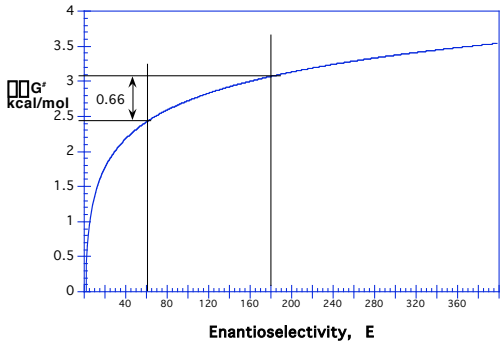


Figure 1.9 The relationship between the *E*-value and the difference in free energy of activation, $\Delta\Delta G^{\#}$, for a resolution of two enantiomers ($\Delta\Delta G^{\#} = -RT\ln E$). The difference in the *E*-value of 180 at an early stage of the reaction (15% conversion) and at the end of the reaction (*E* = 60 at 55% conversion) corresponds to a difference in free energy of activation of 0.66 kcal/mol.

Often enzyme catalyzed kinetic resolutions have been modelled based on the irreversible uni-uni mechanism of the Michaelis-Menten kinetics. This model was also later extended to reversible reactions. The enantioselectivity of irreversible resolutions can be calculated by inserting experimental chromatographic values into the following

equations, where ee_s and ee_p are enantiomeric excess of substrate and product, respectively, and c is the total conversion of the reaction:[42,43]

$$E = \ln [(1-c)(1-ee_s)]/\ln [(1-c)(1+ee_s)]$$
$$E = \ln [1-c(1+ee_p)]/\ln [1-c(1-ee_p)]$$

However, this mechanism is based on one substrate reacting giving one product. Enzyme catalyzed resolutions which involve two substrates resulting in two products are defined as bi-bi reactions. The bi-bi reactions can further be derived into other mechanisms which lead to different functions of reaction rate *vs*. time: The ping-pong mechanism, where the first product is released before the next substrate is bound to the enzyme, and the sequential ordered or random mechanisms, where all substrates must add to the enzyme before any product is released, either in an ordered or in a random way.[51]

Hydrolases are believed to follow the ping-pong bi-bi reaction mechanism.[52] Kinetics of hydrolase-enantioselectivity is well described by van Tol *et al.* and will not be discussed in more detail here.[53] A generalized ping-pong bi-bi reaction is shown in Figure 1.10.[54]

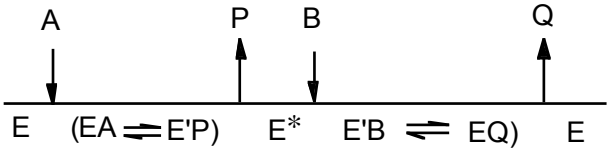
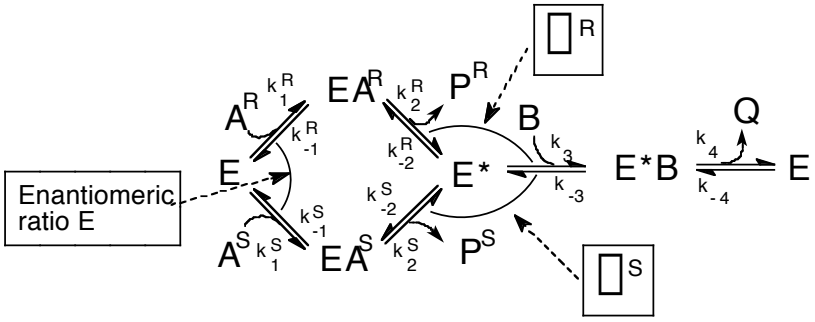


Figure 1.10 Ping-pong bi-bi mechanism (Cleland-diagramme), E = enzyme, E^* = acylenzyme, A and B = substrates, P and Q = products.[54]

In ping-pong bi-bi resolutions three situations are possible, A-P, A-Q and B-Q resolutions. In A-P resolutions the chiral substrate A is converted to the chiral product P (f. inst. hydrolysis). Substrate B (water) and product Q (acid) are not chiral. In the A-Q resolutions the chiral substrate A is converted into the chiral product P. In B-Q

resolutions the chiral substrate B is converted to the chiral product Q (f. inst. transesterification reaction).[55]

In ping-pong bi-bi reactions the selectivity constants β^R and β^S must also be taken into account. β^R and β^S are the ratio of nucleophilic attack of the acylated enzyme by *(R)*-alcohol, *(S)*-alcohol or water in hydrolysis, respectively, as shown in Scheme 1.1. In transesterification β is the selectivity between the acyl donor and the products.



Scheme 1.1

Scheme 1.1 shows a kinetic resolution of *(R,S)*-glycidyl butanoate by porcine pancreas lipase (PPL) where A^R and A^S are the two enantiomers of glycidyl butanoate, P^S is *(S)*-glycidol, P^R is *(R)*-glycidol, B is water, Q is butanoic acid, E is PPL, E* is butyryl-PPL and E*B is the butyryl-PPL-water complex. Partitioning of the acylenzyme complex (E*) is governed by *E* and β^R.[56]

The *E*-values from a ping-pong bi-bi resolution can be calculated from the following equation using measurements of the enantiomeric excess of the product fraction ee_p and the remaining substrate fraction, ee_s, at different degrees of conversion.[57]

$$E = \frac{\ln\left[\frac{ee_p(1-ee_s)}{(ee_p+ee_s)}\right]}{\ln\left[\frac{ee_p(1+ee_s)}{(ee_p+ee_s)}\right]}$$

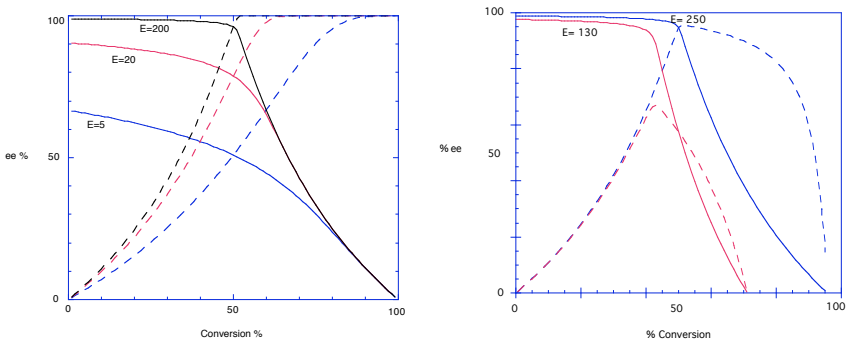
Two computer programs for calculations of *E* are available. *Selectivity Mac 1.0* is based on the uni-uni mechanism of Chen and Sih.[42,43] By inserting the *ee*-values from one experimental conversion the *E*-value is determined. *E & K Calculator 2.03/2.1b0 PPC* is a program where *E* can be calculated from both the uni-uni and the ping-pong bi-bi mechanism.[58,59] It has been observed that the K_{eq} from lipase catalyzed resolution reactions with different acyl donor concentrations is more consistent when the bi-bi mechanism is used. In *E & K Calculator 2.03/2.1b0 PPC* the □-values are determined and taken into account while they are not in the uni-uni calculations. Calculating *E*-values in bi-bi reactions from the simplified equations will of that reason give incorrect values.[53,58]

When the *ee*-values are obtained from separation of the resolved substrates and products on chiral Chirasil-DEX GLC columns the resolution factor (R_s) is different from the resolution factor used in achiral GLC analysis and is given by the following equation:[60]

$$R_S = \frac{1.177\,(t_R(2) - t_R(1))}{(t_{W0.5}(1) + t_{W0.5}(2))}$$

$t_R(1)$ and $t_R(2)$ are the retention times of the first and second eluted enantiomers, respectively, and $t_{W0.5}$ their peak widths at half height. Enantiomeric mixtures with resolution values of 1.5 and above are considered to be effectively base line separated.

By inserting all the experimental *ee*-values from a reaction the *E*-value for the total process is obtained. *Kaleidagraph* plots of ee_p´s and ee_s´s from resolutions of different substrates catalyzed by lipase B from *Candida antarctica* (CAL-B) obtained by *E & K Calculator 2.03/2.1b0 PPC* is shown in Figure 1.11, with irreversible resolutions to the left and reversible reactions to the right.



a) b)

Figure 1.11 a) Enantiomeric excess (% *ee*) plottet against conversion for three irreversible kinetic resolutions giving *E*-values of 200, 20 and 5, respectively. b) Enantiomeric excess (% *ee*) plottet against conversion for two reversible kinetic resolutions giving *E*-values of 250 and 130, respectively. The *ee*-values for the products are shown as whole lines, the *ee´*s of the remaining substrates are plotted as broken lines.

Both in irreversible and reversible reactions an *E*-value of 5 is a result of low *ee´*s both of the product and the remaining substrate and the reaction is considered ineffective. In an irreversible reaction an *E*-value of 20 gives quite low *ee* of the product but the remaining substrate can be obtained in high enantiomeric purity if some yield can be sacrified. This is not the case in a reversible reaction where the ee_s falls drastically between 40-60 % conversion.

E-values in the range 50-200 in irreversible resolution reactions are considered exellent, giving access to high enantiopurities of both product and remaining substrate. *E*-values in the same range obtained in reversible resolutions give access to high enantiopure products, however, the remaining substrate may be obtained in high enantiomeric purity only when K_{eq} is high. In order to obtain the more effective irreversible resolution acyl donors giving leaving groups with low nucleophilisity are employed. (See paragraph 1.5.3, Acyl donors) *E*-values above 200 are difficult to measure accurately due to the small conversions of the slower reacting enantiomer.

It is preferable to make as many measurements as possible throughout a reaction process to avoid errors from chromatographic results. As long as the experimental values from two or more paralelle reactions lie on the generated graphs the resulting *E*-values are considered reproducible. The deviation from experimental points from the generated curves can also be obtained accurately from *E & K Calculator 2.03/2.1b0 PPC*.

1.5 Factors affecting activity and enantioselectivity in hydrolytic resolutions

1.5.1 Substrate structure

The hydrolases (esterases, lipases and proteases) catalyze reactions involving alcohols, glycidyl-, carboxylic, thiol- and phosphoric esters, peptides and substrates with other C–N bonds, amides, glycidyl ethers and carbohydrates.[61,62]

The orientation of the substrate in the active site is the most important factor for the enzyme selectivity, however, it has also been reported that the concentration of the substrate influences the *E*-value.[63]

Compilation of a large number of results from lipase catalyzed resolutions of secondary alcohols show that when the small substituent (S) has lower priority than the large substituent (L) (OH > L > S), esterification of the *(R)*-enantiomer will be the faster reaction. The same preference is appearant in hydrolysis of the corresponding ester.[64,65] (Figure 1.12)

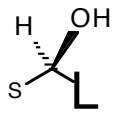


Figure 1.12 Substrate model of the faster reacting enantiomer of a secondary alcohol (*(R)*-enantiomer) in an esterification reaction catalyzed by lipases.[65]

1.5.2 Reaction medium

Aqueous solvent

The natural medium for enzymes is water, and traditionally enzyme catalyzed reactions have been performed in water. The advantage of running enzyme catalyzed reactions in water is that the enzyme maintain its native conformation and natural activity, and moreover, water is an environmentally friendly solvent. However, use of water as solvent in organic chemistry enzyme catalysis is often a problem because most of the substrates are not water soluble. One way to avoid this problem is to perform the reactions in organic solvents or in biphasic media.[66]

Recently it was reported that a biphasic buffer/supercritical carbon dioxide solution effectively kept the unreacted ester in the *Pseudomonas cepacia* lipase catalyzed hydrolysis of a pentenoic acid ethylester while the produced acid was concentrated in the buffer phase.[15]

Organic solvents

Solvation of apolar substrates, easy work-up, equilibrium shifted towards synthesis instead of hydrolysis are some of the benefits of performing biocatalytic reactions in organic solvents compared to aqueous media. Requirements for performing biocatalysis in organic media is that the enzyme is in the active conformation. The catalytically active conformation of the enzymes is maintained by many non-covalent interactions where the polarity of the surrounding water molecules is important. Removing all the water will change the enzyme conformation. However, catalytically active enzymes only need a small layer of water to keep their active conformation while the rest of the water can be replaced by an organic solvent. Most proteins in aqueous systems are surrounded by tightly bound water molecules with different properties compared to those of the bulk water. The bound hydration layer is less mobile and more ordered than the bulk water and has a 10 % greater density. This water layer consist of approximately 0.3 g water per g protein which means almost two water molecules per amino acid residue.[67] The accessibility of solvents to the enzyme surface can be defined by *the*

accessible surface area which is generally described by the center of a solvent molecule of radius 1.4 Å (representative of a water molecule) in van der Waals contact with the molecule.[68]

The amount of water necessary for enzyme activity varies from 50-1000 water molecules per enzyme molecule depending of enzyme and organic solvent.[69,70] Apolar solvents do not interact with substrate and enzyme through hydrogen bonds as water does. The enzyme is not totally hydrated in organic media, and it can possibly form a rigid conformation, resulting in either a decrease or an increase of activity.[70,71] Interactions between substrate and solvent can also cause unfavorable binding of the substrate in active site leading to an increase in activation energy and a drop in enzyme activity.[72,73]

No significant quantity such as the dielectric constants ε and E_T^N, log P or dipole moment[74] is able to describe the quality of solvents for use in biocatalysis, however, the log P value seems to be useful. The partition coeffisient, log P, is defined as the partition of solvent between octanol and water. Generally catalytic activity for enzymes is low in polar solvents with log P < 2, moderate in solvents with log P in the range of 2-4 and high in apolar solvents with log P > 4.[75] It is therefore suggested that enzyme catalysis works best in apolar hydrophobic solvents because they don´t strip water from the enzyme surfaces.[70] However, it is also reported that enzyme selectivity not can be predicted from the log P values of the solvents.[76-78]

Different solvents affect enzyme activity, stability and selectivity both positively and negatively, depending of substrate, enzyme and reaction media.[79-82] It has been observed that the enantioselectivity in lipase catalyzed esterifications is higher when structurally linear solvents are used compared to branched solvents. At high substrate concentrations in both aqueous and organic media the enzyme will comprehend a two phase system, which affect the enzyme either in a positive or in a negative way.[83] A complete reversal of enzyme enantioselectivity upon change of solvent has also been reported.[84,85]

Water activity (a_w) adjustments in organic solvents

Addition of small amounts of water to water immiscible solvents in enzyme catalyzed reactions may affect the enzyme activity and enantioselectivity due to increased hydration of the enzyme. The thermodynamic water activity (a_w) is used as a measure of the hydration level of the enzyme.[86] It is defined relative to pure water, which has a a_w = 1, at constant temperature and presssure.[87] Water is distributed between enzyme, solvent, substrate, carrier (immobilized enzymes) and athmosphere, and should be constant throughout the reaction when equlibrium is achieved.[88]

Adjustments of water activity in solvent systems can be done by addition of pairs of salt hydrates which act as buffers.[86,89-91] The adjusted water activity is maintained under different reaction conditions (substrate concentration, solvent etc.) as long as the capacity of the system is maintained. Pre-equilibration of the system is not necessary thus avoiding a possible deactivation of the enzyme. Table 1.2 shows pairs of salt hydrates for adjustments of different water activities at 30°C.[92,86]

Table 1.2 Water activity (a_w) for different pairs of phosphate salt hydrates.[86]

Salt hydrate 1	Salt hydrate 2	a_w 30°C
Na_2HPO_4 x 2 H_2O	Na_2HPO_4 x 0 H_2O	0.177
Na_2HPO_4 x 7 H_2O	Na_2HPO_4 x 2 H_2O	0.57
Na_2HPO_4 x 12 H_2O	Na_2HPO_4 x 7 H_2O	0.74

Water as a nucleophile competes with other nucleophiles, f. inst. a racemic alcohol, in attacking the acyl enzyme. Extensive research has been done to reveal the effect of water activity on enzyme activity, often the activity of the enzyme increased by increased water activity.[93-105]

The effect of water activity on enzyme enantioselectivity is not well understood. Enzyme, solvent and substrate type must be considered. In some reactions the selectivity increased by increasing water activity,[106] in other cases *E* decreased when a_w

was increased[107-109] finally *E* did not change by changing water activity.[110-112] In transesterification of sulcatol with porcine pancreatic lipase *E* increased from 24 to 100 with dehydrated enzyme compared to undried enzyme.[30] However, the water activity effect has to be seen in connection with the solvent used in the reaction. It has been reported that higher enantioselectivity is obtained in hydrophilic solvents (polar) than in hydrophopic (apolar) ones, although hydrophopic solvents yield higher reaction rates than hydrophilic solvents.[108] It has also been observed that the reaction rate increased with increasing water activity to a certain level and then decreased when the a_w was further increased.[102,89] However, different substrates and different enzymes were used under varying conditions in all of these experiments and no general conclusions have been drawn.

1.5.3 Acyl donors

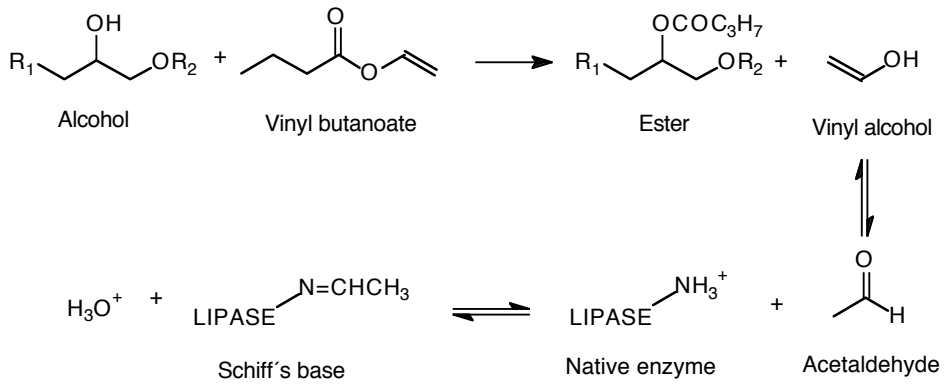
Thermodynamic equlibrium of kinetic resolutions must be avoided because it leads to low yield and low enantiomeric purity of the products. Hydrolytic reactions are irreversible because water is present in large excess. Transesterification reactions are basically reversible because the reactants are present in low concentrations. Another drawback is that the leaving alcohol may inhibit the enzyme leading to a decrease in activity.[113]

To avoid this problem ”activated” esters can be used, *i.e.* esters with electron withdrawing substituents which ensures low nucleophilisity of the leaving alcohol. Examples are cyanometyl- and oxime esters and halogenated ethyl esters.[114]

2,2,2-Trifluoroethyl esters are advantageous bacause of the high degree of ”activation” and because of easy separable by-products. 2,2,2-Trichloroethyl esters lower the expences in large scale reactions, however, trichloroethanol with a boiling point of 151^{o}C is liberated. 2-Chloroethyl esters are used alternatively which forms 2-chloroethanol (bp 130^{o}C). Molecular sieves may be added which attract the leaving alcohol. This makes the system more irreversible and a greater overall yield is the result.

A draw-back with this method is difficulties in enzyme regeneration.[115] Different acyl donor concentrations also affect the *E*-value.[116]

Enol esters make transesterifications completely irreversible because the leaving enol is tautomerized to volatile ketones or aldehydes. Acyl donations from enol esters are shown to react 10 times as slow as hydrolyses but as much as 100 times faster than transesterifications with activated esters.[117] S-Etyl thiooctanoate as acyl donor shifts the equilibrium to the right because of the evaporation of the volatile leaving alcohol.[118] Vinyl butanoate produces acetaldehyde (bp 21°C) after tautomerization of the leaving enol. Although this ensures zero nucleophilisity from the leaving enol, it is known that acetaldehyde may interact with ε-amino groups in lysine residues with high pK_a-values ($pK_a > 12$) producing nucleophiles that may attack the acetaldehyde carbonyl carbon which results in an imine (Schiff´s base). This results in loss of positive charges on the enzyme surface, which in turn may lead to deactivation of the enzyme.[119] (Scheme 1.2)



Scheme 1.2

Candida rugosa lipase has lysine residues around the active site covering lid which may be converted to Schiff´s bases by reacting with acetaldehyde. However, it has been reported that CAL-B is quite stable against acetaldehyde because of the quite shielded lysine residues with pK_a-values up to 11.6.[120] By using enzymes which are immobilized by covalent binding to the ε-amino group in lysine this problem can be avoided.[121]

The acyl part of the acyl donor may affect the enantioselectivity due to production of different acyl enzymes,[43,122,30] however, it has been observed that the enzyme selectivity also depend on the alkyl part of the acyl donor.[30,116,123-126] A series of suitable reagents for reversible and irreversible enzymatic acylations has recently been summarized.[127]

1.5.4 Enzyme inhibition

General

Enzyme inhibitors are substances which decrease the rate of the catalyzed reaction. Reversible inhibitors form an equilibrium system with the enzyme and can be removed by dialysis. Irreversible inhibitors are tightly bound to the enzyme and are not easily removed. If the inhibitor has a structure similar to the substrate, the inhibitor and the substrate may compete for the same binding site. This is called competitive inhibition and it is concentration dependent.

Uncompetitive inhibition is the case when the inhibitor only binds to the enzyme-substrate complex in a different site made due to the conformational change in the enzyme affected by substrate binding. When an inhibitor binds to a site on the enzyme different from the active site it is called non-competitive inhibition. This binding can occur whether a substrate is bound or not. These three types of inhibition describe complete enzyme inhibition where the enzyme-inhibitor complexes are dead-end complexes with no activity, however, partial inhibition is also possible. The enzyme may also be inhibited by the substrate(s) or the product(s) (feedback inhibition).

The type of inhibition which play the most important role in a living cell is the non-competitive allosteric inhibition, which is a type of metabolic regulation. Usually the regulator is bound at a site different from the active site, the allosteric center. In this case the inhibitor induces conformational changes in the enzyme which may alter the binding characteristics.[1]

In general, association between a single ligand molecule (*i.e.* the substrate for the enzyme) and a single site on a protein is relatively simple, but binding of multiple

ligands at multiple sites can lead to very complex behavior. Binding at one site may have no effect on the affinity of the other sites or may increase or decrease them by changing the conformation of the active site which may alter the binding characteristics or the subsequent reaction characteristics. One example is binding of L-tryptophan on the *trp* repressor, which is causing an increased affinity of the repressor for its DNA binding site.[67]

Inhibition of lipases by both substrate and product has been observed. In alcoholysis of methylpropionate with n-propanol catalyzed by CAL-B the alcohol was found to inhibit the enzyme resulting in a dead-end complex.[128] Phosphate and phosphonate containing inhibitors are known to inhibit proteases. Studies of inhibition of CAL-B showed inhibition by diethyl p-nitrophenyl phosphate. The inactivation of the enzyme is due to covalent binding of diethyl p-nitrophenyl phosphate in the active site.[129]

Enantioselective inhibition

Enantioselective inhibition of *Candida rugosa* (*cylindracea*) by dextromethorphan and levomethorphan resulting in an enhanced enantioselectivity has been reported. Kinetic inhibition experiments revealed that the molecular action of the base was a non-competitive inhibition (*i.e*. binding of the base to an allosteric site in the lipase) which caused the inhibition of the transformation of one enantiomer leading to the relative increase in the transformation rate of the other enantiomer.[130]

L-Methioninol was found to increase the hydrolysis rate of the *(R)*-enantiomer in hydrolysis of 3-acetoxy nitriles catalyzed by lipase PS (*Pseudomonas* sp.) while it inhibited the hydrolysis of the *(S)*-enantiomer. It was suggested that the substrate and L-methioninol were combined to the enzyme at different sites and that conformational changes due to the binding of L-methioninol provided a change in the affinity towards the substrates.[131] Asymmetrization of 3-(3,4-dichlorophenyl)glutarate with immobilized CAL-B (Chirazyme L2, Roche) showed a loss of 30 % activity within 18 h of the reaction, which was assumed to be due to product inhibition.[9] The decrease in enantioselectivity in CAL-B catalyzed resolutions of secondary butanoates observed by

Lundhaug *et al.* was also suggested to be caused by inhibition of the enzyme by the enantiopure product alcohol.[132] Akeboshi and co-workers also report that the enantioselectivity gradually declined accompanying the progress of the hydrolysis of benzylprotected primary alcohols and that the alcohol product inhibited the hydrolysis rate of the faster reacting enantiomer.[49]

This phenomenon was first reported in the 1930´s when it was found that strychnine enhanced the human liver esterase catalyzed hydrolysis of methyl L-mandelate but not the D-isomer. These results also indicated an apparent allosteric binding of the enantiopure additive.[133,134]

Kinetic experiments may be used to reveal the type of inhibition. By inserting experimental data to the inverted Michaelis-Menten equation this gives straight line plots which can be extrapolated to yield characterizing constants of the enzyme. An example of a Lineweaver-Burk plot based on the inverted Michaelis-Menten equation is shown in Figure 1.13.[135]

$$V_0 = \frac{V_{max}[S]}{K_m + [S]}$$

$$\frac{1}{V_0} = \frac{K_m}{V_{max}[S]} + \frac{1}{V_{max}}$$

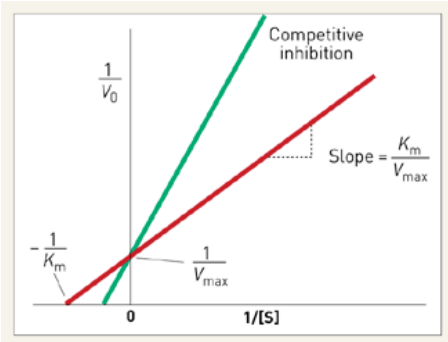


Figure 1.13 The Michalis-Menten equation where V_0 is the initial velocity of the reaction, [S] is the substrate concentration and K_m is the Michaelis constant. The inverted form of this equation is called the Lineweaver-Burk equation and the plot of 1/[S] *vs.* $1/V_0$ is called a Lineweaver-Burk plot. The slope of the line and the x- and y-intercepts determine the Michaelis constant K_m and the maximum velocity, V_{max}.[136]

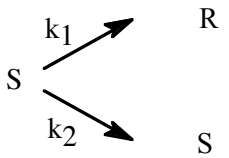
Kinetic studies of the inhibitory effect of 1-butanol in different solvents on lipase B from *Candida antarctica* have been performed, and the competitive inhibition constant (K_i) values obtained correlated with the calculated activity coefficients of the substrate, suggesting that desolvation of the alcohol was the changing condition.[137]

1.5.5 Different additives

Organic bases have been reported to increase the enantioselectivity of lipase-catalyzed reactions in water-saturated organic solvents.[138,139] It was reported that both activity and enantioselectivity of lipase B from *Candida antarctica* (CAL-B/Novozym 435) increased dramatically upon addition of triethylamine (Et_3N) to the water saturated organic reaction medium in a reaction between 2-phenyl-4-benzyloxazol-5(4*H*)-one and butan-1-ol. It was believed that the base was able to make an ion-pair with the acidic by-product thus avoiding the inhibitory effect of the acid by dissolving it and thereby removing it from the microenvironment of the enzyme.[140] Addition of Et_3N, crown ethers and tris-(3,6-dioxaheptyl)amine to Novozym SP 435 catalyzed transesterification of 1-azido-3-phenoxy-2-propanol have also been reported to enhance enantioselectivity and reaction rate.[141]

1.6 Asymmetric synthesis vs. kinetic resolution

An asymmetric synthesis is defined as a synthesis in which an achiral unit or compound is converted to a chiral unit in such a way that the stereoisomers are produced in different amounts. The aim is to achieve the highest possible proportion of the wanted enantiomer, *i.e.* the highest enantiomeric excess, *ee*. (Figure 1.14) There are two different types of achiral substrates which can undergo an asymmetric synthesis: 1) unsaturated compounds (stereoheterotopic) which can undergo addition reactions, and 2) substrates bearing enantiotopic atoms or groups which make the product chiral after conversion of these atoms or groups. The production of a new stereogenic unit in a compound always involves the influence of another chiral group ultimately derived from a naturally occurring compound.[142]



$$e.e. = \frac{\frac{k_1}{k_2} - 1}{\frac{k_1}{k_2} + 1} = \frac{R - S}{R + S}$$

Figure 1.14 A prochiral substrate S is converted to enantiomers P and Q at different reaction rates determined by the rate constants k_1 and k_2. The selectivity is dependent of the rate between k_1 and k_2.

In asymmetric synthesis a prochiral or meso-compound is used as the starting substrate. This gives a possibility of obtaining 100 % yield and 100 % *ee* of the product, depending on the selectivity and activity of the catalyst. In asymmetric synthesis the enantiomeric excess is independent of the degree of conversion, which means that the *ee* of the converted product is constant throughout the reaction process.

In a kinetic resolution the starting substrate is a racemic mixture. The maximum yield of each enantiomer is 50 % and the enantiomeric excess depends on the degree of conversion. Whether to perform an asymmetric synthesis or a kinetic resolution both catalyst, access to suitable substrates and costs must be considered.

1.7 Lipase catalysis

Lipases are classified as triacylglycerol acylhydrolases (E.C. 3.1.1.3) and in Nature they catalyze the hydrolysis of triglycerides to fatty acids and glycerol for use as energy source.

As opposed to other carboxylic ester hydrolases lipases do not follow Michaelis-Menten kinetics. When the substrate concentration is gradually increased the rate of hydrolysis gradually increases until the substrate concentration reaches a certain level. This specific concentration is called critical miscelle concentration (CMC), and the kinetic model is called ”interfacial activity”. It describes the fact that lipases are active only when they are in contact with the interphase between oil and water in a two phase system.[143,144]

An explanation for this phenomenon is that a ”lid” (an α-helix) covers the active site. When the enzyme comes in contact with the oil/water interphase this peptide chain folds back which induces an open entrance to the active site.[144] Hydrophobic substrates are more soluble in organic media than in water. In such cases the enzymes will not come in contact with any interphase and the α-helix will still cover the active site. Of that reason lipases show lower activity in organic media compared to aqueous solutions.[145] However, not all lipases are active due to ”interfacial activity”, f. inst. lipase B from *Candida antarctica* and lipase from *Pseudomonas aeruginosa*.[146]

Lipases from different fungi and bacteria are frequently used in industrial biocatalytic transformations.[147-150,35] Extracted from organisms or expressed in other organisms the purified enzymes can be used (free or often immobilized) in industrial processes as the detergent-, dairy- and food industry. Dow Chemical Company (San Diego) are resolving the racemic compound DOWANOL™ PMA (1-methoxy-2-propanol and -acetate) and several other glycol ether derivatives by lipase B from *Candida antarctica* resulting in high enantiomeric purity of both enantiomers, transforming the *(R)*-enantiomer in both hydrolysis and transesterification.[151] This work was an extension of the work performed on similar substrates.[152,153] Deprotection of *(S)*-1-methoxy-2-propanol gives *(S)*-1,2-propanediol which may be used in the synthesis of cardiovascular drugs such as *(S)*-Verapamil and *(S)*-Gallopamil,[154] and for synthesis of antiviral agents.[155]

1.7.1 Catalytic mechanism of lipases

Lipases show a catalytic mechanism similar to the serine proteases, in which one serine residue, one aspartate (or glutamate) residue and one histidine residue makes the ”catalytic triade”. (Figure 1.15) The arrangement of these three amino acids cause a decrease of the pK-value of the OH-substituent of serine. This will then act as a nucleophile against the carbonyl carbon, either in the substrate (chiral substrate A in hydrolyses) or in the acyl donor (substrate A in transesterifications). A tetrahedral transition intermediate is then formed, which in turn is attacked by an acyl acceptor (chiral substrate B in transesterifications, water (substrate B) in hydrolyses). A second

tetrahedral intermediate is formed, and finally the free enzyme is released together with the product (chiral ester Q in transesterifications, leaving acid Q in hydrolyses).[146,156]

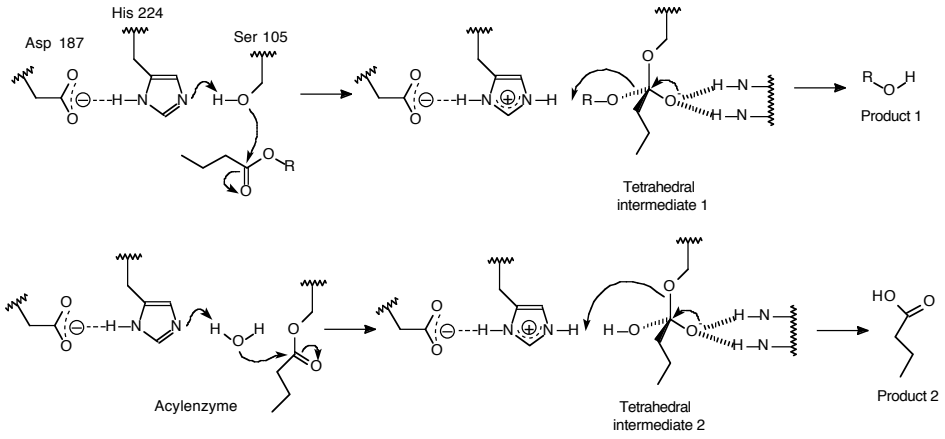


Figure 1.15 Serine hydrolase mechanism for hydrolysis of esters of secondary alcohols.[157]

1.7.2 Candida antarctica lipase B

The yeast *Candida antarctica* produces two different lipases: lipase A and lipase B (classified under lipases E.C.3.1.1.3) Both enzymes have been cloned and expressed in *Aspergillus oryzae*.[158,159] This yeast was first discovered in Antarctis after struggle to find enzymes with extreme properties. The two lipases are quite different. The A-lipase is calsium dependent and thermostable, while the B-lipase is less thermostable and not calsium dependent. The A-lipase is active against triglycerides, while the B-lipase is less active against triglycerides with long fatty acids. However, the B-lipase shows high activity against esters, amides og thioles. The polypeptide chain of the B-lipase is composed of 317 amino acids, has a mass of 33 kD and an isoelectric point (pI) of 6.0.[160]

The sequence of lipase B from *Candida antarctica* (CAL-B) has been determined by crystal structure determination of two crystal forms.[146] Molecular modelling has been performed on the basis of results from esterifications of 1-phenylethyl ethanol[118] and results from hydrolysis of butanoate of 1-(2-phenylethyl)ether and butanoate of 3-methoxy-1,2-propanediol.[46,156] *(R)*-3-Methoxy-2-(2-phenylethoxy)ethylpropyl

butanoate modelled into the acitve site of lipase B from *Candida antarctica* is shown in Figure 1.16.[156]

The primary structure of CAL-B deviates from other lipases and the consensus sequence that is found around the catalytically active serine in other lipases is not the same in CAL-B.

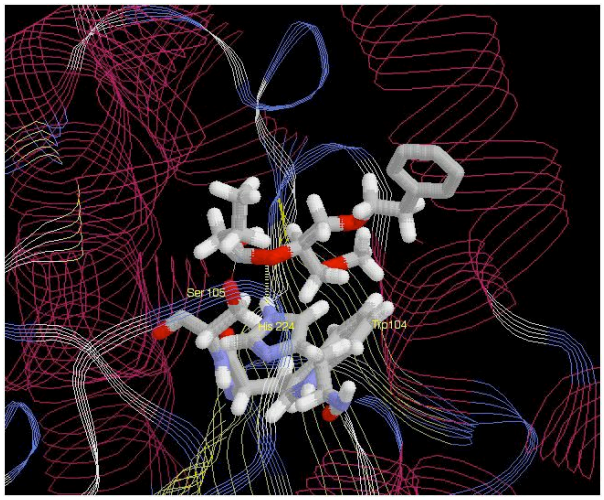


Figure 1.16 *(R)*-3-Methoxy-2-(2-phenylethoxy)ethylpropyl butanoate (the faster reacting enantiomer in the resolution of the racemic ester) modelled into the active site of lipase B from *Candida antarctica.*[156]

Studies of the CAL-B crystal structure show a small lid made up of a short □-helix (□5) above the active site entrance. Nevertheless, lipase B from *Candida antarctica* shows no ”interfacial activity”. For that reason the enzyme is considered as something between a lipase and an esterase. It is believed that the rather restricted entrance to the active site and the absence of the active site covering lid accounts for the high degree of enantioselectivity of this lipase.[146,156,161,144]

The size of CAL-B is ca 30 x 40 x 50 Å. As many other hydrolases the protein is folded in a so called □,□-hydrolasefold.[162] The optimal pH for an enzyme catalyzed reaction is generally 7, however, CAL-B is stable in aqueous media in a pH range of 3.5-9.5.

Immobilized CAL-B is also quite thermostable and can be used continiously in a temperature range of 60-80^{o}C without loss of activity.[163,164]

CAL-B, like other lipases, shows a catalytic mechanism somewhat similar to the serine proteases, with the Ser105-His224-Asp187 triade as the catalytic reaction centre. (Figure 1.15) The substrate can enter the active site via a narrow channel. The catalytically active serine is situated in the bottom of a channel which is approximately 10 x 4 Å wide and 12 Å deep measured from the serine oxygen to the surface. The channel walls consist of mostly aliphatic residues and are very hydrophobic. Trp104 is the only aromatic residue in the channel.[146] The active site consists of two pockets: one fitting the alcohol part and the other fitting the acyl part of the substrate.[156]

It is believed that CAL-B follows the multi substrate ping-pong bi-bi mechanism like other lipases.[165,55]

1.7.3 Substrate requirements of CAL-B catalyzed reactions

A model of the active site shows that the enzyme selectivity depends on substrate size[166] as well as electronegative effects of the small substituent, which is usually situated in the stereospecificity pocket.[167] The Trp104 residue in immobilized CAL-B has been mutated as to increase the size of the stereospecificity pocket. This resulted in lower activity and almost no enantioselectivity when control experiments were performed.[168]

In CAL-B catalysis of secondary alcohols it has been shown that the large substituent of the faster reacting enantiomer is situated outwards to the entrance of the active site and the small substituent is situated in the stereospecificity pocket.[169,166,118] Esterification of 3-octanol with S-ethyl thiooctanoate and CAL-B in hexane showed an *E*-value of 340.[166] In a transesterification reaction of 4-decanol with the same acyl donor and CAL-B it was observed that $E = 10$. In a similar reaction with 5-undecanol as substrate the *E*-value was found to be 3.[166] The two substituents attached to the stereocenter in the latter alcohol is quite similar and a low *E*-value would be expected.[64,65]

The *E*-value in transesterification reactions of different halohydrins (small substituent -CH_2Br or -CH_2Cl, see Figure 1.17) was lower than for the corresponding methyl substituted alcohols, despite the similar size of the bromo and methyl substituents.[170] The explanation may be that the electronegative effects in the bromo and chloro substituents interact with the functional groups of the amino acids in the stereospecificity pocket with a resulting repulsion of the halogens. However, high *E*-values have been observed in halohydrins where the bromo and chloro substituents appear as the large substituent and a methyl group is the small substituent.[167] Resolution of 3-chloro-1-(2-thienyl)-1-propanol catalyzed by CAL-B in hexane gave an *E*-value of 300.[171] Akeboshi and co-workers have studied kinetic resolutions of a series of primary alcohols with a branched small group catalyzed by CAL-B and report *E*-values from 1-37.[49]

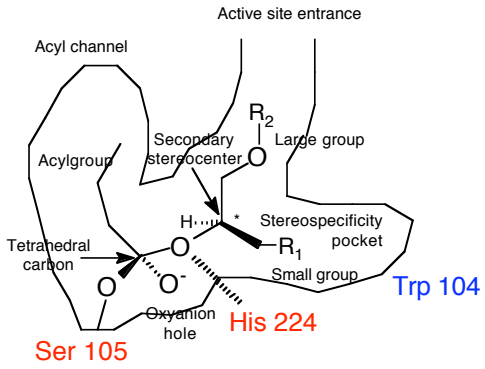


Figure 1.17 Model of the tetrahedral transition intermediate of the faster esterified enantiomer of a 1,2-diol derivative in active site of CAL-B.

It has also been observed that the *E*-values were quite different in CAL-B catalyzed resolutions of structurally related secondary alcohols/butanoates where the size of the large substituent was changed as follows: R_2 = phenoxy-, benzyloxy- or phenyletoxy- according to the substrate model in Figure 1.17.[46,116,153,172-174] In every experiment the benzyloxy compounds showed lower *E*-values than the respective phenoxy- og phenyletoxy compounds.

Plotting reaction time against concentration of each enantiomer at different degrees of conversion (see paragraph 1.8, Comparison of reaction progresses), showed that the slower reacting enantiomer in the benzyloxy compounds was reacting faster than the slower reacting enantiomer of the phenoxy compounds.[170,172] This was probably caused by a more favorable binding of the slower reacting enantiomer during catalysis due to more freedom in the rotation of the larger substituent.

1.8 Comparison of reaction progresses

When enzyme selectivity changes in kinetic resolutions of a compound as the reaction conditions change, this may be due to increased or decreased reaction rate of one of the enantiomers. It can also be caused by a change of reaction rate of both the enantiomers. The reaction rate of each of the enantiomers can be determined by calculating the concentration of the two enantiomers at different degrees of conversion from the equations below.[175]

$$c_{\cdot F} = c \text{ x } (1 + ee_p)$$

$$c_{\cdot S} = c \text{ x } (1 - ee_p)$$

$c_{\cdot F}$ and $c_{\cdot S}$ are the conversions of the faster and the slower reacting enantiomer (% converted of total concentration of substrate enantiomer), respectively, c is conversion of the racemic substrate and ee_p is enantiomeric excess of the product. The results are plotted in *Kaleidagraph 3.0* and the progress curves of different reactions can be compared. It was observed that the decrease in E-value from the CAL-B catalyzed resolution of 3-fluoro-1-phenoxy-2-propanol with vinyl butanoate (E = 490) compared to an equally performed resolution of 3-benzyloxy-1-fluoro-2-propanol (E = 17) was due to a higher reaction rate of the slower reacting enantiomer of 1-benzyloxy-3-fluoro-2-propanol.[170] (Figure 1.18)

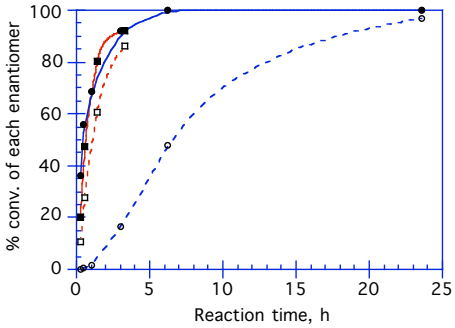
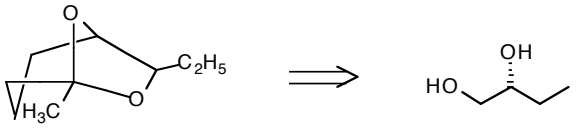


Figure 1.18 Conversion of each enantiomer plotted against reaction time from the resolutions of 3-fluoro-1-phenoxy-2-propanol (E = 490) and 1-benzyloxy-3-fluoro-2-propanol (E = 17) with CAL-B and vinyl butanoate. 3-Fluoro-1-phenoxy-2-propanol (circles), 3-benzyloxy-1-fluoro-2-propanol (squares). Filled symbols: faster reacting enantiomer, open symbols: slower reacting enantiomer.[170]

1.9 Industrial use of derivatives of 1,2-alkanediols

Enantiopure 1,2-alkanediols are widely used as synthetic building blocks for natural products, pharmaceuticals and fine chemicals.[154,155,176-178] Enzyme catalyzed synthesis of pure enantiomers of 1-phenoxy-2-butanol, 1-phenoxy-2-pentanol, 1-phenoxy-2-hexanol, 1-benzyloxy-2-butanol, 3-bromo-1-phenoxy-2-propanol and 3-chloro-1-phenoxy-2-propanol and corresponding butanoates (*(R)*- and *(S)*-**1a/b**-**6a/b**) have been one goal of the work presented here. Enantiopure halohydrins as *(R)*-3-chloro- and *(R)*-3-bromo-1-phenoxy-2-propanol substituted in the phenylring are synthons in the production of various □-blockers[179,180] and compounds with potential anti-viral activity.[177,181]

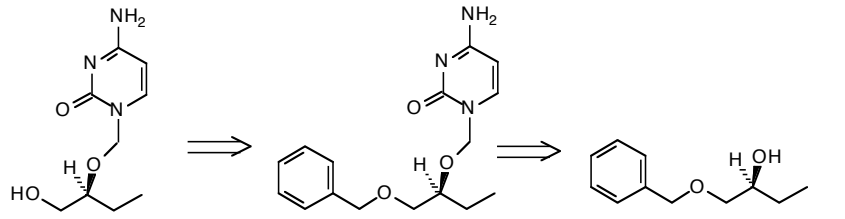
The active enantiomer of the pheromone brevicomin contains a 1,2-butanediol subunit with *R*-configuration which may be made from *(R)*-1-phenoxy-2-butanol (*(R)*-**1a**). (Scheme 1.3) (1*R*, 5*S*, 7*R*)-*exo*-Brevicomin is produced by females of the western pine beetle *Dendroctonus brevicomis,* as a male attractant. The chemical name of *exo*-brevicomin is (*1R*, *5S*, *7R*)-(+)-*exo*-7-ethyl-5-methyl-6,8-dioxabicyclo[3.2.1]octane, and it is only this enantiomer which is biologically active.[182]



exo-brevicomin *(R)*-1,2-butanediol

Scheme 1.3

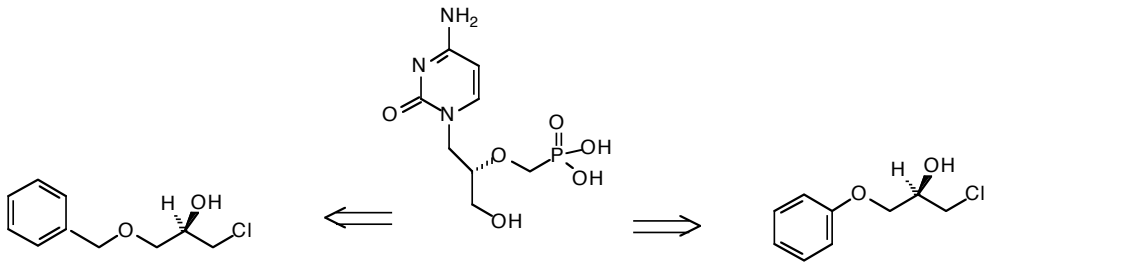
(S)-1-Benzyloxy-2-butanol (*(S)*-**4a**) is a potential building block in the synthesis of the compound 1-[(*(S)*-1-hydroxy-2-butoxy)methyl]cytosine which has been regarded as a potential anti-HIV drug. (Scheme 1.4) It has previously been synthesized by a multi-step synthetic route from *(S)*-*O*-benzylglycidol.[183]



1-[(*(S)*-1-hydroxy-2-butoxy)methyl]cytosine *(S)*-1-Benzyloxy-2-butanol, *(S)*-**4a**

Scheme 1.4

The anti-viral drug cidofovir (*S*-configuration) has previously been synthesized by a multi-step synthetic route starting from *(R)*-glycidol.[184,185] This compound may also be synthesized from *(R)*-1-benzyloxy-3-chloro-2-propanol, *(R)*-3-chloro-1-phenoxy-2-propanol (*(R)*-**6a**) or from *(R)*-1-benzyloxy-3-bromo-2-propanol. (Scheme 1.5)



(R)-1-benzyloxy-3-chloro-2-propanol Cidofovir *(R)*-1-phenoxy-3-chloro-2-propanol, (*R*)-**6a**

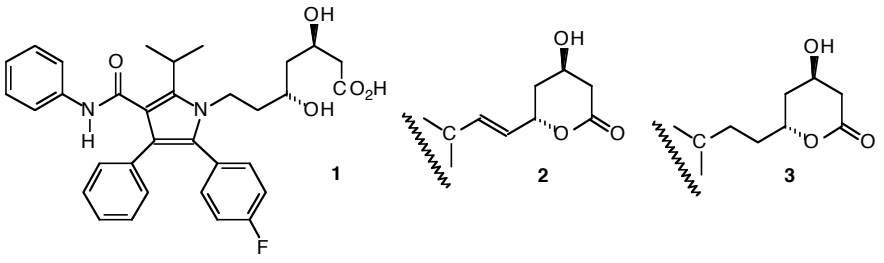
Scheme 1.5

The aryl group in the alcohols **1a-6a** protects the 1-position hydroxy group from acylation in enzyme catalyzed transformation and ensures the most effective organization of the enantiomers in the active site of the enzyme. One way to remove the phenyl group is with trimethyl phenylsilan and iodine.[186] The benzyl group can be removed by nucleophilic attack of thiophenolation made by *in situ* K_2CO_3-catalyzed synthesis from thiophenol.[187] Alternatively the benzyloxy group may be transformed to a hydroxy-function by palladium catalyzed hydrogenation.[76] From a synthetic view it is easier to remove the benzyl group than the phenyl group.

The separation of ester and alcohol enantiomers after enzyme catalyzed kinetic resolutions is usually performed by column chromatography. Sulfation of enantiomerically pure esters by sulfurtrioxide pyridine complex and subsequent extraction from the aqueous layer has been reported to enable effective isolation of the esters from the alcohols. This method would be especially advantageous in a large scale.[188]

1.10 Industrial use of enantiomerically pure derivatives of monoesters of glutarate

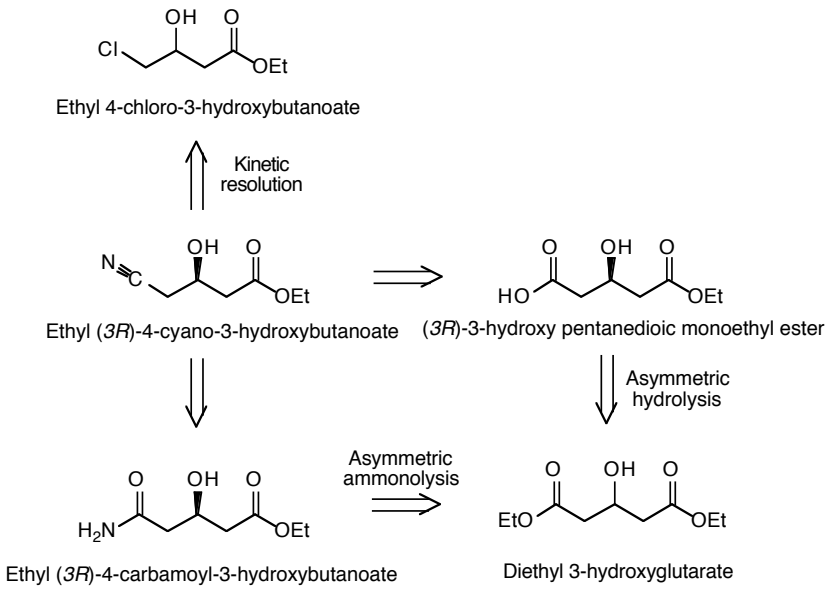
In the cholesterol-lowering drugs Lipitor (atorvastatin) (**1**), Crestor (rosuvastatin, approved by FDA August 2003) and Zocor (simvastatin) (**2** and **3**, respectively) the side-chain with *(R,R)*-configuration of the oxygen functions are quite similar.[27,189-191] (Scheme 1.6)



Scheme 1.6

There are several routes to obtain ethyl *(3R)*-4-cyano-3-hydroxybutanoate, the chiral starting material for these building blocks: By chemical modification of sodium erythronate (iso-vitamin C),[192] by enzymatic kinetic resolution of ethyl 4-chloro-3-hydroxybutanoate[193] or by enzyme catalyzed asymmetric synthesis from prochiral diesters of 3-hydroxyglutarate. (Scheme 1.7) The syntesis of ethyl *(3R)*-4-cyano-3-hydroxybutanoate from sodium erythronate involves harmful reagents as HBr and HCl, which are not regarded as "Green chemistry".

By enzymatic kinetic resolution of ethyl 4-chloro-3-hydroxybutanoate the highest *E*-value (E >100) was obtained for the *tert*-butyl ester with vinyl propanoate as acyl donor and lipase from *Rhizomucor miehei* as catalyst. However, the synthesis of the ester substrate was not trivial starting with diketene. Moreover, since the target nitrile is an ethyl ester a transesterification reaction would be necessary in order to convert the *tert*-butyl ester to the ethyl ester.



Scheme 1.7

Enzymatic conversion of diethyl 3-hydroxyglutarate and dimethyl 3-hydroxyglutarate is an environmentally friendly reaction and have been performed with use of different enzymes under different conditions. Most of the asymmetrizations resulted in the *(S)*-monoesters, apart from □-chymotrypsin which gave *(3R)*-3-hydroxy pentanedioic monoethyl ester.[194-200] In the asymmetrization of diethyl 3-acetoxyglutaric acid by □-chymotrypsin the *ee* of the monoester was analyzed on chiral HPLC and found to be 99 % with *R*-configuration. These and similar reactions have now been patented.[201]

One challenge in asymmetric synthesis of these prochiral glutaric diesters has been to determine the enantiomeric excess and the absolute configuration of the products. Previous workers have to a large extent relied on optical rotation for this purpose with well known problems and inaccuracies, especially due to the low optical rotation of the produced enantiomers. The high *ee*-value and configuration of the chymotrypsin catalyzed monomethyl and monoethyl esters[194] were based on comparison with a previously reported optical rotation value for the monomethyl ester produced by classical resolution via the cinchonidine salt.[202] This data have later been referred to by the scientists who have worked with enzymatic asymmetrizations of prochiral glutaric diesters.

Enantiopure *(3R)*-3-hydroxy pentanedioic monoethyl ester could be obtained from enzymatic hydrolysis. The monoester could in turn be converted to the nitrile ethyl *(3R)*-4-cyano-3-hydroxybutanoate using chlorosulphonylisocyanate.[203] The enantiopure product from the enzyme catalyzed ammonolysis of diethyl 3-hydroxyglutarate, ethyl *(3R)*-4-carbamoyl-3-hydroxybutanoate may in turn be converted to the target nitrile using 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide.[204] Production of the chiral side chain with two stereocenters starting from ethyl *(3R)*-4-cyano-3-hydroxybutanoate (one stereocenter) has been described by Brower *et al.*[205] It is preferable to introduce the chirality in a molecule in an early step in the synthesis. Introduction of the chiral side chain in the synthesis of atorvastatin is introduced in step 3. The molecule is then treated with acid and used as the calsium salt in the drug Lipitor.[206]

2 Summary of results

2.1 General

The results of related topics are sorted together. This may deviate from the order of publishing or submitting dates of papers and manuscripts.

Experimental data for enzymatic and non-enzymatic syntheses and spectroscopic data for synthesis products are described in the appended papers and manucripts 1-6.

When the substrate alcohol have been produced by more than one method, the alcohol from the most successful reaction was used in the enzymatic reactions. Only these methods were described experimentally in the papers.

The exact *E*-values obtained in the enzyme catalyzed resolutions of alcohols and esters are given in this chapter although the *E*-values above 200 are uncertain due to small conversions of the slower reacting enantiomer.

1-Phenoxy-2-butanol (**1a**) was resolved with three different concentrations of vinyl butanoate and with 5 times excess of the other acyl donors used, substrate **2a**-**6a** were resolved with 5 times excess of the four different acyl donors. The *E*-values of all the resolutions were determined from at least 3 measurements with standard deviations less than 4. Solvents used in the resolution reactions were dried over Union Carbide molecular sieves 4Å and the low water concentration was confirmed by the Karl Fischer method using a Metrohm 756 KF coulometer.

In determination of *E*-values by *E & K Calculator 2.03/2.1b0 PPC ee*-curves for the whole reaction progress were generated even though *ee*-values were not determined for the whole process. The generated curves are shown as they appear from the computer program.

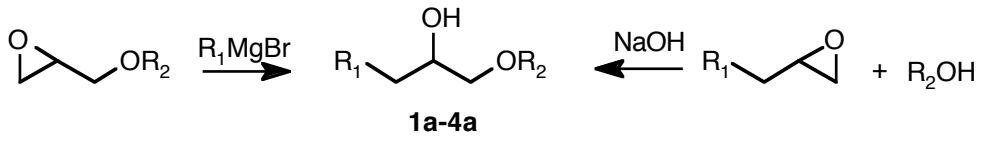
The *ee*-values determined from a resolution process with high *E*-value at conversions lower than 10 % and higher than 80 % are considered unreliable due to low conversions of one of the enantiomers. When *E*-values from those conversions are given for comparison this consideration must be taken into account.

2.2 Synthesis of substrates

2.2.1 Derivatives of 1,2-alkanediols

The secondary alcohols 1-phenoxy-2-butanol (**1a**),[207] 1-phenoxy-2-hexanol (**3a**),[208,209] and 1-benzyloxy-2-butanol (**4a**)[183] were synthesized by base catalyzed regioselective opening of the corresponding 1,2-epoxy alkanes with phenol and benzylalcohol as nucleophiles, respectively, and tetrabutylammonium hydrogen sulphate (TBAHS) as phase transfer catalyst.[210] (Scheme 2.1)

After purification by column chromatography of **1a**, **3a** and **4a** the purities were 97, 99 and 100 %, respectively (GLC). The yields were 35, 40 and 24 %, respectively, due to difficulties with separation of the produced regioisomers (5-10 %) from the products. The NMR data of **1a** and **3a** were consistent with the reported data of 1-phenoxy-2-butanol and 1-phenoxy-2-hexanol made from the corresponding ketones.[209] The alcohols **1a** and **2a** were also synthesized by a Grignard reaction with lithium tetrachloro cuprate as complexing agent[183] in 32 % yield (purity 96 %) and 48 % yield (purity 100 %), respectively. (Scheme 2.1)

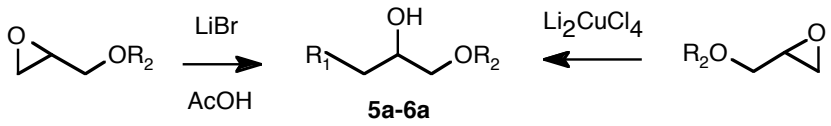


1a-4a

1a $R_1 = CH_3$, $R_2 = Ph$
2a $R_1 = CH_2CH_3$, $R_2 = Ph$
3a $R_1 = CH_2CH_2CH_3$, $R_2 = Ph$
4a $R_1 = CH_3$, $R_2 = CH_2Ph$

Scheme 2.1

Synthesis of 3-bromo-1-phenoxy-2-propanol (**5a**) was performed using phenyl glycidyl ether and acetic acid dissolved in dry THF with addition of dry LiBr.[211] After purification by flash chromatography the yield was 85 % and the purity 99 %. Synthesis of 3-chloro-1-phenoxy-2-propanol (**6a**) was performed from a solution of dilithium tetrachloro cuprate (Li_2CuCl_4) in dry THF which was added to a stirred solution of phenyl glycidyl ether dissolved in dry THF. (Scheme 2.2) After kugelrohr distillation (0.1 mm Hg, 94°C) the yield was 86 % with a purity of 99 %.

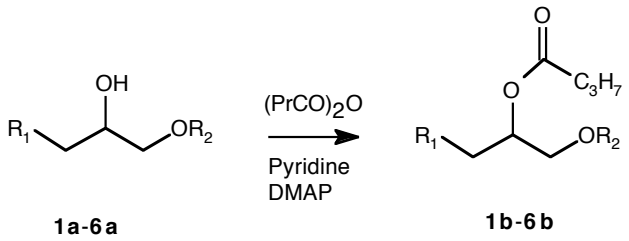


5a R_1 = Br, R_2 = Ph
6a R_1 = Cl, R_2 = Ph

Scheme 2.2

Recently it was reported that benzyl ethers were synthesized in high yield and purity by benzylation of alcohols with bis(acetylacetonato)copper ($Cu(acac)_2$) in an effective, fast and cheap way.[212]

The butanoates **1b-6b** were synthesized from the secondary alcohols **1a-6a** by standard conditions in 44, 93, 24, 16, 37 and 42 % yield and 97, 100, 98, 99, 99 and 98 % purity (GLC), respectively.[170,213] (Scheme 2.3)



Scheme 2.3

2.3 Synthesis of enantiopure reference compounds

2.3.1 (R)-Phenyl glycidyl ether

(R)-Phenyl glycidyl ether was synthesized in 55 % yield with 98 % purity (GLC) from *(S)*-epichlorohydrin and phenol.[210] Takano and co-workers suggested that this reaction took place with retention of configuration, however, Partali *et al.* have shown that the reaction proceeds with inversion of the configuration of *(S)*-epichlorohydrin.[173]

2.3.2 Synthesis of (R)-**1a**, (R)-**2a** and (R)-**3a**

Enantiopure reference compounds *(R)*-**1a**, *(R)*-**2a** and *(R)*-**3a** were synthesized by a Grignard reaction using *(R)*-phenyl glycidyl ether and methyl iodide, ethyl bromide, and propyl bromide, in 56, 43 and 65 % yield and 98, 95 and 100 % purity, respectively.[183] The enantiomeric excess (*ee*) of the purified enantiomers *(R)*-**1a**-**3a** were all > 99 % as determined by chiral GLC analysis.

Determination of absolute configurations

The absolute configurations of the faster reacting enantiomers from the biocatalytic resolutions were verified by comparison with *(R)*-**1a**-**3a** synthesized as described above. (Table 2.1) The properties of *(S)*-**4a** were compared with reported data.[183] Optical rotation of *(S)*-**5a** has been reported earlier.[170] The absolute configuration of **6** was not determined directly, but assigned by comparing relative retention times on chiral GLC supported by the known enantiopreference of CAL-B.

Table 2.1 The absolute configurations of the enantiopure alcohols and esters from the biocatalytic resolutions were determined by comparison of optical rotation with enantiopure reference compounds synthesized as described in paragraph 2.3. The optical rotation of **4a** was compared to reported data.[183] The optical rotation of **5a** has been determined earlier.[170]

Compound	*ee*, %	Concentration/solvent	Optical rotation
(R)-**1a**	96	c 1.37, $CHCl_3$	$[\alpha]_D^{25} = -6.57$
(R)-**1a** (ref.)	99	c 1.40, $CHCl_3$	$[\alpha]_D^{25} = -6.44$
(S)-**1a**	99	c 1.40, $CHCl_3$	$[\alpha]_D^{25} = +5.84$
(S)-**1b**	99	c 1.50, $CHCl_3$	$[\alpha]_D^{25} = -6.57$
(R)-**2a**	99	c 1.14, $CHCl_3$	$[\alpha]_D^{30} = -12.25$
(R)-**2a** (ref.)	99	c 1.17, $CHCl_3$	$[\alpha]_D^{20} = -6.86$
(R)-**3a** (ref.)	99	c 0.90, $CHCl_3$	$[\alpha]_D^{25} = -5.55$
(R)-**4a**	94	c 2.95, EtOH	$[\alpha]_D^{25} = +4.74$
(S)-**4a**	100	c 2.20, $CHCl_3$	$[\alpha]_D^{25} = +4.03$
(S)-**4a**	100	c 2.20, EtOH	$[\alpha]_D^{25} = -4.03$
(S)-**4a** (ref.)[183]	100	c 4.50, EtOH	$[\alpha]_D^{25} = -4.35$

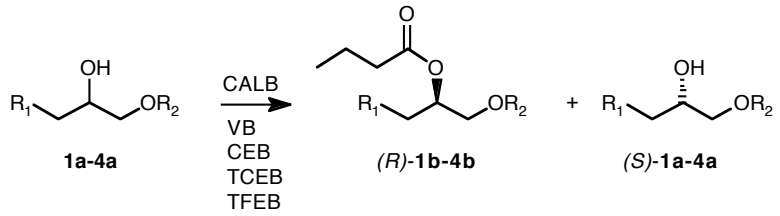
These observations showed that lipase B from *Candida antarctica* used in resolutions of **1a**/**b**-**6a**/**b** preferentially transformed the *(R)*-enantiomers of **1a/b-4a/b** and the *(S)*-enantiomers in the halohydrins **5a/b-6a/b**. This is in accordance with the selectivity preference rule formulated by Kazlauskas.[65]

2.4 Biocatalytic kinetic resolutions

*2.4.1 Significance of substrate structure and acyl donor of CAL-B resolutions (**Paper 1**)*

To gain a better understanding of the active site in lipase B of *Candida antarctica* esterifications of the secondary alcohols 1-phenoxy-2-butanol (**1a**), 1-phenoxy-2-pentanol (**2a**) and 1-phenoxy-2-hexanol (**3a**) were performed in hexane with the four different acyl donors vinyl butanoate (VB), 2,2,2-trichloroethyl butanoate (TCEB), 2,2,2-trifluoroethyl butanoate (TFEB) and 2-chloroethyl butanoate (CEB) with CAL-B as the catalyst. In these substrates the small group (cf. Kazlauskas´ substrate model)[65] increases from **1a**-**3a**.

Resolutions of 1-benzyloxy-2-butanol (**4a**, related to **1a**) were performed in order to reveal any influence of the large group on *E*. (Scheme 2.4)



1a-4a *(R)*-**1b-4b** *(S)*-**1a-4a**

1a $R_1 = CH_3$, $R_2 = Ph$
2a $R_1 = CH_2CH_3$, $R_2 = Ph$
3a $R_1 = CH_2CH_2CH_3$, $R_2 = Ph$
4a $R_1 = CH_3$, $R_2 = CH_2Ph$

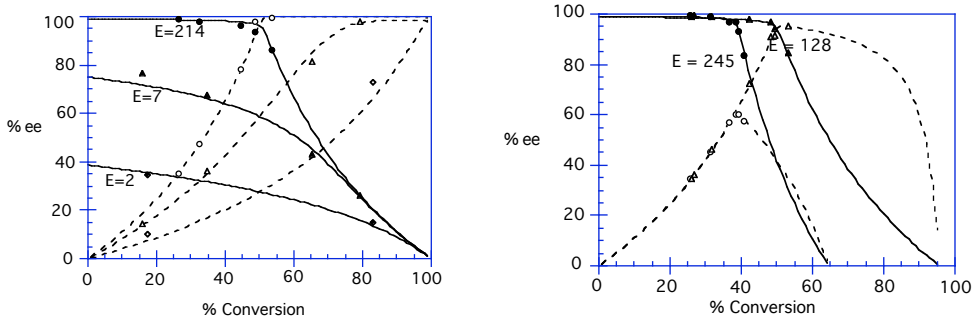
Scheme 2.4

When the substrate was changed from **1a** to **3a,** the enantiomeric ratio decreased with increasing size of R_1, which was expected from the known structure of the active site of CAL-B. Resolution of **4a** gave a significantly lower *E*-value compared to the resolution of **1a**. (See Figure 2.1 b) From this it is clear that the large group in the 1-position of the alcohol also affects the *E*-value. The *E*-value aslo changed by use of different acyl donors in the esterifications of the alcohols **1a**-**4a**. (Table 2.2)

Table 2.2 *E*-values change by size of the small substituent, the large substituent and by change of acyl donor in CAL-B catalyzed transesterifications of 1-phenoxy-2-butanol (**1a**), 1-phenoxy-2-pentanol (**2a**), 1-phenoxy-2-hexanol (**3a**) and 1-benzyloxy-2-butanol (**4a**). (Cf. Kazlauskas´s substrate model for secondary alcohols.[65]) The acyl donors were vinyl butanoate (VB), 2-chloroethyl butanoate (CEB), 2,2,2-trichloroethyl butanoate (TCEB) and 2,2,2-trifluoroethyl butanoate (TFEB).

Substrate	VB		CEB		TCEB		TFEB	
	E	K_{eq}	***E***	K_{eq}	***E***	K_{eq}	***E***	K_{eq}
1a	214	>10000	245	0.27	293	4.79	233	5.72
2a	7	>10000	21	0.63	31	6.35	40	>10000
3a	2	>10000	1.7	1.02	2.1	>1000	2.5	0.40
4a	13	>10000	84	0.41	106	6.59	128	3.33

The consequences for the *ee* of the produced *(R)*-butanoates and the remaining *(S)*-alcohols in the reactions of **1a**-**3a** with vinyl butanoate as acyl donor are shown in Figure 2.1 a. Both *(R)*-**1b** and *(S)*-**1a** are close to 100 % *ee* at 50 % conversion, while *(S)*-**2a** does not reach high *ee* until 75 % conversion, *i.e.* the yield will be low. Neither of the products from the resolution of **3a** could be isolated with high enantiopurity due to the low *E*-value of this reaction.



a) b)

Figure 2.1 a) Reaction plots of CAL-B catalyzed esterifications of **1a**, **2a** and **3a** with VB as acyl donor giving *E*-values of 214, 7 and 2, respectively. b) Reaction plots of the CAL-B catalyzed transesterifications of **1a** (CEB as acyl donor) and of **4a** (TFEB as acyl donor) giving *E*-values of 245 and 128, respectively. The *ee*-values for the product esters are plotted with filled markers, the *ee*´s of the remaining alcohols are shown as open symbols.

Lower enantioselectivities were observed in the resolutions of **4a** with all the four acyl donors compared to the selectivities of **1a** with the same acyl donors. Figure 2.1.b shows reaction plots of the CAL-B catalyzed transesterifications of **1a** with CEB as acyl donor and of **4a** with TFEB as acyl donor which gave *E*-values of 245 and 128, respectively. Figure 2.2 shows the conversion of each enantiomer from the resolutions of **1a** and **4a** with CAL-B and vinyl butanoate in hexane and it is clear that the slower reacting enantiomer of **4a** is reacting faster than the slower enantiomer of **1a** and the faster reacting enantiomer of **4a** is reacting slower than the faster reacting enantiomer of **1a**. It is possible that the benzyloxy group in **4a** makes the orientation of the two enantiomers quite equal due to a more favorable binding of the slower reacting

enantiomer during catalysis. This will cause a smaller difference in reaction rate between the enantiomers resulting in lower enantioselectivity. This effect has also been observed previously.[172,153,116,174,46,173]

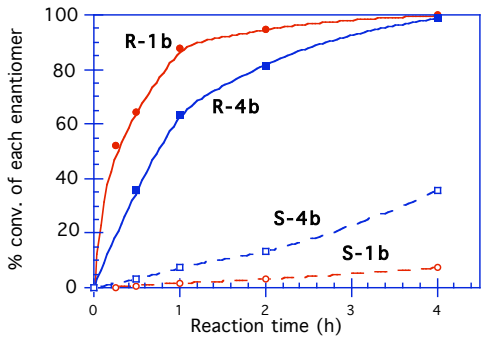


Figure 2.2 The percentage conversion of each enantiomer of **1a** and **4a** plotted against reaction time in CAL-B catalyzed resolutions with vinyl butanoate. The selectivities were E = 214 (**1a**) and E =13 (**4a**). The *ee*-curves for *(R)*-**1b** is shown as a full line with circles, *(S)*-**1b** is shown as a broken line with open circles, *(R)*-**4b** is shown as a full line with squares and *(S)*-**4b** is shown as a broken line with open squares.

Four different acyl donors were used in the transesterification reactions of **1a**-**4a**, all giving different E-values in resolutions of substrate **1a**, **2a** and **4a** although the reactions were resolved under similar conditions. (Table 2.2) These acyl donors all produce the same acyl enzyme which should not affect the individual reaction rates of the two enantiomers of the alcohols. However, this effect have been observed previously during CAL-B catalyzed resolution of similar substrates.[116,170]

The total esterification rates of **1a**-**4a** were also different with respect to the different acyl donors. The resolution of **1a** with VB reached 50 % conversion in 4 hours, in the reactions with TCEB, CEB and TFEB the reaction time to 50 % conversion were 8 h, 14 h and 48 h, respectively. Reaction time to 50 % conversion differed also in the reactions of **4a**, ranging from 4 hours with TCEB, 3 hours for the reaction with VB, 48 hours with TFEB and finally 4 days with CEB. In the resolutions of **3a** the reaction with

vinyl butanoate reached 25 % conversion after 1 hour while the reactions with the other three acyl donors needed from 2-15 days to reach 25 % conversion.

Transesterification reactions of **4a** with vinyl butanoate (VB), 2,2,2-trifluoroethyl butanoate (TFEB) and 2-chloroethyl butanoate (CEB) are shown in Figure 2.3. The resolution of **4a** with 2,2,2-trichloroethyl butanoate (TCEB) was similar. Vinyl butanoate is the only acyl donor giving an irreversible reaction. However, 2,2,2-trifluoroethyl butanoate is the best acyl donor for this reaction regarding enantioselectivity and thus the possibility to obtain high enantiopurity of both product and remaining substrate.

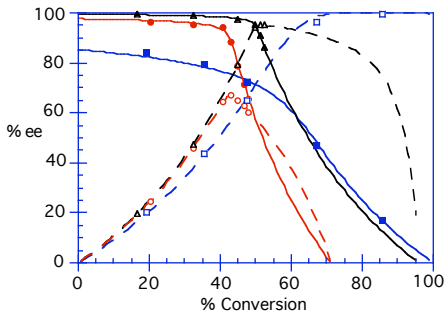


Figure 2.3 Transesterifications of **4a** catalyzed by CAL-B with three different acyl donors:, 2,2,2-trifluoroethyl butanoate (TFEB, triangles), 2-Chloroethyl butanoate (CEB, circles) and vinyl butanoate (VB, squares). Filled symbols represent ee_p-values (*(R)*-**4b**), while open symbols are the *ee*-values of the remaining substrate fraction *(S)*-**4a**.

From the reaction profile of the resolutions of **4a** with vinyl butanoate and 2-chloroethyl butanoate it is shown that the faster reacting enantiomer in the reaction with vinyl butanoate is reacting slower than the faster reacting enantiomer in the reaction with 2-chloroethyl butanoate. The same relative reaction rate is also seen for the slower reacting enantiomers in the two reactions. (Figure 2.4)

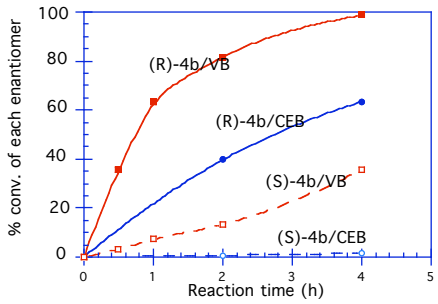


Figure 2.4 The percentage conversion of each enantiomer of **4a** plotted against reaction time in CAL-B catalyzed resolutions with vinyl butanoate (E = 13, squares) and 2-chloroethyl butanoate (E = 84, circles).

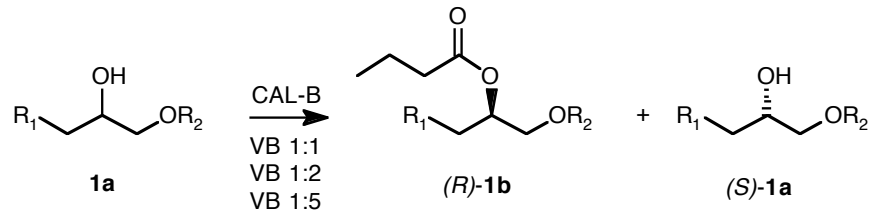
When comparing E-values from the resolutions of substrate **1a** (E = 214-293, depending on acyl donor) with previous results for 3-bromo-1-phenoxy-2-propanol (**5a**) (E = 37-58, depending on acyl donor),[170] it is possible to estimate the influence of electronegative effects on the E-value. These two substrates are of comparable size, but the electronegativity of the small substituents, $-CH_2R_1$, are different. The results indicate that these properties are important for the enantiomeric ratio.

As mentioned above, modelling reveals that the small group of the faster reacting enantiomer is located in the stereospecificity pocket and furthermore that a tryptophane residue (Trp104) limits the size of this pocket.[156] This residue is probably also responsible for unfavorable interactions with an electron rich R_1-group. Esterifications of 3-chloro-1-phenoxy-2-propanol (**6a**) with vinyl butanoate and CAL-B gave E-values of 11 (in hexane)[170] and 33 (in toluene),[214] respectively. However, resolution of 3-chloro-1-(2-thienyl)-1-propanol (similar small group as **6a**, different large group) catalyzed by CAL-B in hexane with vinyl butanoate gave an E-value of 300.[171] In spite of this, discrimination of enantiomers by CAL-B is not yet fully understood.

*2.4.2 Transesterification of **1a** with different concentrations of vinyl butanoate*

It has been observed that 0.6 and 1 equivalents of acyl donor to substrate concentration in transestrification reactions of secondary alcohols with CAL-B caused an enhancement of the enantioselectivity compared to 5 equivalents. It was shown that the

acyl donor itself caused the decrease in selectivity and not the liberated alcohols. However, the low acyl donor concentration caused unfavorable equilibrium positions.[116] To reveal the effect of acyl donor concentration in CAL-B resolutions of 1-phenoxy-2-butanol (**1a**) the alcohol was resolved with equimolar, 2 and 5 times excess of vinyl butanoate in hexane, respectively. (Scheme 2.5)



Scheme 2.5

The acyl donor concentration is set in the *E & K Calculator 2.03/2.1.b0 PPC* before minimization of the *ee*-values. It can be seen from Table 2.3 that the enantioselectivity of **1a** decreased by decreasing acyl donor concentrations, *i.e.* the opposite effect of what was reported by Hoff *et al*.[116]

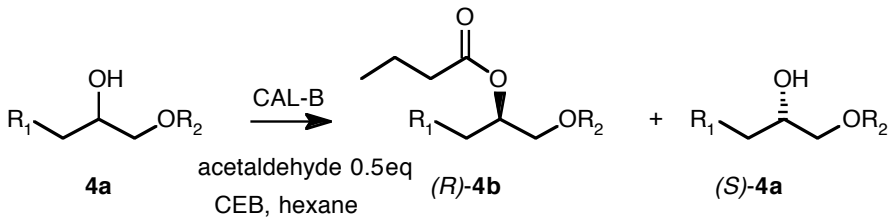
Table 2.3 *E*- and K_{eq}-values in the CAL-B catalyzed esterification of 1-phenoxy-2-butanol (**1a**) with different vinyl butanoate concentrations, hexane as the solvent.

1a:VB	*E*	K_{eq}	Time to 50 % conv.
1:5	214	> 10 000	4 h
1:2	132	9 900	2 h
1:1	89	9 800	3 h

However, an *E*-value of 132 from the reaction with 2 times acyl donor concentration is exellent, in addition the reaction time to 50 % conversion decreased from 4 h in the reaction with 5 times acyl donor concentration to 2 h in the reaction with 2 times acyl donor concentration.

2.4.3 *Effect of acetaldehyde on CAL-B enantioselectivity*

To reveal the possible influence of acetaldehyde on the *E*-value in CAL-B catalyzed resolutions of secondary alcohols with vinyl butanoate the following transesterification reactions were performed: 1-Benzyloxy-2-butanol (**4a**), 2-chloroethyl butanoate and CAL-B in hexane with i) addition of 0.5 eqv. of acetaldehyde (substrate:additive) and ii) with use of enzyme pre-treated with vinyl butanoate for 24 hours before washing with hexane. (Scheme 2.6) The water activity was adjusted to $a_w = 0.18$ by addition of equal amounts of different salt hydrate pairs. (See Table 2.6)



4a (*R*)-**4b** (*S*)-**4a**

4a $R_1 = CH_3$, $R_2 = CH_2Ph$

Scheme 2.6

The *E*-values in these reactions were from 5-8 % lower (*E* = 77 and 80, respectively) compared to the ordinary reaction of **4a** with CEB without addition of acetaldehyde (*E* = 84). (Table 2.4)

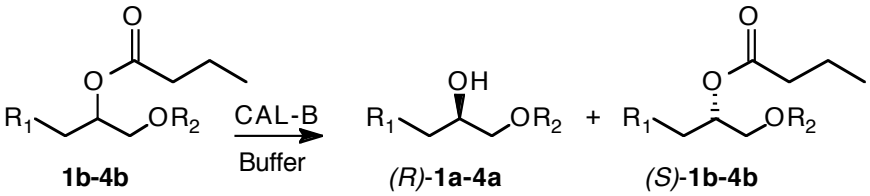
Table 2.4 CAL-B catalyzed resolutions of **4a** with VB and CEB and resolution of **4a** with CEB with i) addition of acetaldehyde and ii) pre-treated CAL-B with VB.

Acyl donor	a_w	Additive/treatment	*E*	K_{eq}
VB	□0	None	13	>10000
CEB	□0	None	84	0.41
CEB	0.18	Acetaldehyde 0.5 eqv.	77	0.35
CEB	0.18	VB 24 h	80	0.34

However, this deviation is not in the range of the decrease in *E*-value of the resolutions of **4a** when vinyl butanoate was used (*E* = 13) instead of 2-chloroethyl butanoate (*E* = 84), which gave a decrease in *E* of 86 %. It can be concluded that the formed acetaldehyde was not the reason for the low selectivity observed in the CAL-B catalyzed resolutions of secondary alcohols with vinyl butanoate as acyl donor.

*2.4.4 Hydrolysis of butanoates **1b**, **2b**, **3b** and **4b***

Hydrolysis of the butanoates **1b**-**4b** catalyzed by CAL-B were performed in phosphate buffer in a shaker incubator at 30°C. (Scheme 2.7 and Table 2.5) The *ee*-plots from the hydrolyses of **1b** and **2b** are shown in Figure 2.6.



1b $R_1 = CH_3$, $R_2 = Ph$
2b $R_1 = CH_2CH_3$, $R_2 = Ph$
3b $R_1 = CH_2CH_2CH_3$, $R_2 = Ph$
4b $R_1 = CH_3$, $R_2 = CH_2Ph$

Scheme 2.7

Table 2.5 Hydrolysis of butanoates **1b**-**4b** catalyzed by CAL-B in phosphate buffer.

Substrate	Phosphate buffer conc.	*E*	Conv. /rx. time
1b	0.05 M, pH 7.00	158	51 % / 24 h
2b	0.1 M, pH 7.00	326	46 % / 96 h
3b	0.1 M, pH 7.00	7	-
4b	0.1 M, pH 7.00	600	49 % / 8 h

The *E*-value from hydrolysis of the butanoate of 1-phenoxy-2-butanol (**1b**) was lower than the *E*-values obtained during the transesterifications of the corresponding alcohol **1a** (*E* = 214-293). This is the opposite observation compared to similar substrates where

the hydrolysis reactions showed much higher *E*-values than the esterifications of the corresponding alcohols.[215,216,213,217]

However, the hydrolysis of **1b** was performed in 0.05 M phosphate buffer while hydrolysis of **2b**-**4b** were performed in 0.1 M phosphate buffer. Since hydrolyses of **1b**-**4b** not were performed in a pH stat, the low buffer concentration in the hydrolysis of **1b** possibly caused an incomplete neutralization of the produced acid which could possibly inactivate the enzyme resulting in the lower *E*-value.

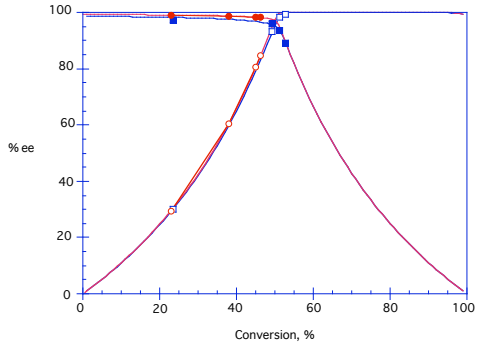


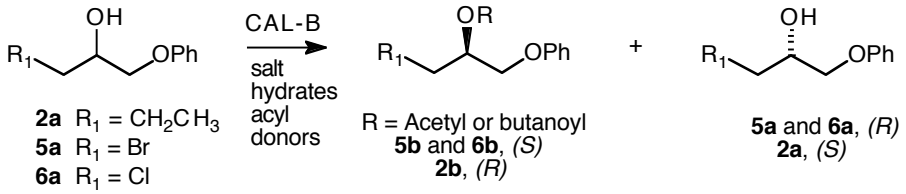
Figure 2.6 CAL-B catalyzed hydrolysis of **1b** (squares), and **2b** (circles) in phosphate buffer giving *E*-values of 158 and 326, respectively.

The hydrolysis of **2b** showed higher enantioselectivity (*E* = 326) compared to the transesterification reactions of the corresponding alcohol **2a** (*E* = 7-40, depending of acyl donor). The hydrolysis of **4b** also showed higher selectivity (*E* = 600) compared to the transesterification reactions of **4a** (*E* = 13-128, depending of acyl donor). Hydrolysis of **3b** resulted in an *E*-value of 7 after 3 weeks of reaction.

*2.4.5 Effect of water activity in solvents of different polarity on CAL-B selectivity (**Paper 2**)*

It has been reported that the water activity (a_w) influences the enantioselectivity depending on the lipase used.[102,218] In order to test the influence of water activity on the enantioselectivity and reaction rate obtained of CAL-B catalyzed resolutions, the following experiments were performed: i) Resolutions of **2a** in hexane with a_w = 0.18,

a_w = 0.39 and a_w = 0.65 with vinyl butanoate, 2-chloroethyl butanoate, 2,2,2-trichloroethyl butanoate and 2,2,2-trifluoroethyl butanoate, ii) transesterification reactions of **5a** with vinyl butanoate in 1,4-dioxan, acetonitrile, tetrahydrofuran, diethyl ether, hexane, carbontetrachloride, toluene and benzene with a_w = 0.18, a_w = 0.39 and a_w = 0.65 and iii) transesterification reactions of **6a** with vinyl butanoate and vinyl acetate in toluene with the three different water activities. All of the reactions were catalyzed by CAL-B Novozym 435. The water activity was adjusted with addition of equal amounts of the salt hydrate pairs listed in Table 2.6. (Scheme 2.8)



Scheme 2.8

Table 2.6 Pairs of salt hydrates for adjustment of water activity in the transesterification reactions of **2a**, **5a** and **6a**.[89,91]

Salt hydrate 1	Salt hydrate 2	a_w 30°C
Na_2HPO_4 x 2 H_2O	Na_2HPO_4 x 0 H_2O	0.18
$CaSO_4$ x 2 H_2O	$CaSO_4$ x 0.5 H_2O	0.39
Na_2HPO_4 x 7 H_2O	Na_2HPO_4 x 2 H_2O	0.65

In the transesterification reactions of **2a** with vinyl butanoate in hexane the *E*-value increased slightly by increasing water activity (*E* = 12-16). (Table 2.7) However, when the acyl donors 2-chloroethyl butanoate (CEB), 2,2,2-trichloroethyl butanoate (TCEB) and 2,2,2-trifluoroethyl butanoate (TFEB) were used, the *E*-value decreased (or the reaction stopped) when the water activity reached 0.65. From the esterification of **2a** with different acyl donors it is clear that the acyl donor also affected the rate of reaction in general. Esterification of **2a** with VB reacted much faster than **2a** with CEB and moreover, when **2a** was esterified with VB and CEB in hexane the reaction was faster

when a_w = 0.18 compared to the same reaction in hexane with a_w = 0.39. (Figure 2.7) The *E*-values in the resolutions of **6a** in toluene with vinyl acetate and vinyl butanoate also decreased by increasing water activity.

Table 2.7 *E*-values of the kinetic resolutions of **2a** catalyzed by CAL-B in hexane with different water activity esterified with 2-chloroethyl butanoate (CEB), 2,2,2-trichloroethyl butanoate (TCEB), 2,2,2-trifluoroethyl butanoate (TFEB), vinyl butanoate (VB) and **6a** esterified with vinyl butanoate (VB) and vinyl acetate (VA) with CAL-B in toluene with different a_w.

Solvent	Substrate	Acyl donor	a_w = 0.18	a_w = 0.39	a_w = 0.65
Hexane	**2a**	CEB	20	17	conv. <1 %
Hexane	**2a**	VB	12	15	16
Hexane	**2a**	TCEB	30	32	9
Hexane	**2a**	TFEB	23	19	conv. <1 %
Toluene	**6a**	VB	33	25	21
Toluene	**6a**	VA	13	n.d.	11

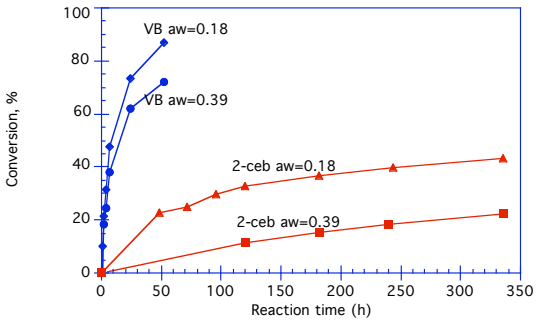


Figure 2.7 Esterification of **2a** with VB in hexane with a_w = 0.18 (diamonds) and a_w = 0.39 (circles) and CEB in hexane with a_w = 0.18 (triangles) and a_w = 0.39 (squares). It is clearly shown that the esterification of **2a** is much slower when CEB is used as acyl donor than when VB is used, both in the reaction with a_w = 0.18 and the reaction with a_w = 0.39.

In resolutions of **5a** the *E*-values increased with increasing a_w in all of the solvents. (Table 2.8) In the reactions of **5a** in 1,4-dioxan, acetonitrile and tetrahydrofuran a significant increase of *E* was observed when going from a_w = 0.18 to a_w = 0.39, however

the reactions with a_w = 0.39 stopped at 30 % conversion. When the a_w was adjusted to 0.65 the reactions did not exceed 1 % conversion. When water immiscible solvents were used, *E* increased moderately when a_w was increased from 0.18 to 0.65. The reactions were slow when a_w = 0.65, but did not stop until 40-50 % conversion was achieved.

Table 2.8 Esterifications of **5a** catalyzed by CAL-B with vinyl butanoate as acyl donor in media with controlled water activity.

Solvent	**Log P**	**a_w = 0.18**	**a_w = 0.39**	**a_w = 0.65**
1,4-Dioxan	-1.1	25	47	conv. <1%
Acetonitrile	-0.3	40	69	conv. <1%
THF	0.5	30	73	conv. <1%
Et_2O	0.9	23	24	conv. <1%
Benzene	2.0	22	31	48
Toluene	2.5	35	42	52
CCl_4	3.0	52	63	69
Hexane	3.5	16	25	55

CAL-B catalyzed resolutions of **5a** with vinyl butanoate in carbontetrachloride with a_w = 0.18 and a_w = 0.65 and in tetrahydrofuran with a_w = 0.18 and a_w = 0.39 are shown in Figure 2.8. It is clear that a a_w of 0.39 in THF has a more significant influence on the CAL-B selectivity in the resolution of **5a** than a a_w of 0.65 has on the enzyme selectivity of the same reaction in CCl_4. The reaction of **5a** with vinyl butanoate in THF with a_w = 0.39 stopped at 22 % conversion. (Figure 2.8)

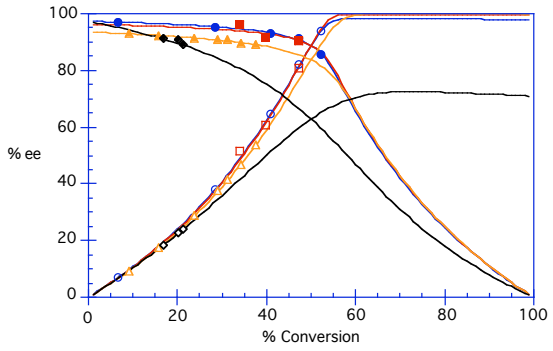


Figure 2.8 Esterifications of 3-bromo-1-phenoxy-2-propanol (**5a**) with vinyl butanoate in CCl_4 with a_w = 0.18 (circles) and a_w = 0.65 (squares) and in THF with a_w = 0.18 (triangles) and a_w = 0.39 (diamonds). Open symbols represent the *ee´*s of the substrates, filled symbols are the *ee´*s of the products. Esterification of **5a** did not take place in THF with a_w = 0.65.

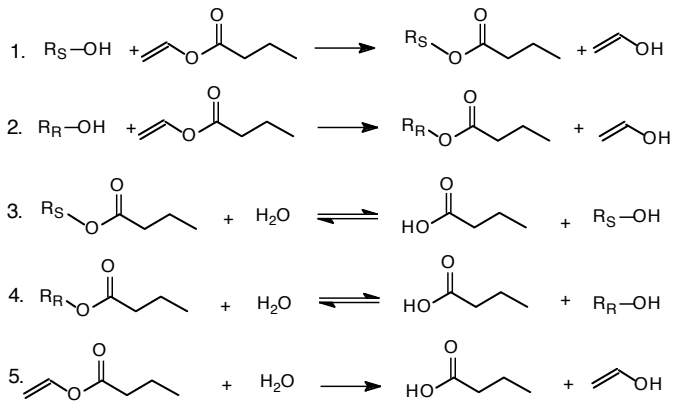
The water content can affect the enzyme catalyzed kinetic resolution by esterification in organic solvents either by influencing the *E*-value or by hydrolyzing esters present in the reaction media.[215] The reason for the different reaction rates in the esterifications of **5a** is probably that the water content at the same water activity is considerably higher in 1,4-dioxan, acetonitrile, tetrahydrofuran and diethyl ether compared to water immiscible solvents. A Karl Fischer coloumeter (Metrohm 756 KF) was used to determine the actual water content in the reaction media adjusted by salt hydrate pairs (listed in Table 2.6). (Table 2.9) The solvents (p.a. grade) were dried over molecular sieves, (Union Carbide 4Å, 48 h) and stored under N_2. THF was dried over Al_2O_3. This was regarded as the ”start water activity”, $a_w \approx 0$, when the salt hydrate pairs were added.

The water content in all of the solvents increased slightly from a_w = 0.18 to a_w = 0.39 and in polar solvent it increased significantly from a_w = 0.39 to a_w = 0.65. It is well known that polar solvents need higher water content to reach the same water activity as compared to apolar solvents,[219] which is actually determined in acetonitrile, 1,4-dioxan, tetrahydrofuran and diethyl ether. Water miscible solvents have the lowest log P values. Other solvent parameters were not considered in this case.

Table 2.9 Water content (□g water per g solvent) at different water activity in different solvents, as average values from 3 measurements at 30°C by a Karl Fischer 756 coloumeter.

Solvent	Log P	a_w=0.18	a_w=0.39	a_w=0.65	a_w □ 0
1,4-Dioxan	-1.1	360	433	5820	30
Acetonitrile	-0.3	1641	1862	12391	27
THF	0.5	882	904	13003	32
Et_2O	0.9	471	543	7980	14
Benzene	2.0	134	179	627	20
Toluene	2.5	138	142	510	12
CCl_4	3.0	45	55	84	2
Hexane	3.5	27	28	74	3

The increased selectivity due to increased water activity can be explained by the fact that the enzyme bind more water which may induce conformational changes or increased polarity of the active site which in turn may influence *E*. However, in a transesterification reaction of a racemic alcohol performed in different water containing organic solvent there are at least five equilibria to consider. When esterification of a racemic mixture of alcohols is performed by use of irreversible acyl donors, f. inst. vinyl butanoate, there are three irreversible esterification reactions (reaction 1, 2 and 5) and two reversible hydrolysis reactions (reactions 3 and 4). (Scheme 2.9)



Scheme 2.9

Water in the medium can hydrolyze the formed enantiomers at different rates. Thus the ester formed at a higher rate will also be hydrolyzed at a higher rate. Hence the *ee* of both the ester product and the remaining alcohol substrate will decrease. If the acyl donor is hydrolyzed (reaction 5), the esterifications (1 and 2) will be slowed down and will finally stop. This was actually observed when **5a** was esterified with vinyl butanoate in 1,4-dioxan, acetonitrile, tetrahydrofuran and diethyl ether with $a_w = 0.65$. (Table 2.8) Even after addition of 10 equivalents of vinyl butanoate in acetonitrile, the degree of conversion did not exceed 6 %. Moreover, formation of butanoic acid would take place (reactions 3, 4 and 5).

Esterifications of **5a** in carbontetrachloride with $a_w = 0.65$ and in tetrahydrofuran with $a_w = 0.39$ showed a great difference of the rate of the reactions, as seen in Figure 2.9. However, the relative reaction rates of the two enantiomers in these two media are almost equal. ($E = 69$ and $E = 73$, respectively).

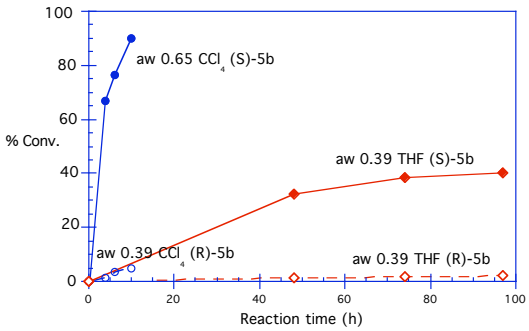


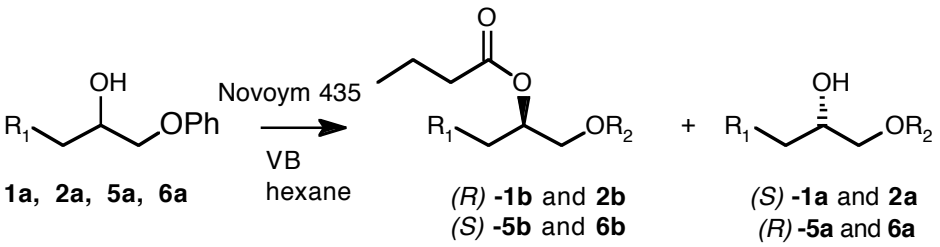
Figure 2.9 Esterifications of the two enantiomers of **5a** with vinyl butanoate (VB) in CCl_4 with $a_w = 0.65$ shows that the faster reacting enantiomer *(S)*-**5b** (filled circles) and the slower reacting enantiomer *(R)*-**5b** (open circles) both react quite fast compared to the same enantiomers in the similar esterification of **5a** performed in THF with $a_w = 0.39$. The faster reacting enantiomer *(S)*-**5b**: Filled diamonds. The slower reacting enantiomer *(R)*-**5b**: Open diamonds.

The best reaction conditions for the enantioselectivity of CAL-B for **2a**, **5a** and **6a** was apolar solvents with water activity from 0.39 to 0.65 and vinyl esters as acylating agents.

2.4.6 *The influence on CAL-B enantioselectivity by addition of enantiopure secondary alcohols (**Paper 3 and 4**)*

E-values from the CAL-B catalyzed resolution reactions have been calculated using *E & K Calculator 2.03/2.1.b0 PPC*.[59] Usually all experimental *ee*-values from a reaction are minimized in the same calculation giving an *E*-value for the total process. However, when calculating one experimental point at the time from a repetitive resolution of 1-phenoxy-2-butanol (**1a**) with Novozym 435 this showed a decrease in *E* by conversion. Increasing *E*-values by conversion in CAL-B catalyzed hydrolysis of secondary esters have earlier been observed in our group,[46] and in transesterifications of sulcatol with *Pseudomonas cepacia* the *E*-value increased in chlorinated solvents.[47]

It was interesting to see if the deviating *E*-value was consistent throughout the series of alcohol substrates, and new transesterification reactions of **1a**, **2a**, **5a** and **6a** (Scheme 2.10) and hydrolysis of their corresponding butanoates **1b**, **2b**, **5b** and **6b** catalyzed by Novozym 435 were performed. Calculation of the *E*-values were done by inserting one *ee*-value at a time in *E & K Calculator 2.03/2.1b0 PPC*.

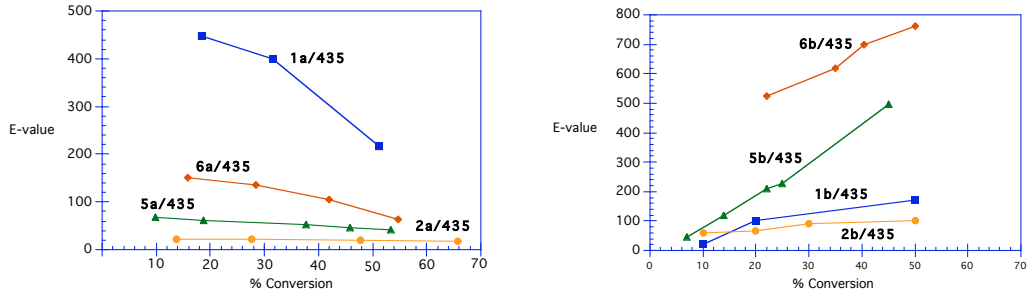


1a $R_1 = CH_3$
2a $R_1 = CH_2CH_3$
5a $R_1 = Br$
6a $R_1 = Cl$

Scheme 2.10

The *E*-values in the transeserification reactions decreased by conversion in resolutions of the alcohols **1a**, **2a**, **5a** and **6a**, however the decrease in *E*-value in the resolution of **2a** was small, *E* = 21-16. (Figure 2.10 a) Increasing *E*-value with conversion was

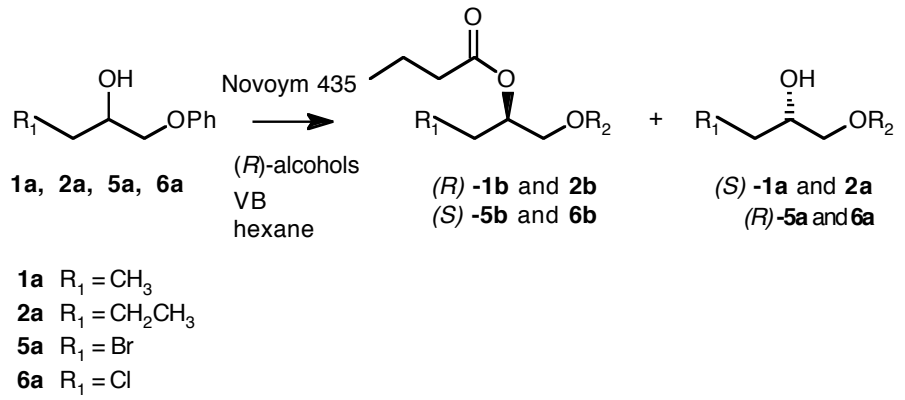
observed in the hydrolyses of the corresponding butanoates **1b**, **2b**, **5b** and **6b**. (Figure 2.10 b)



a) b)

Figure 2.10 a) *E*-values at different degrees of conversion in transesterification reactions with vinyl butanoate (VB) of **1a**, **2a**, **5a** and **6a** catalyzed by Novozym 435. b) *E*-values at different degrees of conversion in hydrolysis of **1b**, **2b**, **5b** and **6b** catalyzed by Novozym 435.

One hypothesis concerning these observations was that the effect was connected to the changing concentration of the enantiomeric esters. However, no effect on *E* was observed when enantiopure *(R)*-**1b**, the faster reacting enantiomer of the butanoate of 1-phenoxy-2-butanol, was added to the transesterification reaction of 1-phenoxy-2-pentanol (**2a**). A similar result was obtained when enantiopure *(R)*-**6b**, the slower reacting enantiomer of the butanoate of 3-chloro-1-phenoxy-2-propanol, was added to the transesterification reaction of 1-phenoxy-2-butanol (**1a**). It was concluded that the effect of the changing *E*-value was not connected to the changing concentration of the ester. In order to verify this 3-chloro-1-phenoxy-2-propanol, **6a**, (Scheme 2.11) was esterified with vinyl butanoate with addition of an enantiopure *(R)*-alcohol at approximately 30 % conversion. (Figure 2.11)



Scheme 2.11

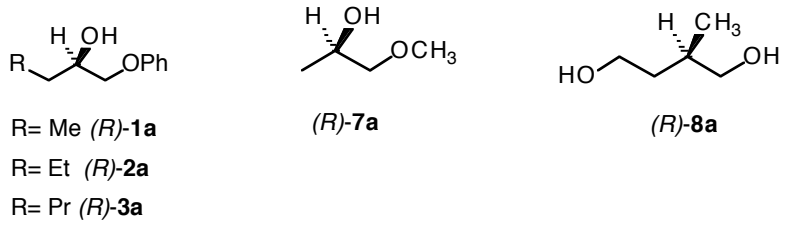


Figure 2.11 Enantiopure alcohols *(R)*-**1a**, *(R)*-**2a**, *(R)*-**3a**, *(R)*-**7a** and *(R)*-**8a** added to the transesterification reactions of **6a** at approx. 30 % conversion in order to reveal an effect of the additives on the CAL-B selectivity.

The reason for adding a different, but structurally related *(R)*-alcohol was that analysis and *E*-value calculations would be influenced by the added *(R)*-alcohol of the actual substrate. In the esterification of **6a** it was observed a decrease in *E* from 160 at 9 % conversion to 94 at 30 % conversion. (This decrease of *E* equals a difference in $\Delta\Delta G^{\#}$ of 0.37 kcal/mole, cf. Paragraph 1.4.2 and Figure 1.9) However, when *(R)*-**1a** was added to the transesterification reaction of **6a** at 30 % conversion the *E*-value increased from 94 to 205 before it decreased again. (Figure 2.12)

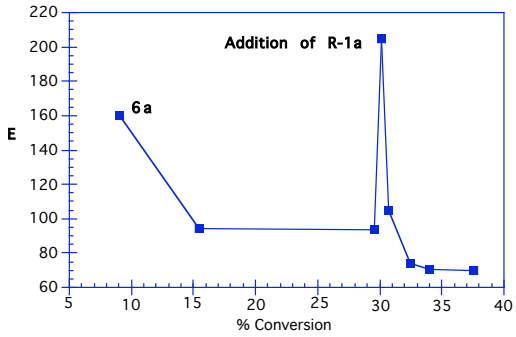


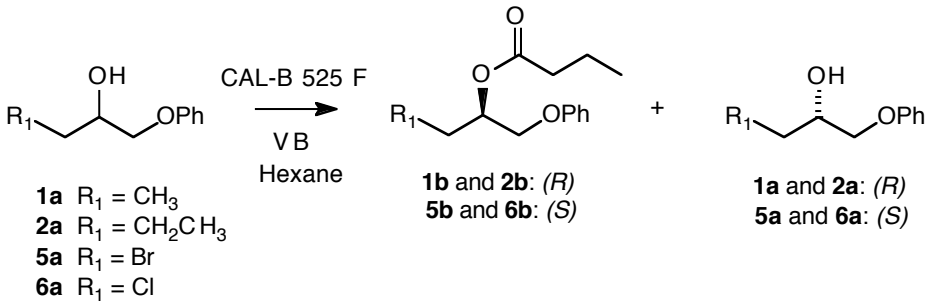
Figure 2.12 *E*-values at different degrees of conversion during transesterification of **6a**. *(R)*-**1a** was added at 30 % conversion and the *E*-value increased from 94 to 205 and then decreased again.

Esterifications of **6a** with addition of *(R)*-1-phenoxy-2-hexanol (*(R)*-**3a**) and *(R)*-1-methyl-1,2-butanediol (*(R)*-**8a**) showed similar results as visualized in Figure 2.12. Addition of 1-phenoxy-2-pentanol (*(R)*-**2a**) and *(R)*-phenyl ethanol to the resolution of **6a** showed similar results, however with smaller increase of *E*. Addition of *(R)*-1-methoxy-2-propanol (*(R)*-**7a**) did surprisingly not affect the selectivity.

It was also surprising that the *E*-value in the case of *(R)*-1-phenoxy-2-hexanol (*(R)*-**3a**) increased and also dropped to the low level quickly after addition. We have previously shown that **3a** is esterified by vinyl butanoate and CAL-B with almost no selectivity (Paper 1).[213] Of that reason it seems unlikely that the disappearance of the selectivity enhancement was only due to esterification of the alcohol. However, it is possible that the enantiopure alcohols after addition were bound to the enzyme, and after a while brought into the bulk solution by the solvent. The observed quick drop of effect after addition may therefore be a combination of removal of the *(R)*-alcohol by esterification or by solvation.

Since this effect was observed with the immobilized preparation of CAL-B (Novozym 435) it was interesting to perform the same reactions with pure protein preparations of CAL-B to see if the immobilization of the enzyme was the reason for the changing *E*-value. The formulated CAL-B Novozym 525 F is a water solution of the *Candida*

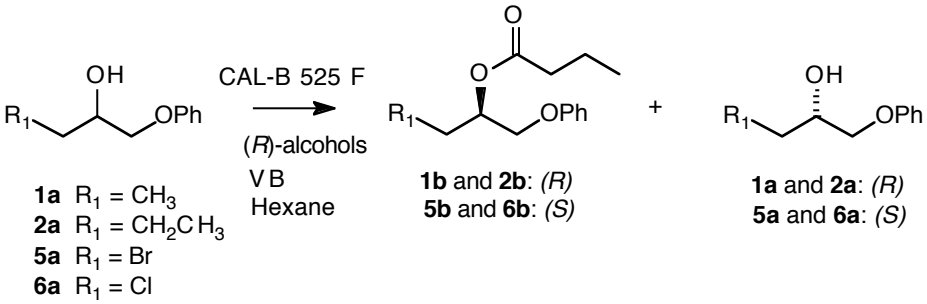
antarctica B lipase containing 1-10 % pure protein. After freeze drying at – 80°C this dry protein powder was used to catalyze the transesterification reactions of **1a**, **2a**, **5a** and **6a** with vinyl butanoate in hexane. (Scheme 2.12)



Scheme 2.12

The *E*-values in all of the reactions decreased by increasing conversion. Figure 2.13 shows the *E*-values from the esterification of **6a** with Novozym 525 F (**6a**/525 F) plotted together with the resolution of **6a** with Novozym 435 (**6a**/435). *E*-values from resolutions of **6a** with Novozym 525 F were quite similar to the *E*-values obtained from the resolution of **6a** with Novozym 435.

Subsequently resolutions of 3-chloro-1-phenoxy-2-propanol (**6a**) were performed with Novozym 525 F in hexane with vinyl butanoate and addition of the pure *(R)*-alcohols *(R)*-**1a**, *(R)*-**2a**, *(R)*-**3a,** *(R)*-**7a** and *(R)*-**8a** at approximately 30 % conversion. (Scheme 2.13) Figure 2.14 shows the reactions of **6a** catalyzed by Novozym 435 and Novozym 525 F with addition of *(R)*-**1a** to both reactions. The enantiopure alcohol shows a very clear selectivity enhancing effect suddenly after addition, however, the effect decreased after a short while also in these reactions.



Scheme 2.13

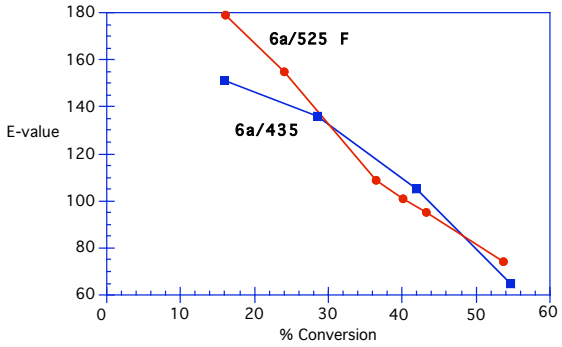


Figure 2.13 *E*-values from the transesterification reaction of **6a** with Novozym 435 (**6a**/435, circles) and **6a** with Novozym 525 F (**6a**/525 F, squares) in hexane with vinyl butanoate as acyl donor. The *E*-value decreased by conversion in both reactions.

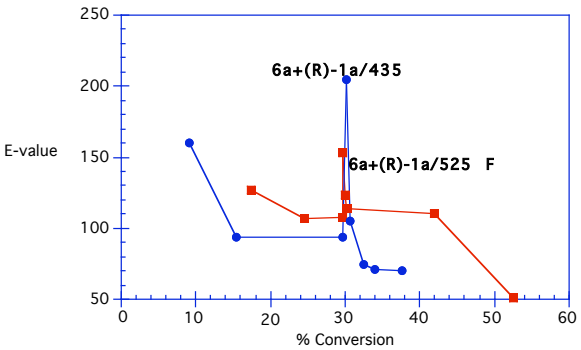


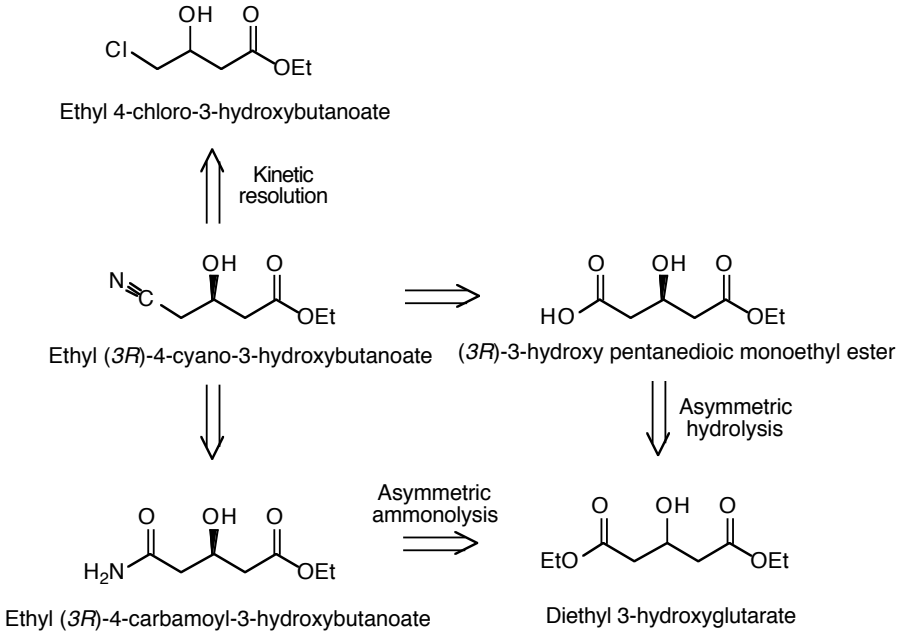
Figure 2.14 a) Resolutions of 3-chloro-1-phenoxy-2-propanol (**6a**) performed with Novozym 435 (**6a**/435, circles) and Novozym 525 F (**6a**/525 F, squares) in hexane with vinyl butanoate and addition of *(R)*-**1a** at 30 % conversion.

Since addition of the enantiopure *(R)*-alcohols increased the selectivity of CAL-B, it is likely that it is causing a conformational change in the enzyme, possibly due to an allosteric effect. However, an allosteric center is not mentioned in the structure details for CAL-B. Investigations in order to find the reason for this enantioselectivity enhancement effect in lipase B from *Candida antarctica* are in progress.

*2.5 Biocatalytic asymmetrization (**Paper 5 and 6**)*

2.5.1 Hydrolysis and ammonolysis of diethyl and dimethyl 3-hydroxyglutarate

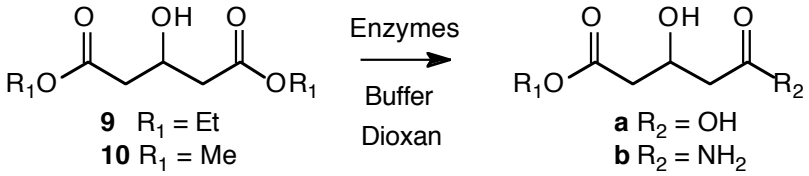
The primary goal of this project was to synthesize ethyl *(3R)*-4-cyano-3-hydroxy butanoate by enzymatic asymmetrization of diethyl 3-hydroxyglutarate. However, the pure enantiomers of 3-hydroxy pentanedioic monoethyl ester and 3-hydroxy pentanedioic monomethyl ester are also valuable synthons, f. inst. in the synthesis of pimaricin, compactin, L-carnitine and carbapenem.[194-200] Retrosynthesis is shown in Scheme 2.14.



Scheme 2.14

Hydrolysis of prochiral diethyl 3-hydroxyglutarate (**9**) and dimethyl 3-hydroxyglutarate (**10**) with lipase B from *Candida antarctica* (CAL-B Novozym 435) as catalyst gave the monoesters *(3S)*-3-hydroxy pentanedioic monoethyl ester (*(S)*-**9a**) and *(3S)*-3-hydroxy pentanedioic monomethyl ester (*(S)*-**10a**) with *ee*-values of 91 and 90 % respectively. (Scheme 2.15 and Table 2.11)

Ammonolysis of **9** and **10** with CAL-B as catalyst with ammonia in dioxan (Scheme 2.15) both showed an *ee* of the products ethyl and methyl *(3S)*-4-carbamoyl-3-hydroxybutanoate (*(S)*-**9b** and *(S)*-**10b**, respectively) of 98 %. Attempted ammonolysis of **9** and **10** using PLE or α-chymotrypsin were unsuccessful. Other enzymes were also used both in hydrolysis and ammonolysis, the results are shown in Table 2.11.



Scheme 2.15

Table 2.11 Enzymatic asymmetrization by hydrolysis and ammonolysis of diethyl 3-hydroxyglutarate (**9**) and dimethyl 3-hydroxyglutarate (**10**).

Prod.	Enzyme	Activity	% ee	% yield	$[\alpha]_D^{20}$	Conf.
9a	CAL-B	7 PLU/mg	91	80	+ 1.8 (c 11.5, acetone)	*(S)*
9a	CAL-A		91	77	+ 1.8 (c 11.5, acetone)	*(S)*
9a	CLEC-CAL-B	17 U/mg	86	80		*(S)*
9a	HLL		72	89		*(S)*
9a	RML	60 U/g	74	89		*(S)*
9a	PLE	15 U/mg	35	76	+ 0.2 (c 11.5, acetone)	*(S)*
9a	α-Chymotrypsin	70 U/mg	50	65		*(R)*
9a	*A. lwoffii*	(cell cult.)	56			*(S)*
10a	CAL-B	7 PLU/mg	90	70	+ 0.8 (c 11.5, acetone)	*(S)*
10a	PLE		22	75		*(S)*
10a	α-Chymotrypsin		45	59		*(R)*
10a	MCL	cell prep.	75	70		*(S)*
9b	CAL-B	7 PLU/mg	98	95	– 6.9 (c 10.0, dioxan) – 6.5 (c 1.3, $CHCl_3$)	*(S)*
10b	CAL-B	7 PLU/mg	98	95	− 2.0 (c 3.5, dioxan)	*(S)*

Lipases from *Rhizomucor miehei (RML)*, *Humicola lanuginosa (HLL) (Now: Thermomyces lanuginosa)* gave lower *ee´*s of **9a** than the reactions catalyzed by CAL-B Novozym 435. The reactions with these enzymes, and also with lipase A from *Candida antarctica* (CAL-A), were slower than the reactions with Novozym 435. Also the cross-linked CAL-B preparation from Altus Biologics Inc. (CLEC-CAL-B) gave lower *ee* of **9a** compared with Novozym 435 from Novozymes.

The *S*-configuration was predominant in all of the lipase asymmetrizations which is the opposite of the hydrolysis product of **9** and **10** catalyzed by α-chymotrypsin. A cell culture of *Acinetobacter lwoffii* (ATCC-17925, purchased from American Type Culture Collection) was also used in order to obtain the *R*-configuration of the hydrolysis product from **9**, however this enzyme system (an esterase)[198] also predominantly selected the pro-*R* enantioface of **9** giving *(S)*-**9a** in 56 % *ee*.

The asymmetrization of dimethyl 3-hydroxyglutarate (**10**) with a cell preparation of lipase from *Mucor circinelloides* (MCL)[220-222] resulted in an *ee* of *(S)*-**10a** (*(3S)*-3-hydroxy pentanedioic monomethyl ester) of 75 %. Attempts to obtain *(R)*-**9a** by hydrolysis of **9** with proteases from *Aspergillus oryzae*, *Streptomyces griseus, Bacillus polymyxa, Rhizopus* species, *Aspergillus sojae*, *Bacillus licheniformis* (Subtilisin Carlsberg), *Aspergillus saitoi* (Aspergillus acid protease) and Papaya showed no conversion after 3 days.

The immobilized catalyst CAL-B was filtered from the hydrolysis reaction mixtures and re-used for hydrolysis more than ten times with retention of high activity and enantioselectivity. (Figure 2.15)

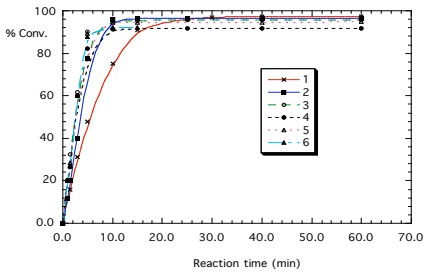


Figure 2.15 Reaction time plotted against conversion in six subsequent CAL-B catalyzed hydrolysis reactions of diethyl 3-hydroxyglutarate (**9**). The reaction time increased from reaction 1 to 6.

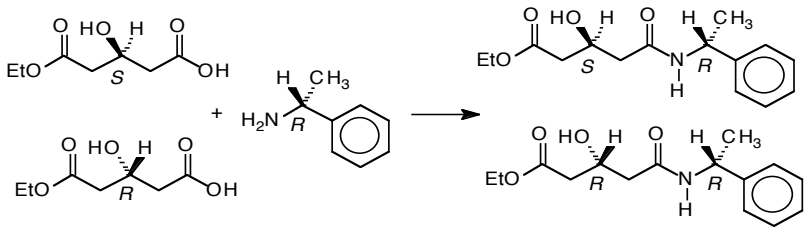
2.5.2 Determination of the configuration of (3S)-3-hydroxy pentanedioic acid monoethyl ester

Enzyme catalyzed asymmetrization of diethyl and dimethyl 3-hydroxyglutarate (**9** and **10**, respectively) or kinetic resolution of alkyl 4-chloro-3-hydroxybutanoate have been reported earlier.[10,193,196,198,199] The *(S)*-enantiomer of the monoesters has been the predominant product from most of the asymmetrizations.

Optical rotation determination and HPLC and NMR analyses for determination of the amount of the produced enantiomers from the different asymmetrization reactions have been reported.[194,199,223] However, the *(3S)*-3-hydroxy pentanedioic monoethyl- and

monomethyl esters show low optical rotation values which may lead to false results if used to determine absolute configurations and enantiomeric excess (*ee*) of the reaction products. For simplification of the *ee* determination of these monoesters a GLC separation method has been developed (Paper 5).[10] Several names have been used for the enantiopure monoesters from these asymmetrization reactions, however, *(3S)*-3-hydroxy pentanedioic monoethyl ester is the CAS name of *(S)*-**9a**.

The mixtures of enantiomers of amides **9b** and **10b** were separated as trifluoroacetic esters on the chiral column CP Chirasil-DEX G-TA with the temperature program 90°(1)-105°/1° (0), column pressure 6.0 psi and 60mL/min split flow. The monoesters **9a** and **10a** were converted to diastereomeric derivatives using *(R)*-α-phenylethylamine and analyzed at 253°C on an achiral DBWAX-N30 column. (Scheme 2.16)



Scheme 2.16

Figure 2.16 shows the separation of the diastereomeric derivatives of the enantiomers of **9a** on DBWAX-N30 from the asymmetrizations of **9** with α-chymotrypsin, lipase B from *Candida antarctica* (CAL-B) and pig liver esterase (PLE), respectively. The optical rotation values of the products were compared with the reported values in order to determine the configuration of **9a** and **10a**[194,223] and **10b**.[199]

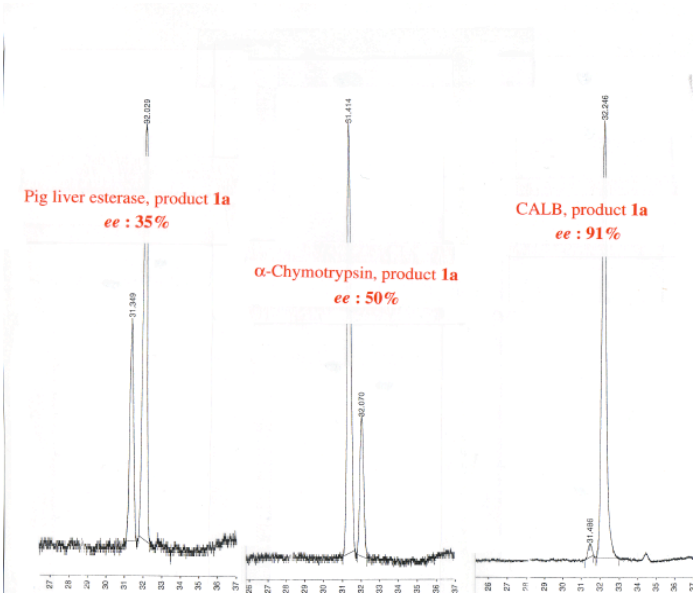


Figure 2.16 The reaction mixtures from asymmetrizations of **9** catalyzed by 1: α-Chymotrypsin, 2: lipase B from *Candida antarctica* and 3: pig liver esterase, derivatized with *(R)*-α-phenylethylamine separated on a DBWAX-N30 column. Asymmetrization of **9** giving **9a** is denoted **1a** on the figure.

The absolute configuration of *(3S)*-3-hydroxy pentanedioic monoethyl ester (*(S)*-**9a**) was in additon determined by X-ray crystallography (OSCAIL).[224] Salt crystals were formed in the reaction of equal amounts of *(S)*-**9a** (*ee* of 91 %) and *(R)*-phenylethylamine. The known configuration of the amine confirmed the configuration of (*S*)-**9a**. (Figure 2.17) We assume that the amides **9b** and **10b** also have the *(S)*-configuration.

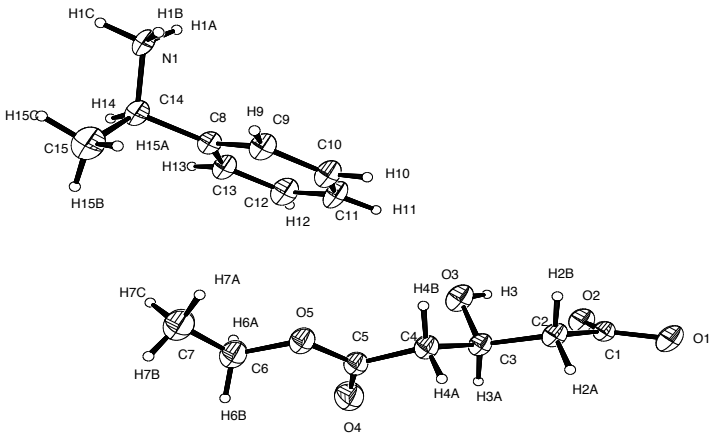
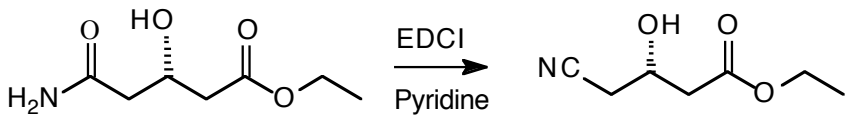


Figure 2.17 X-ray crystal structure of co-crystal of *(R)*-phenylethylamine and the monoethyl ester *(S)*-**9a** produced by hydrolysis catalyzed by CAL-B. The stereocenter of *(R)*-phenylethylamine is labeled C-14 and it is inferred that it has *(R)*-configuration. The carboxylate group of *(S)*-**9a** is labeled C-1 and O-1, O-2, the ethyl ester group is C-6, C-7. From the X-ray structure it is clear that the stereocenter at C-3 has *(S)*-configuration.

*2.5.3 Synthesis of ethyl (3S)-4-cyano-3-hydroxybutanoate, (S)-**11***

Ethyl *(3S)*-4-cyano-3-hydroxybutanoate (*(S)*-**11**) was produced from ethyl *(3S)*-4-carbamoyl-3-hydroxybutanoate (*(S)*-**9b**, 98 % *ee*) in dichloromethane with pyridine and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrogen chloride (EDCI) as a dehydration agent.[204] (Scheme 2.17) Inversion of the configuration of *(S)*-**9b**/*(S)*-**11** could be performed by several methods, f. inst. Mitsunobu esterification or via the mesylate ester.[225]



(S)-**9b** *(S)*-**11**

Scheme 2.17

The yield of *(S)*-**11** was 90 % and the *ee* was maintained at 98 % verified by chiral GLC analysis of the TFA derivative of *(S)*-**11** analyzed on a Varian 3400 gas chromatograph equipped with a chiral CP-Chiralsil Dex CB column (25m, 0.32mm i.d, 0.25 m film thickness). The temperature program was 90-95^{o}/0.2^{o}(0) with column pressure 7.5 psi and split flow 60 mL/min. The retention time for the *(R)*-enantiomer was 15.4 min, for the *(S)*-enantiomer it was 15.9 min. The resolution, R_S, was 1.6.

3 References

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Enhanced selectivity in Novozym 435 catalyzed kinetic resolution of secondary alcohols and butanoates caused by the (*R*)-alcohols

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Abstract—In esterifications of secondary alcohols catalyzed by immobilized lipase B from *Candida antarctica* (Novozym 435) the *E*-values decreased during the reaction. Hydrolysis of the corresponding butanoates showed the opposite effect. When an enantiopure (*R*)-alcohol, related but different, was added to the transesterification reaction, the *E*-value was significantly enhanced.

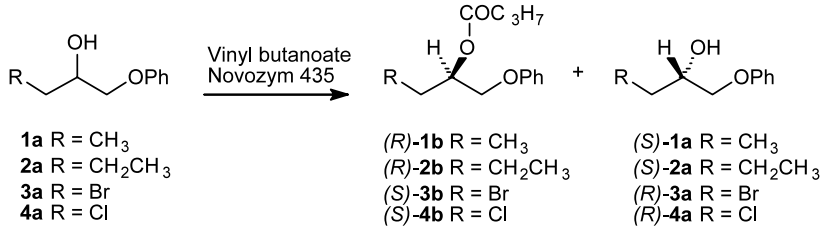
The enantiomeric ratio *E*, or often termed the enantioselectivity in kinetic resolutions, is the relative rate of reaction of the two enantiomers. It is related to the difference in free energy of activation $\Delta\Delta G^{\#}$ and to the ratio of the specificity constants k_{cat}/K_M for the enantiomers in enzyme catalyzed reactions.

$$\Delta\Delta G^{\#} = -RT\ln E \qquad E = \frac{\left(\frac{k_{cat}}{K_M}\right)_R}{\left(\frac{k_{cat}}{K_M}\right)_S}$$

The *E*-value can be calculated by measuring the enantiomeric excess of the product fraction ee_p, and the remaining substrate fraction ee_s, at a certain degree of conversion.[1,2]

$$+E = \frac{\ln\frac{[ee_p(1-ee_s)]}{(ee_p+ee_s)}}{\ln\frac{[ee_p(1+ee_s)]}{(ee_p+ee_s)}}$$

Provided that $\Delta\Delta G^{\#}$ is not influenced during the reaction, *E* will remain constant.[3] We have discovered that the *E*-value can increase by increasing degree of conversion in lipase catalyzed kinetic resolutions by hydrolysis of secondary esters. We suggested that the deviation from the theory might be due to change of the reaction medium during the reaction. The medium, taken in a wider sense, not only consists of the solvent, but also of the reactants and products present in the solvent. During lipase catalyzed hydrolysis the concentration of the



Scheme 1. Kinetic resolutions of (**1a**–**4a**) by transesterification of vinyl butanoate catalyzed by Novozym 435, lipase B from *Candida antarctica* (CAL-B). The faster reacting enantiomer is the same with respect to relative size of the groups at the stereocenter, however, the (*R*)- and (*S*)-notation changes when going from **1a** and **2a** to **3a** and **4a** and the corresponding esters.

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faster reacting enantiomer, usually the (*R*)-ester, is decreasing and the (*R*)-alcohol is formed and its concentration is increasing.[4,5] Consequently the medium is changing. The significance of the solvent for the *E*-value has been discussed.[6] Similar effects in esterifications catalyzed by *Pseudomonas cepacia* have been observed in chlorinated solvents.[7] Since each single product was shown not to cause the effect, it was suggested that complex interactions in the medium or on the enzyme between substrate and solvent and two or more products, improve enzyme selectivity. Inhibition of CAL-B by different primary alcohols has also been reported.[8] It has been reported that enantioselectivity increased when acetone was added to the medium when CAL-B was used to hydrolyse 3-chloro-1-phenylmethoxy-2-propyl butanoate. Addition of co-solvent solvates the liberated (*S*)-alcohol thus preventing the effect of inhibition.[9] It has been reported that strychnine enhances the hydrolysis of methyl L-mandelate catalyzed by human liver esterase and not the D-isomer. The results indicate an apparent allosteric activation.[10] Enantioselective inhibition of *Candida rugosa* (*cylindracea*) by dextromethorphan and levomethorphan has also been reported.[11]

We have now expanded the range of substrates in order to elucidate the nature of the effect of the changing *E*-value. Esterification of the secondary alcohols (**1a**–**4a**) (Scheme 1) and hydrolysis of their corresponding butanoates (**1b**–**4b**) catalyzed by Novozym 435, have been carried out. Synthesis, analysis and spectroscopic data of **1a**, **2a**, **1b** and **2b** have been described,[12] this also applies to **3a**, **4a**, **3b** and **4b**.[13]

E-values for each degree of conversion were determined using the method devised by Rakels et al.[2] The results for esterification are shown in Figure 1 and for hydrolysis in Figure 2. The esterifications of the alcohols **1a**–**4a** applying a transesterification reaction with vinyl butanoate as acyl donor all showed a decrease of selectivity ranging from 27 to 57%, when the reactions were monitored up to 65% conversion.

When the butanoates **1b**–**4b** were hydrolyzed in phosphate buffer up to 50% conversion, an increase of *E*-value ranging from 18% (**4b**) to 90% (**3b**) was observed (Fig. 2).

Our first hypothesis concerning these observations, that the *E*-value was decreasing during esterification and increasing during hydrolysis, was that the effect was connected to the changing concentration of the enantiomeric esters. When enantiopure (*R*)-**1b**, the faster reacting enantiomer, was added to the transesterification reaction of 1-phenoxy-2-pentanol (**2a**), no effect on *E* was observed. The same negative result was obtained when enantiopure (*R*)-**4b**, the slower reacting enantiomer, was added to the esterification of 1-phenoxy-2-butanol (**1a**). We therefore concluded that the effect of the changing *E*-value had to be connected to the changing concentration of the alcohol. In order to verify this we planned the following experiment: (a) monitor an esterification reaction of a racemic alcohol; (b) at a certain degree of conversion, add enantiopure (*R*)-alcohol, produced in a

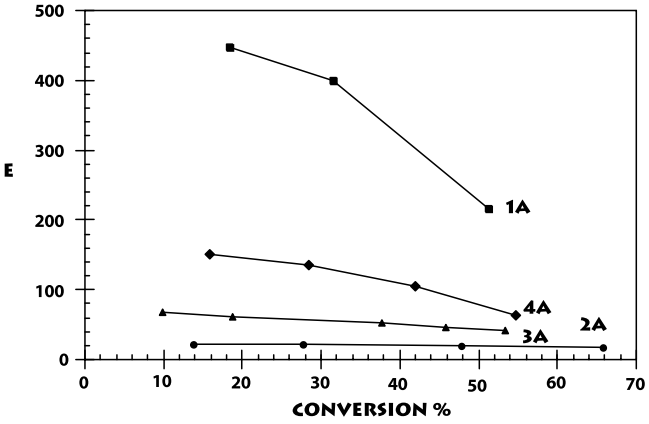

Figure 1. *E*-values at different degrees of conversion in esterifications of **1a**–**4a**.

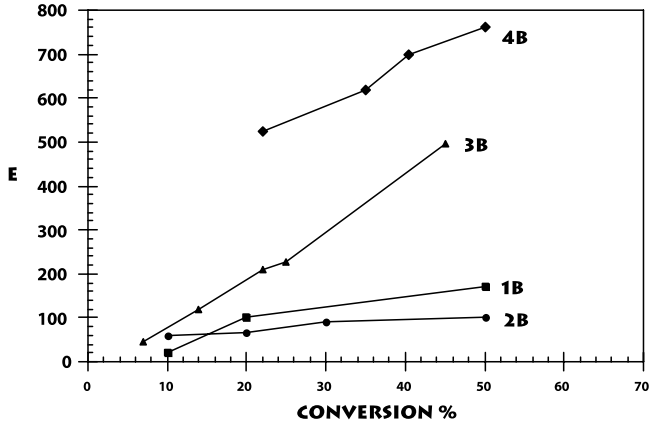

Figure 2. *E*-values at different degrees of conversion in hydrolysis of **1b**–**4b**.

different resolution reaction, and observe an increase of the *E*-value. However, analysis and *E*-value calculations would be influenced by the added (*R*)-alcohol and we therefore decided to add a different, but structurally related (*R*)-alcohol. We monitored carefully the esterification of **4a** and observed a decrease of *E* from 160 at 9% conversion to 94 at 30% conversion (Fig. 3). How-

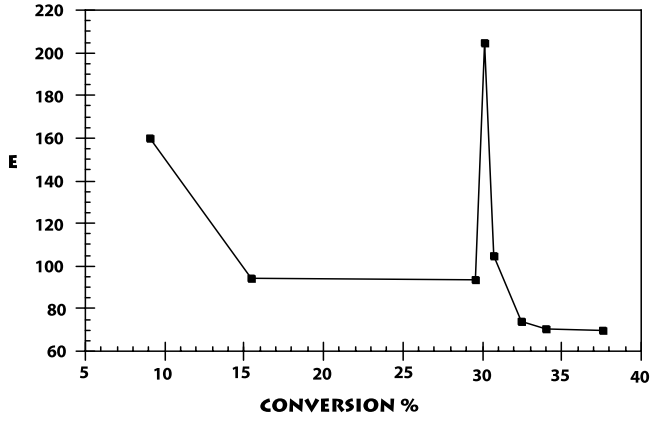

Figure 3. *E*-values at different degrees of conversion during transesterification of **4a**, (*R*)-**1a** was added at 30% conversion and the *E*-value increased from 94 to 205.

ever, when (*R*)-**1a** was added to the transesterification reaction at 30% conversion the *E*-value increased from 94 to 205 (Fig. 3). It is remarkable that the *E*-value dropped quickly.[14] We assumed that the reduction of the effect was due to (*R*)-**1a** being esterified under these conditions to give (*R*)-**1b**.

Addition of (*R*)-**2a** and (*R*)-phenyl ethanol gave similar results, however with smaller increase of *E*. In a final experiment we added (*R*)-1-phenoxy-2-hexanol as a possible selectivity enhancer. The results were almost identical to what is shown in Figure 3. However, it was surprising, that the *E*-value also in this case dropped to the low level quickly after addition. We have previously shown that 1-phenoxy-2-hexanol is esterified by vinyl butanoate and CAL-B very slowly and with almost no selectivity.[12] Hence, it seems unlikely that the disappearance of the selectivity enhancement, was due to esterification of the alcohol. It is more reasonable to believe that (*R*)-1-phenoxy-2-hexanol immediately after addition is bound to the enzyme, and after a short while brought into the bulk solution by the solvent. The observed quick drop of effect after addition may therefore be a combination of removal of (*R*)-alcohol by esterification or by solvation.

Since addition of the enantiopure (*R*)-alcohols increased the selectivity of CAL-B, it is likely that it is causing a conformational change in the enzyme, possibly due to an allosteric effect. Further studies towards the mechanism of this activation effect on various CAL-B preparations are in progress.

Acknowledgements

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14. 1-Phenoxy-2-pentanol (**2a**) (0.024 g, 1.31×10^{-4} mol), (*R*)-**1b** (0.125 g, 5.30×10^{-4} mol) and vinyl butanoate (0.075 g, 6.55×10^{-4} mol) were added to dry hexane (3 mL) and the reaction was started by addition of lipase (20 mg). 1-Phenoxy-2-butanol (**1a**) (0.0146 g, 8.79×10^{-5} mol), (*R*)-**4b** (0.010 g, 3.9×10^{-5} mol) and vinyl butanoate (0.050 g, 4.40×10^{-4} mol) were added to dry hexane (3 mL) and the reaction was started by addition of lipase (20 mg). The chloro alcohol **4a** (0.0243 g, 1.31×10^{-4} mol) and vinyl butanoate (0.075 g, 6.55×10^{-4} mol) were added to dry hexane (3 mL) and the reaction was started by addition of lipase (20 mg). When the reaction had reached 30% conversion, (*R*)-**1a**, (*R*)-**2a**, (*R*)-phenyl ethanol and (*R*)-1-phenoxy-2-hexanol ($3.9–6.5\times10^{-5}$ mol) was added.

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Immobilization does not influence the enantioselectivity of CAL-B catalyzed kinetic resolution of secondary alcohols

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Abstract—Decreasing enantioselectivity (*E*-value) by increasing conversion has been observed in transesterification reactions of secondary alcohols catalyzed by a pure protein formulation of lipase B from *Candida antarctica* (Novozym 525 F). Addition of a range of enantiopure alcohols caused a temporary increase in selectivity of the transesterification reaction of 3-chloro-1-phenoxy-2-propanol with vinyl butanoate. The corresponding immobilized lipase B, (Novozym 435) showed a similar relationship between the *E*-value and degree of conversion.

1. Introduction

Previously we have reported that the enantioselectivity (*E*) decreased during esterifications of a range of secondary alcohols **1**–**4** catalyzed by immobilized lipase B from *Candida antarctica* (Novozym 435) and that addition of enantiopure (*R*)-alcohols, (*R*)-**1**, (*R*)-**2**, (*R*)-**5**, (*R*)-**6** and (*R*)-**7**, induced an increase in the *E*-value of the esterification of 3-chloro-1-phenoxy-2-propanol **4**.[1] We suggested that the increase in enantioselectivity was caused by inhibition of the slower reacting enantiomer due to an allosteric binding of the enantiopure additive. Enantioselective inhibition of *Candida rugosa* (*cylindracea*) by dextromethorphan and levomethorphan resulting in an enhanced enantioselectivity has been reported.[2] Inhibition experiments revealed that the action of the base was non-competitive inhibition, that is, binding of the base to an allosteric site in the lipase. This caused inhibition of the transformation of one enantiomer leading to increased selectivity. Hydrolysis of 3-acetoxy nitriles catalyzed by lipase PS (*Pseudomonas* sp.) with the addition of L-methioninol showed an increasing hydrolysis rate of the (*R*)-enantiomer and a decreasing hydrolysis rate of the (*S*)-enantiomer. It was suggested that the substrate and L-methioninol were bound to the enzyme at different sites and consequently that conformational changes provided a change of reaction rates.[3] This observation was first reported in 1930 when it was found that strychnine enhanced the human liver esterase catalyzed hydrolysis of methyl L-mandelate but not the D-isomer. These results indicated an allosteric binding of the enantiopure additive.[4,5]

Recently it was reported that the changing *E*-value in esterifications of 4-methyloctanoic acid catalyzed by Novozym 435 was due to substrate sorption into the polymer matrix of the immobilized enzyme.[6] Hence it was interesting to investigate whether immobilization was the reason for the changing *E*-value in our experiments. In addition to the immobilized CAL-B, Novozym 435, we used lipase B from *C. antarctica*, Novozym 525 F that was not immobilized, for comparison.

2. Results and discussion

Esterifications of the alcohols 1-phenoxy-2-butanol **1**, 1-phenoxy-2-pentanol **2**, 3-bromo-1-phenoxy-2-propanol **3** and 3-chloro-1-phenoxy-2-propanol **4** catalyzed by a freeze dried pure preparation of lipase B from *C. antarctica*, Novozym 525 F, with vinyl butanoate as acyl donor, were performed in hexane. The predominantly formed esters and remaining unreacted alcohols are shown in Scheme 1.

As for the reactions with the immobilized CAL-B, Novozym 435[1] the Novozym 525 F that was not immobilized showed a decrease in *E* with conversion (Fig. 1).

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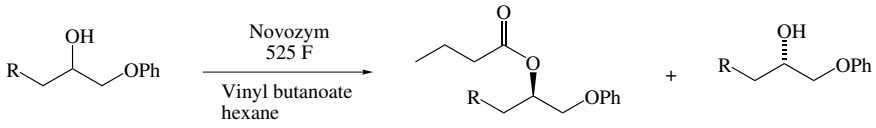


1 R = CH_3
2 R = CH_2CH_3
3 R = Br
4 R = Cl

(*R*)-1a and **(*R*)-2a**
(*S*)-3a and **(*S*)-4a**

(*S*)-1 and **(*S*)-2**
(*R*)-3 and **(*R*)-4**

Scheme 1.

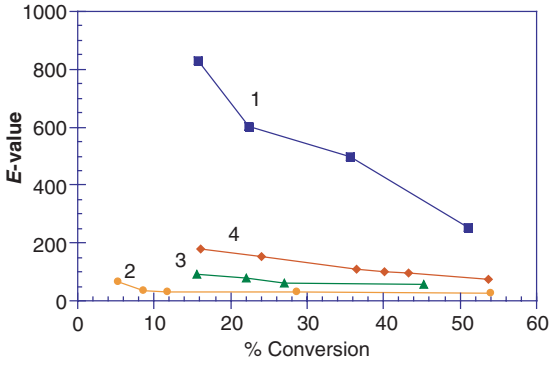


Figure 1. Decrease of enantioselectivity with conversion in the transesterification reactions of **1**–**4** with vinyl butanoate catalyzed by CAL-B Novozym 525 F. (■) = **1**, (●) = **2**, (▲) = **3**, (◆) = **4**.

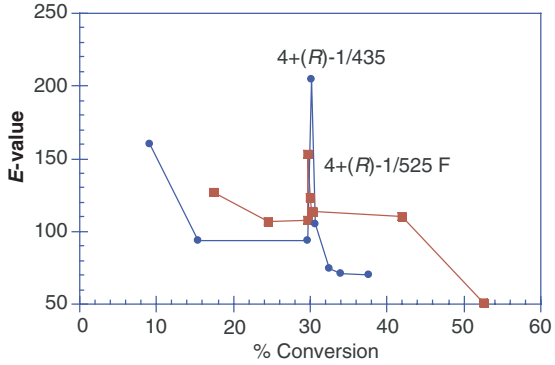


Figure 3. Transesterification of 3-chloro-1-phenoxy-2-propanol **4** catalyzed by CAL-B 435 (●) and CAL-B 525 F (■) in hexane with vinyl butanoate as acyl donor and with addition of (*R*)-**1** at 30% conversion.

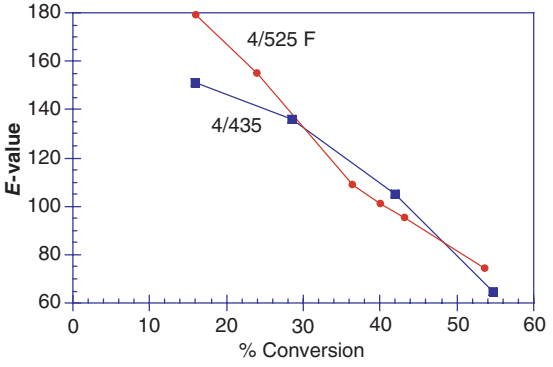


Figure 2. Comparison of the decreasing enantioselectivity (*E*-value) in the transesterification reactions of **4** catalyzed by CAL-B Novozym 525 F (●) and CAL-B Novozym 435 (■).

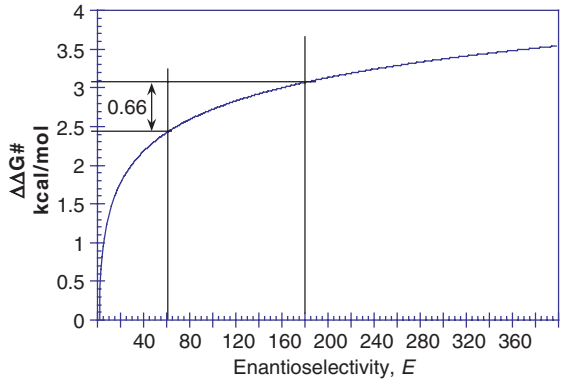
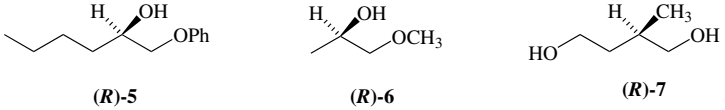


Figure 4. The relationship between the *E*-value and the difference in free energy of activation, $\Delta\Delta G^{\#}$, for the reaction with the two enantiomers ($\Delta\Delta G^{\#} = -RT\ln E$). The exemplified reaction is the transesterification of **4** catalyzed by CAL-B Novozym 525 F (see Fig. 2). The difference in the *E*-value of 180 at an early stage of the reaction (15% conversion) and at the end of the reaction ($E = 62$, 55% conversion) corresponds to the difference in free energy of activation of 0.66 kcal/mol.

The largest effect was observed with **1**, however, it is difficult to measure extremely large values of *E* accurately, hence we chose **4** as the candidate for comparative studies. The results of the esterification of **4** with the two different catalysts are shown in Figure 2. In fact, the graphs relating the *E*-values with conversion were virtually identical for the immobilized and the not immobilized preparations of CAL-B.

Moreover, when enantiopure (*R*)-**1** was added to the Novozym 525 F catalyzed esterification of **4** at 30% conversion, a similar burst of increase in *E*-value was observed (Fig. 3). Also when (*R*)-**2**, (*R*)-**5**, (*R*)-**6** and (*R*)-**7** were used as additives, similar results were obtained.

The drop in enantioselectivity in these reactions corresponds to a change in free energy of activation, $\Delta\Delta G^{\#}$,



(*R*)-5 **(*R*)-6** **(*R*)-7**

for the reaction with the two enantiomers of 0.66 kcal/mol (Fig. 4). The significance of this relatively small number is uncertain. However, it indicates that a small change in enzyme conformation may lead to considerable effects on the selectivity of the enzyme.

3. Conclusions

Resolutions of 1-phenoxy-2-butanol **1**, 1-phenoxy-2-propanol **2**, 3-bromo-1-phenoxy-2-propanol **3** and 3-chloro-1-phenoxy-2-propanol **4** catalyzed by Novozym CAL-B 435 and Novozym CAL-B 525 F both showed a significant decrease in *E*-values by increasing conversion. Addition of the (*R*)-alcohols (*R*)-**1**, (*R*)-**2**, (*R*)-**5**, (*R*)-**6** and (*R*)-**7** at 30% conversion to the resolution of **4** with both enzymes induced a temporary increase in the enantioselectivity of the reactions. It can be concluded that the decrease in *E*-value by increasing conversion in resolutions of **1**–**4** is not due to the immobilization preparation of the lipase B from *C. antarctica* as in Novozym 435 as reported by Heinzman et al. for the esterification of 4-methyloctanoic acid catalyzed by Novozym 435.

4. Experimental

4.1. General

Immobilized lipase B from *C. antarctica* (CAL-B Novozym 435) had an activity of 10 PLU/mg and a water content of 2% w/w. The pure enzyme preparation of lipase B from *C. antarctica* (CAL-B Novozym 525 F) was a water solution with 1–10% protein content. Both enzyme preparations were gifts from Novozymes, Bagsværd, Denmark. Chemicals were purchased from Fluka. Column and flash chromatography were performed using silica gel 60 from Fluka, with pore size 0.0663–0.2000 mm and 0.035–0.070 mm, respectively. Optical rotations were determined using an Optical Activity Ltd. AA-10 automatic polarimeter, concentrations are given in g/100 mL. Chiral analyses were performed using a Varian 3400 gas chromatograph equipped with CP-Chirasil-Dex CB columns from Chrompack (25 m, 0.25 or 0.32 mm i.d., 0.25 μm film density). For syntheses of racemic substrates with NMR data and chromatographic parameters of the resolution products see Refs. 7 and 8. Enantiomeric ratios, *E*, were calculated based on ping-pong bi-bi kinetics using the computer program E&K Calculator 2.1b0 PPC.[9]

4.2. Enzymatic reactions

4.2.1. Transesterification reactions. Substrates **1**–**4** (1.31×10^{-4} mol) and an acyl donor (6.55×10^{-4} mol) were added to hexane (3 mL). The reactions were started by the addition of Novozym 525 F (30 mg) and performed in an Infors shaker incubator at 30 °C. Chiral GLC analyses gave ee_s- and ee_p-values from which the degree of conversion was determined according to $c = ee_s/(ee_s + ee_p)$. In controlled experiments under the reaction conditions without an enzyme, no acylation was observed.

4.2.2. Transesterification reactions with the addition of enantiopure alcohols. Substrate **4** (1.31×10^{-4} mol), an acyl donor (6.55×10^{-4} mol) and Novozym 525 F (30 mg) were added to hexane (3 mL) and performed in the same way as the original reaction of **4** but with the addition of (*R*)-**1** (0.0099 g, 5.96×10^{-5} mol), (*R*)-**2** (0.0097 g, 5.38×10^{-5} mol), (*R*)-1-phenoxy-2-hexanol, (*R*)-**5**, (0.0045 g, 2.33×10^{-5} mol), (*R*)-1-methoxy-2-propanol, (*R*)-**6**, (0.0117 g, 1.30×10^{-4} mol) and (*R*)-2-methyl-1,4-butanediol, (*R*)-**7**, (0.0083 g, 7.97×10^{-5} mol) at approximately 30% conversion.

4.3. Synthesis of enantiopure alcohols

(*R*)-1-Phenoxy-2-butanol (*R*)-**1**: The butanoate of 1-phenoxy-2-butanol **1a**, (0.8691 g, 3.68 mmol) was hydrolyzed by addition of CAL-B Novozym 435 (0.105 g) in phosphate buffer (0.1 M, 183.5 mL). The enantiopure alcohol (*R*)-**1** was separated from the remaining butanoate on silica with acetone/hexane, 2:8, as eluent with a yield of 0.137 g (15.75%), purity 100% (GLC), and an ee of 96%, $[\alpha]_D^{25} = -6.6$ (*c* 1.369, $CHCl_3$).

(*R*)-1-Phenoxy-2-pentanol (*R*)-**2**: The butanoate of 1-phenoxy-2-pentanol **2a**, (1.47 g, 5.89 mmol) was hydrolyzed by addition of CAL-B Novozym 435 (0.20 g) in phosphate buffer (0.05 M, 100 mL). The enantiopure alcohol (*R*)-**2** was separated from the remaining butanoate on silica with acetone/hexane, 3:7, as eluent with a yield of 0.279 g (19%), purity 95% (GLC) and an ee of 99.3%, $[\alpha]_D^{30} = -12.25$ (*c* 1.142, $CHCl_3$).

(*R*)-1-Phenoxy-2-hexanol (*R*)-**5** was synthesized from (*R*)-phenyl glycidyl ether as described in Ref. 7. The yield was 0.630 g (65.5%) with a purity of 100% (GLC) and an ee higher than 99% $[\alpha]_D^{25} = -5.55$ (*c* 0.90, $CHCl_3$).

(*R*)-1-Methoxy-2-propanol (*R*)-**6** and (*R*)-2-methyl-1,4-butanediol (*R*)-**7** were purchased from Fluka.

4.4. Determination of absolute configurations

The absolute configurations of the faster reacting enantiomers of **1**–**3** were determined by comparisons of the specific rotation and of the retention times on GLC with (*R*)-**1**, (*R*)-**2** and (*S*)-**3** synthesized by a two-step procedure from (*R*)-phenyl glycidyl ether made from (*S*)-epichlorohydrin and phenol.[10,11] The synthesized enantiopure alcohols had the following properties: (*R*)-**1** ee >99%, $[\alpha]_D^{25} = -6.4$ (*c* 1.40, $CHCl_3$), (*R*)-**2**: ee >99%, $[\alpha]_D^{20} = -6.9$ (*c* 1.17, $CHCl_3$) and (*S*)-**3** ee = 96%, $[\alpha]_D^{22} = +5.3$ (*c* 1.71, EtOH). The absolute configuration of **4** was not determined directly, but assigned by comparing relative retention times on chiral GLC supported by the known enantiopreference of CAL-B.

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Enantioselective enzymatic preparation of chiral glutaric monocarboxylic acids and amides

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Abstract

Enantioselective hydrolyses and ammonolyses of diethyl-3-hydroxyglutarate (**1**) and dimethyl-3-hydroxyglutarate (**2**) gave a maximum of 91 and 98% enantiomeric excess (ee), respectively, using immobilized lipase B from *Candida antarctica*. The ees were determined using chiral GLC of the monoamides and achiral GLC of diasteromeric derivatives of the monocarboxylic acids. The catalyst was re-used more than 10 times with retention of high activity and selectivity.

Keywords: Monoamides; Monocarboxylic acids; Enantioselective hydrolyses; Ammonolysis; Chiral GLC

1. Introduction

Enantiopure ethyl- and methyl-3-hydroxyglutaric monocarboxylic acids are precursors for synthesis of molecules of biological interest such as pimaricin, L-carnitine, carbapenem and compactin. These chiral building blocks have been obtained by hydrolysis of prochiral diethyl- and dimethyl-3-hydroxy glutarates catalyzed by various proteases and esterases [1–7].

2. Results and discussion

Hydrolysis of prochiral diethyl-3-hydroxyglutarate (**1**) and dimethyl-3-hydroxyglutarate (**2**) with immobilized lipase B from *Candida antarctica*, CALB, Novozyme 435, as catalyst gave **1a** and **2a** with enantiomeric excess, ee, of 91 and 90%, respectively (Scheme 1 and Table 1). The (*S*)-configuration was predominant which is the opposite of the hydrolysis product of **1** and **2** catalyzed by α-chymotrypsin. Other enzymes gave lower ee's and slower reactions or no reaction (LPS). The immobilized catalyst CALB has been re-used more than 10 times for hydrolysis with retention of high activity and selectivity. Ammonolysis of **1** with CALB as catalyst showed an ee of the product ethyl (*S*)-4-carbamoyl-3-hydroxybutanoate (**1b**) of 98%. Attempted ammonolysis of **1** and **2** using PLE or α-chymotrypsin were unsuccessful.

During our work with enzyme catalysis, efficient and accurate chiral analyses have been crucial [8]. Also, in the present work, we have used GLC to determine ee. Previous workers have, to a large extent, relied on optical rotation for this purpose with well-known problems and inaccuracies [9–11]. We have developed GLC methods that are simple and

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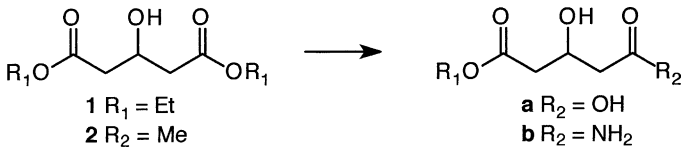

Scheme 1.

Table 1
Asymmetrization by hydrolysis and ammonolysis of **1** and **2**

Product	Enzyme	Activity	ee (%)	Yield (%)	$[\alpha]_D^{20}$	Configuration
1a	CALB	7 PLU/mg	91	80	+1.8 (c 11.5, acetone)	(*S*)
1a	CALA		91	77	+1.8 (c 11.5, acetone)	(*S*)
1a	CLEC-CALB	17 U/mg	86	80		(*S*)
1a	HLL		72	89		(*S*)
1a	RML	60 U/g	74	89		(*S*)
1a	PLE	15 U/mg	35	76	+0.2 (c 11.5, acetone)	(*S*)
1a	α-Chymotrypsin	70 U/mg	50	65		(*R*)
2a	CALB	7 PLU/mg	90	70	+0.8 (c 11.5, acetone)	(*S*)
2a	PLE		22	75		(*S*)
2a	α-Chymotrypsin		45	59		(*R*)
1b	CALB	7 PLU/mg	98	95	−6.9 (c 10.0, dioxane), −6.5 (c l.3, $CHCl_3$)	(*S*)
2b	CALB	7 PLU/mg	98	95	−2.0 (c 3.5, dioxane)	(*S*)

quick to perform. The mixtures of enantiomers of amides **1b** and **2b** were separated directly on a chiral column as TFA-esters, while the monocarboxylic acids **1a** and **2a** were derivatized to diastereomeric mixtures using (*R*)-α-phenylethylamine and analyzed on an achiral column. The optical rotation values of the products were compared with known values in order to determine the configuration of **1a** and **2a** [1,9], and **2b** [6].

The ee of the hydrolysis product of **1** with CALB as catalyst, was optimized by lowering the substrate concentration (Table 2). However, the reaction time increased with decreasing substrate concentration.

Table 2
Variation of concentration of **1** hydrolyzed by CALB

Substrate concentration (g/ml)	ee (%)	Reaction time (min)
0.75	85	20
0.23	87	25
0.11	91	30
0.06	90	35
0.03	91	40

(The amount of enzyme was the same in all the experiments.)

3. Experimental

3.1. General

Immobilized lipase B from *C. antarctica* (CALB, Novozyme 435) from Novozymes had a water content of 1–2% (w/w), lipase A from *C. antarctica* (CALA), *Humicola lanuginosa* lipase (HLL), *Rhizomucor miehei* lipase (RML) from Novozymes were immobilized on Accurel, lipase from *Pseudomonas cepacia* (LPS) was purchased from Amano Pharmaceutical Co. Ltd. ChiroCLEC-CALB was a gift from Altus Biologics Inc., Cambridge, MA, USA and α-chymotrypsin and porcine liver esterase (PLE) were purchased from Fluka and Sigma, respectively. Other chemicals were purchased from Fluka. For enzyme activities, see Table 1 (PLU: palm oil lipase units). The hydrolyses were performed in phosphate buffer (pH 7.0, 0.1 M) using a Metrohm pH-Stat 718 Titrino. For the ammonolyses, a G24 environmental

incubator shaker from New Brunswick Co. Inc., Edison, NJ, USA, was used. Optical rotation and NMR spectra were measured as described earlier [12].

3.2. Hydrolysis

Diethyl-3-hydroxyglutarate (**1**) (3.0 g, 14.7 mmol) was suspended in phosphate buffer (20 ml, pH 7.0, 0.1 M), CALB (0.5 g) was added, and the reaction mixture was stirred for approximately 0.5 h. The reactions were stopped after addition of 14.5 ml 1.0 M NaOH. The enzyme was filtered off and washed with CH_2Cl_2 for re-use. The water phase was extracted with Et_2O (5 × 30 ml). The organic phase was dried over $MgSO_4$ and the solvent was removed to give the product. Similar reactions of **1** (1.0 g, 4.9 mmol) were performed using α-chymotrypsin (0.4 g, reaction time 48 h), PLE (0.15 g, reaction time 6 h), ChiroCLEC-CALB (50 μl, 100 mg enzyme/ml susp.), HML (0.2 g, reaction time 5 h), RML (0.1 g, reaction time 5 h) and CALA (0.1 g, reaction time 4 h). Hydrolyses of dimethyl-3-hydroxyglutarate (**2**) (1.00 g, 5.67 mmol) were performed as for **1**.

3.3. Ammonolysis

NH_3 was bubbled through 1,4-dioxane (7 ml) at 0 °C for 10 min, after which **1** and **2** (0.4 g, 1.96 and 2.3 mmol, respectively) and CALB (20 mg) were added. The mixture was shaken at 30 °C and 20 rpm over night. The enzyme was filtered off and washed with CH_2Cl_2 for re-use. The organic solvents were evaporated to obtain the products.

3.4. Analyses

Chiral analyses were performed using Varian 3800 and 3400 gas chromatographs equipped with a chiral CP-Chirasil-DEX G-TA column from Chrompack (10 m, 0.25 mm i.d., and 0.25 μm film density) and an achiral DBWAX-N30 (25 m, 0.25 mm i.d., and 0.25 μm film density) from J&W Scientific, respectively. *GLC* (*amides*): Ethyl and methyl 1-4-carbamoyl-3-hydroxybutanoate as trifluoroacetic anhydride derivatives (**1b** and **2b**) were separated on Chirasil-DEX G-TA at 90(1)-105/1(0)-150/15(2), column pressure 6.0 psi and splitflow 60 ml/min. **1b**: RT_1 = 10.40, RT_2 = 10.90, R_s = 1.8. **2b**: RT_1 = 9.10, RT_2 = 9.40, R_s = 1.50.

GLC (*monocarboxylic acids*): To **1a** and **2a** (20 μl) was added $SOCl_2$ (20 μl) and dimethylformamide (20 μl), dissolved in Et_2O (2 ml) and the mixture was shaken for 5 min. (*R*)-α-Phenylethylamine (40 μl) was added and the mixture shaken for another 5 min during which HCl gas was formed. The Et_2O was evaporated and the remaining mixture dissolved in CH_2Cl_2 (2 ml). The solution was washed with water and saturated Na_2CO_3, dried over $MgSO_4$ and separated on DBWAX-N30 at 235 °C isothermic 35 min, column pressure 12 psi, splitflow 60 ml/min. **1a**: RT_1 = 31.4, RT_2 = 32.1, R_s = 2.9. **2a**: RT_1 = 31.6, RT_2 = 32.2, R_s = 2.5. ^{1}H NMR **1a**: 1.27 (3H, t), 2.59 (4H, dd), 4.17 (2H, q), 4.49 (1H, m), 6.97 (2H, br. s). ^{13}C **1a**: 14.2, 40.6, 40.7, 61.2, 64.8, 172.2, 176.3. ^{1}H NMR **2a**: 2.60 (4H, dd), 3.70 (3H, s), 4.50 (1H, m), 6.80 (2H, br. s). **1b**: 1.27 (3H, t), 2.42–2.57 (4H, dd), 3.70 (1H, OH), 4.16 (2H, q), 4.49 (1H, m), 6.05 (1H, NH), 6.45 (1H, NH). **2b**: 2.43–2.59 (4H, dd), 3.72 (3H, s), 4.16 (1H, br. s), 4.45 (1H, m), 6.05 (1H, NH), 6.45 (1H, NH).

4. Conclusion

Lipase B from *C. antarctica* is a suitable catalyst for asymmetrization of prochiral diesters, both in hydrolysis and ammonolysis. Lowering of substrate concentration in the hydrolysis of **1** gave higher ee of the product. The catalyst can be re-used more than 10 times without loss of activity. Cross-linked enzyme crystals (ChiroCLEC-CALB) increased the rate of the hydrolysis, but with apparent reduction of ee.

Acknowledgements

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Absolute configurations of monoesters produced by enzyme catalyzed hydrolysis of diethyl 3-hydroxyglutarate

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Abstract—Biocatalytic asymmetrizations of diethyl 3-hydroxyglutarate furnish a route to the enantiomers of ethyl 4-cyano-3-hydroxybutanoate. The enantiopreference of different enzymes has been established by chiral chromatography. Conclusive evidence for absolute configurations has been provided by X-ray crystallographic structure determination of co-crystals of the predominant monoester with (*R*)-phenylethylamine. The predominant enantiopure monoester produced by ammonolysis of diethyl 3-hydroxyglutarate catalyzed by immobilized lipase B from *Candida antarctica* (Novozym 435) was ethyl (3*S*)-4-carbamoyl-3-hydroxybutanoate. This was converted to ethyl (3*S*)-4-cyano-3-hydroxybutanoate in high yield and enantiomeric excess. Growing cells of *Acinetobacter lwoffii* gave low ee and predominance of the (*S*)-enantiomer when used for hydrolysis of diethyl 3-hydroxyglutarate as opposed to previous reports. When Novozym 435 was used for hydrolysis it could be re-used 10 times without loss of activity and selectivity.

1. Introduction

Lipitor has for several years been one of the best selling drugs. It is used to reduce the natural synthesis of cholesterol in patients who suffer from high cholesterol level in blood. Lipitor acts by inhibiting the enzyme hydroxymethyl co-enzyme A reductase, which is essential for the biosynthesis of cholesterol.[1] The active ingredient in lipitor, atorvastatin (Fig. 1), is a member of a family of statins, which also comprises lovastatin,[2] compactin, simvastatin, fluvastatin, cerivastatin and rosuvastatin.[1] These all contain a C-7 side chain with two stereocentres. This C-7 moiety has, for the synthesis of atorvastatin, been made from ethyl (3*R*)-4-cyano-3-hydroxybutanoate **1** and has been the focus of developing efficient syntheses for this chiral building block in its enantiopure (*R*)-form. A frequently used way of producing chiral building blocks is starting with enantiopure natural products. Starting with iso-vitamin C,[3] enantiopure nitrile **1** was synthesized using a series of non-'Green chemistry' steps. Based on lactose as the starting material a range of chiral building blocks are commercially available; among them starting materials for **1**.[4] Biocatalysis may also offer a greener way to many, in particular chiral, building blocks. This has been demonstrated in recent years both in the production of bulk chemicals such as acryl amide and in the fine chemicals and drug industry.[5]

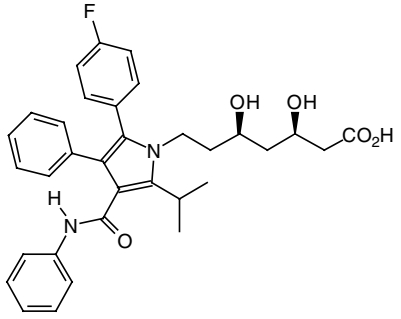

Figure 1. Atorvastatin.

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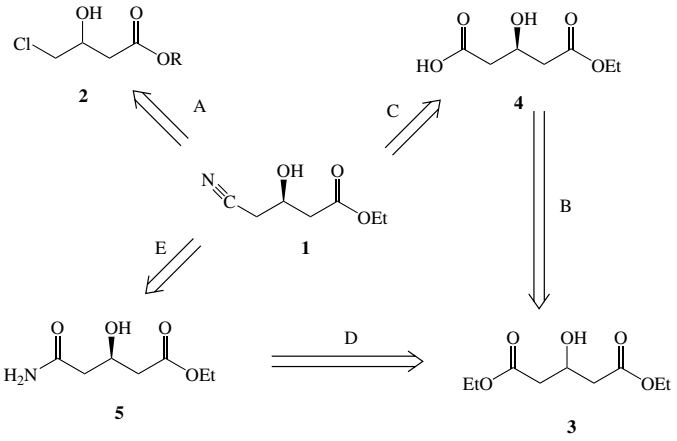

Figure 2. Retrosynthetic analysis for the synthesis of enantiopure target molecule **1** by biocatalysis. Route A, resolution of racemic chlorohydroxy ester **2**; route B, enzyme catalyzed asymmetrization by 'half'-hydrolysis of diethyl ester **3**, followed by route C, conversion of the enantiopure monoester **4** to nitrile **1**; route D ammonolysis of prochiral diester **3** to give enantiopure amide **5**, followed by route E, conversion of amide **5** to target nitrile **1**.

2. Results and discussion

We analyzed the possibilities of making **1** by biocatalysis based on the retrosynthetic scheme shown in Figure 2. Our first approach was to make **1** from racemic chlorohydroxy ester **2** by enzyme catalyzed kinetic resolution (route A).[6] The drawback with this resolution is that only 50% of the right enantiomer could be obtained. This may not be serious since quantitative conversion to one single enantiomer is possible using the technique of stereoinversion.[7,8] We started a systematic approach by varying the R-groups, solvents, acyl donors and enzymes. The highest *E*-value of >100 was obtained for the *tert*-butyl ester with vinyl propanoate as the acyl donor and lipase from *Rhizomucor miehei* as the catalyst. However, the synthesis of the ester substrate was not trivial starting with the diketene. Moreover, target nitrile **1** is an ethyl ester and a transesterification would be necessary in order to convert the *tert*-butyl ester to the ethyl ester. Hence we looked for an alternative method. By asymmetric synthesis instead of resolution, a theoretical yield of 100% of one enantiomer may be obtained directly, however, with enzyme catalysis, it is not guaranteed that this will be the wanted enantiomer.

An obvious starting material for the asymmetric synthesis would be diethyl 3-hydroxyglutarate **3**, a prochiral substrate. Enantiopure monoester **4** could be obtained by route B using enzymatic hydrolysis and this in turn could be converted to the nitrile using chlorosulfonylisocyanate (route C).[9] An even simpler method would be enzyme catalyzed ammonolysis via route D to the enantiopure monoamide **5**, which in turn could be converted to the target nitrile **1** using 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (route E).[10]

All of the mentioned strategies were tried using a range of enzymes.[11] By far the highest ee-values were obtained using lipase B from *Candida antarctica* (CALB). Indeed, the ammonolysis reaction gave ee = 98% and 95% yield. Chymotrypsin catalyzed hydrolysis gave much lower ee-values (≈50%). It has previously been reported that the (*R*)-monoethyl ester is predominant in hydrolysis catalyzed by chymotrypsin, and moreover, that the reaction gives the product with very high ee.[12] This reported high ee-value and configuration is based on comparison with a previously reported specific rotation value for the monomethyl ester produced by classical resolution via the cinchonidine salt.[13] It has been reported that growing cells of *Acinetobacter lwoffii* gives predominance of the (*R*)-enantiomer when used for hydrolysis of diethyl 3-hydroxyglutarate with an ee of 80%.[14] In our hands the same organism (ATCC-17925) gave the (*S*)-enantiomer and with low ee (56%).

Since the specific rotation of the monoethyl ester **4** is a very small numerical value, we wanted to check the absolute configuration of the monoester produced by CALB not only by comparison of the $[\alpha]_D$ values, but with a more reliable method. The monoethyl ester produced by CALB was co-crystallized with (*R*)-phenylethylamine with the crystal structure determined to be as shown in Figure 3. This structure determination shows conclusively that the monoester produced by CALB is the (*S*)-enantiomer and so we consider it safe to assume that amide **5** has the same configuration. Based on very reliable chiral GLC analyses we also find that chymotrypsin gives a predominance of the (*R*)-enantiomer **4**. However, in our hands the ee values obtained were much lower than previously reported[12] both for the diethyl and dimethyl esters.[11] Recently, a patent for a process for producing the monoethyl ester of diethyl 3-acetoxyglutarate using chymotrypsin, has been reported. The ee-values are very high and the configuration (*R*) as wanted for synthesis of **1**.[15]

After use in the first hydrolysis of diethyl and dimethyl 3-hydroxyglutarate, the immobilized catalyst CALB was re-used more than 10 times with retention of high activity and enantioselectivity. In fact, the activity of the enzyme increased significantly from the first to the second batch of hydrolysis of **3** (Fig. 4).

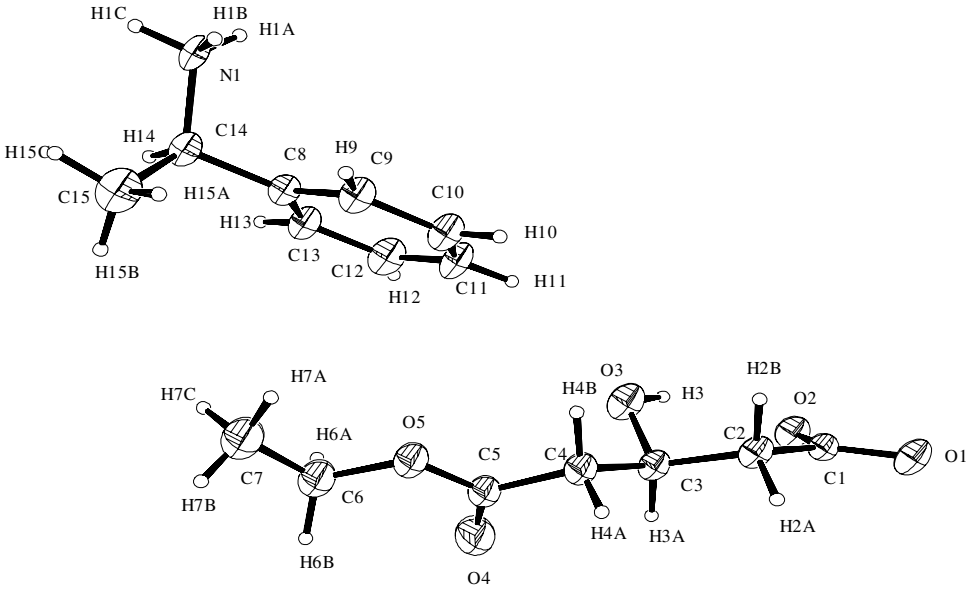

Figure 3. X-ray crystal structure of the co-crystal of (*R*)-phenylethylamine and the monoethyl ester produced by hydrolysis catalyzed by CALB. The stereocentre of (*R*)-phenylethylamine is labelled C-14 and it is inferred that it has (*R*)-configuration. The carboxylate group of the monoester is labelled C-1 and O-1, O-2; the ethyl ester group is C-6, C-7. From the X-ray structure it is clear that the stereocentre at C-3 has an (*S*)-configuration.

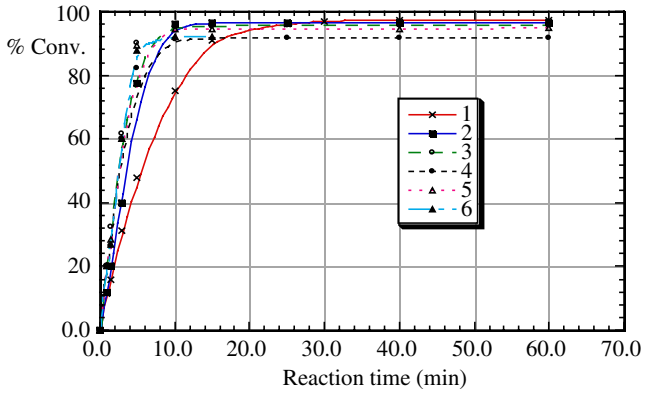

Figure 4. Conversion in six subsequent CALB catalyzed hydrolyses of diethyl 3-hydroxyglutarate **3**. The reaction time increased from reaction 1 to 6.

3. Experimental

3.1. Ethyl (3*S*)-4-cyano-3-hydroxybutanoate 1

Ethyl (3*S*)-4-carbamoyl-3-hydroxybutanoate (1.0 g, 5.7 mmol) was added to CH_2Cl_2 (15 mL), pyridine (0.89 g, 11.4 mmol) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide, EDCI (1.78 g, 11.4 mmol). The mixture was stirred at room temperature for 4 days and the reaction monitored using GLC. After the addition of water (10 mL) and shaking, the water layer was removed and the solution dried over $MgSO_4$. The solvent was removed under vacuum, yield: 0.81 g (90%) MS: M+1 158, *m/z* 130, 112, 88, 84, 43; ^{1}H NMR (Bruker DPX 300, 300 MHz; $CDCl_3$): 1.22 (t, 3H), 4.15 (q, 2H), 4.30 (m, 1H), 2.60 (m, 4H), ^{13}C NMR (Bruker DPX 300, 75 MHz, $CDCl_3$): 174.71, 41.40. 64.41, 25.65, 117.73, 61.53, 14.5; enantiomeric excess by chiral GLC 98% (see below), same as amide **5**, $[\alpha]_D^{20} = +31.5$ (*c* 1.0, $CHCl_3$), for the (*R*)-enantiomer $[\alpha]_D^{20} = -31.3$ (*c* 1.0, $CHCl_3$).[16]

3.2. Determination of enantiomeric excesses

The product from the above reaction, predominantly ethyl (3*S*)-4-cyano-3-hydroxybutanoate **1**, was derivatized with trifluoroacetic anhydride and analyzed using Varian 3400 gas chromatograph equipped with a chiral CP-Chirsil Dex CB column (25 m, 0.32 mm i.d., 0.25 μm film thickness), temperature program 90–95 °C, 0.2 °C/min, 95–180 °C, 15 °C/min, column pressure 7.5 psi and split flow 60 mL/min. Retention times (*R*) = 15.4 min, (*S*) = 15.9 min, resolution $R_S = 1.6$. Chiral GLC of 4-ethoxycarbonyl-3-hydroxybutanoic acid and ethyl 4-carbamoyl-3-hydroxybutanoate have been described earlier.[11]

Cells of *A. lwoffii* (ATCC-17925) were grown and used as described previously.[14] The resulting monoester was analyzed as described earlier[11] with the results showing that the (*S*)-enantiomer was formed with an ee of 56%.

3.3. Determination of the absolute configuration of monoethyl ester

Monoethyl ester produced by CALB hydrolysis (0.5 g, 2.8 mmol) was dissolved in Et_2O (15 mL) and (*R*)-phenylethylamine (0.34 g, 2.8 mmol) was added. After cooling on ice, crystals were formed. The X-ray crystallographic structure is shown in Figure 3 and proves the identity as being (3*S*)-3-hydroxypentanedioic monoethyl ester.

3.4. X-ray crystallography

Diffraction data were collected on a CAD-4 diffractometer ($\theta_{max} = 23°$) using graphite monochromated Mo-K_α radiation ($\lambda = 0.71069$ Å). The structure was solved by direct methods and refined using the integrated program package OSCAIL.[17]

3.5. X-ray data

Orthorhombic space group $P2_12_12_1$ with $a = 5.9796(16)$, $b = 14.546(5)$, $c = 19.477(6)$ Å, $V = 1694.1(9)$ Å^3 and one molecule in the asymmetric unit. The structure was refined to $R = 0.086$ and $R_w = 0.27$ (the crystal was of poor quality) using 1394 unique reflections. H-atom parameters were not refined. CCDC 235996.[18]

3.6. Re-use of Novozym 435

To diethyl 3-hydroxyglutarate **3** (3.0 g, 14.7 mmol) was added buffer (15 mL) and CALB (0.5 and 0.05 g) and the reaction monitored using a pH-stat. The amount of added base (1 M NaOH) expressed the rate of reaction. The catalyst was separated by filtration and re-used 10 times under the same conditions.

Acknowledgements

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18. Crystallographic data have been deposited with the Cambridge Crystallographic Data Centre. Copies of the data can be obtained free of charge on application to CCDC, 12 Union Road, Cambridge CB2 1EZ [fax: +44(0)-1223-336033 or e-mail: deposit@ccdc.cam.ac.uk].

Biocatalytic resolution of saphenic acid. Substrate preferences for lipases A and B from *Candida antarctica*

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Dedicated to Professor Torbjörn Norin on his 75th birthday

Abstract

Efficient methods for kinetic resolution of saphenic acid (**2**) and phenyl-(2-naphthyl)-methanol (**6**) have been developed using lipases B and A from *Candida antarctica,* respectively, as catalysts. Lipase B from *Candida antarctica* showed high enantioselectivity in esterification with vinyl butanoate of the alcohol **2**. By subsequent crystallization, the enantiomeric excess, *ee*, of (*S*)-saphenic acid ((*S*)-**2**) was higher than earlier reported. The butanoate (*R*)-**4** was obtained in high enantiopurity by esterification of the alcohol **2**. The enantiopure alcohol (*R*)-**2** and the butanoate (*S*)-**4** were synthesized by CALB catalyzed hydrolysis of **4** (the butanoate of **2**).

Lipase A from *Candida antarctica* showed high enantioselectivity in esterification of the alcohol **6** with vinyl acetate, and both (*S*)-phenyl-(2-naphthyl)-methanol ((*S*)-**6**) and the (*R*)-acetate of phenyl-(2-naphthyl)-methanol ((*R*)-**7**) were obtained in high enantiomeric excess. The corresponding (*R*)-phenyl-(2-naphthyl)-methanol ((*R*)-**6**) and the (*S*)-acetate of phenyl-(2-naphthyl)-methanol ((*S*)-**7**) were obtained by CALA-catalyzed hydrolysis of the acetate of phenyl-(2-naphthyl)-methanol (**7**).

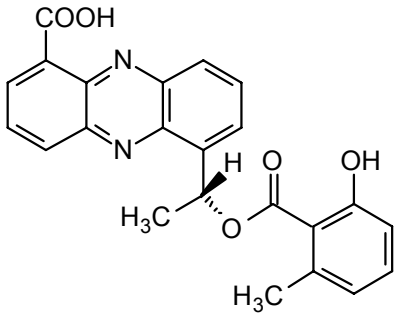
Keywords: Kinetic resolution, lipases A and B from *Candida antarctica*, saphenic acid, saphenamycin, *E*-value

Introduction

The secondary metabolite saphenamycin (yellow pigment) (**1**) has been isolated from a *Streptomyces* species, and it shows activities against Gram positive and Gram negative bacteria, antitumor activity,[1] antitrichomonal activity and lethal activity against midge/mosquito larvae.[2] Saphenamycin analogues show similar antimicrobial effect.[3] It is believed that the antibiotic effect of saphenamycin is due to intercalation of the drug with the duplex DNA with formation

of a non-covalent complex. This will result in inhibition of DNA replication and/or transcription, probably due to deformation of the double helix.[3]

The tricyclic phenazine structure of **1** has been found in several secondary metabolites isolated from other bacteria, and many of these also show antibiotic or antiviral effects. The DNA destroying mechanism seems to be the same for these compounds.[4] Saphenamycin isolated from microorganisms shows low optical activity, however, it has been suggested that it racemizes during work-up.[1] Saphenic acid (**2**) is the main building block of saphenamycin.



(*R*)-Saphenamycin, (*R*)-1

Both enantiomers of **1** have been synthesized by crystallization with (–)-brucine and their absolute configurations have been determined by X-ray crystallography co-crystallized with (–)-brucine. It has been suggested that **1** is biosynthesized as the (*R*)-enantiomer due to the analogy of the biosynthesis of saphenic acid (**2**). Saphenamycin has been synthesized from (*R*)-**2** and 6-methyl salicylic acid with retention of enantiomeric excess and configuration.[4]

The butanoate of saphenic acid (**4**) (Scheme 1) is found to be active against several types of microorganisms, however, it has only been tested in racemic form and it is reasonable to believe that the enantiomers will show different activity.[5]

Esterifications of several secondary alcohols have been catalyzed by lipase B from *Candida antarctica* (CALB) with high enantioselectivity.[6-10] Lipase A from this fungus (CALA) is reported to show broad catalytic activity towards a diversity of sterically hindered alcohols, including both secondary and tertiary alcohols.[11-16] It was interesting to investigate whether this lipase could catalyze resolutions of secondary alcohols with phenyl and naphthyl groups and other bulky groups attached to the stereocenter. The 3D structure of CALA has not been published, however, the structure of the lipase has recently been predicted by comparative modeling and site directed mutagenesis.[17]

Results and Discussion

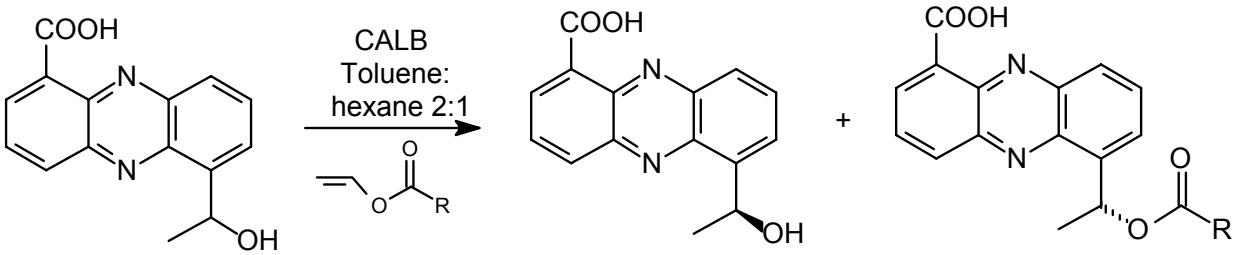
Attempts to resolve saphenic acid (6-(1-hydroxyethyl)-phenazine-1-carboxylic acid) (**2**) and phenyl-(2-naphtyl)-methanol (**6**) were performed by use of different lipases, esterases and

acylases. (See Table 1). Immobilized lipase B from *Candida antarctica* (CALB/Novozym 435) and immobilized lipase from *Rhizomucor miehei* (Lipozyme RM IM) catalyzed the esterification of **2**, the other enzymes did not give any reaction products after one week. Lipozyme RM IM showed low enantioselectivity toward saphenic acid (**2**) (*E*-value = 37), and the reaction was not scaled up. Novozym 435 was chosen as the best catalyst with *E*-values > 200, both with vinyl acetate and vinyl butanoate as acyl donor. (Scheme 1a) However, the acetates of saphenic acid, (*R*)-3 and (*S*)-3, were not properly separated neither by chiral GLC nor by HPLC. Vinyl butanoate was chosen as the acyl donor.

Table 1. Enzymes used in resolutions of saphenic acid (**2**) and phenyl-(2-naphtyl)-methanol (**6**)

Enzyme	Specifications
Lipase A from *Candida antarctica* (CALA)	E.C. 3.1.1.3, Novozym 735
Lipase B from *Candida antarctica* (CALB)	E.C. 3.1.1.3, Novozym 435, LC-200204
Lipase fra *Thermomyces lanuginosus*	E.C. 3.1.1.3, Lipozyme TL IM
Lipase fra *Rhizomucor miehei*	E.C. 3.1.1.3, Lipozyme RM IM
Lipase from *Candida rugosa*	
Lipase from wheat seeds	E. C. 3.1.1.3, Type 1, Sigma, L-3001
Lipase from Papaya	E.C. 3.1.1.3, Type III, Sigma, P-4880
Pig liver esterase	E.C. 3.1.1.3, Type II, Sigma, L-3126
Acylase 1 from *Aspergillus melleus*	E. C. 3.5.1.14, Sigma, A-2156

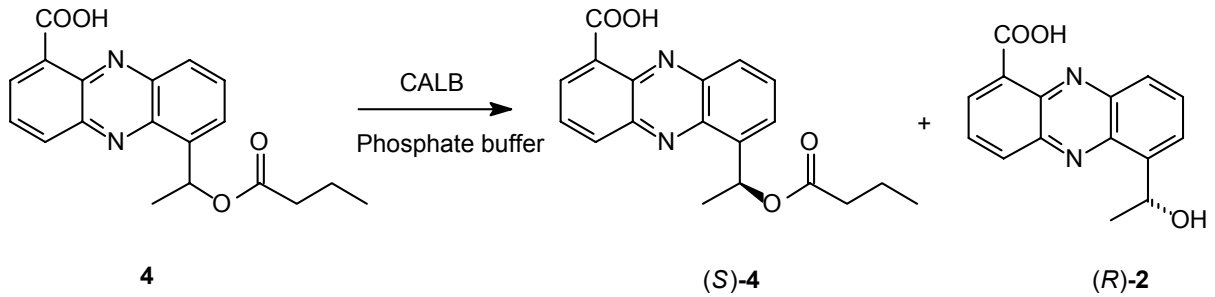
The (*R*)-butanoate of saphenic acid ((*R*)-**4**) was obtained in 41 % yield and 96.4 % *ee* while *(S)*-saphenic acid ((*S*)-**2**) was obtained in 25 % yield and 100 % *ee* after recrystallization. Recrystallization of (*R*)-**4** did not increase the *ee*.



rac-**2** (*S*)-**2** (*R*)-**3** R = CH_3 (*R*)-**4** R = C_3H_7

Scheme 1a

(*R*)-Saphenic acid ((*R*)-**2**) was obtained by CALB catalyzed hydrolysis of saphenic acid butanoate (**4**) in 96.6 % *ee*. (Scheme 1b) After crystallization, the *ee* was improved to 100 %. The (*S*)-enantiomer of the butanoate of saphenic acid ((*S*)-**4**) was obtained in 36 % yield and 81 % *ee*. The *ee* was not improved by crystallization.

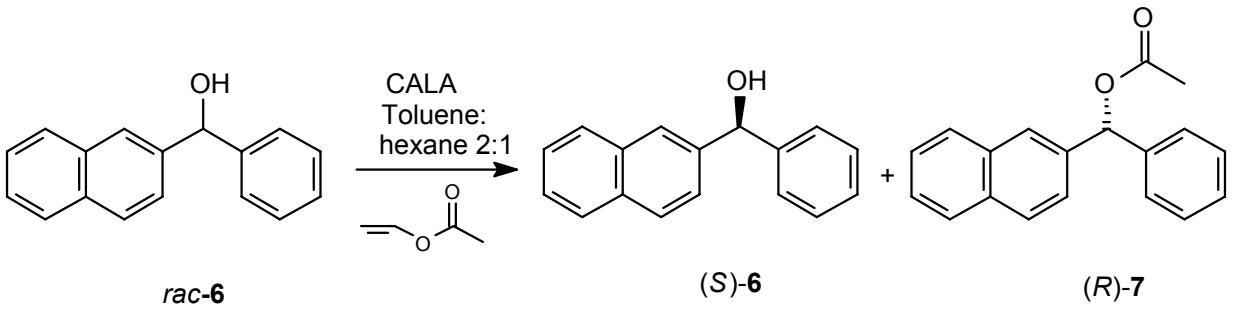


4 (*S*)-**4** (*R*)-**2**

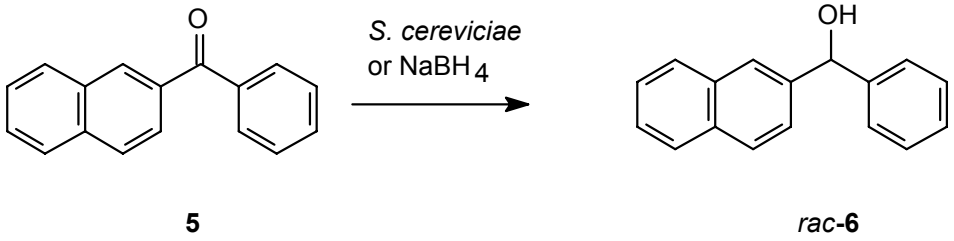
Scheme 1b

The absolute configurations of the enantiomers of saphenic acid (**2**) were verified by comparison of optical rotation values.[4] The optical rotation values measured by us, deviated slightly from the reported values, however, it may be due to different *ee´*s. The reported optical rotation for (*R*)-**2** with *ee* 74 % is $[\alpha]_D^{22} = -19.70$ (c 0.13, $CHCl_3$),[4] measured by us (96.6 % *ee*): $[\alpha]_D^{20} = -15.91$ (c 0.41 $CHCl_3$). The reported optical rotation for (*S*)-**2** with *ee* 80 % is $[\alpha]_D^{22} = +20.00$ (c 0.41, $CHCl_3$),[4] determined by us (100 % *ee*): $[\alpha]_D^{20} = +16.82$ (c 0.41 $CHCl_3$). Phenyl-(2-naphthyl)-methanol (**6**) and the acetate **7** were used as model substrates in order to compare substrate preferences for several lipases.

Lipase catalyzed kinetic resolution of phenyl-(2-naphthyl)-methanol (**6**) were performed by the enzymes listed in Table 1, except for the lipase from papaya and Acylase A from *Aspergillus melleus*. Vinyl acetate was used as acyl donor. Only *Candida antarctica* lipase A (CALA) (Scheme 2) gave reaction products with an *E*-value = 67. (*S*)-Phenyl-(2-naphthyl)-methanol ((*S*)-**6**) was obtained in 47.3 % yield and 94.5 % *ee*. The (*R*)-acetate of phenyl-(2-naphthyl)-methanol ((*R*)-**7**) was obtained in 35.8 % yield and 95.0 % *ee*. Optical rotation of the alcohol is reported for both enantiomers[18] and the absolute configurations have been determined by X-ray crystallography. However, optical rotation values of the (*R*)-acetate ((*R*)-**7**) has not been reported and the configuration of this ester is based on comparison of the chromatographic retention times (HPLC) with the alcohol enantiomers. Phenyl-(2-naphthyl)-methanol (**6**) was prepared by reduction of phenyl-(2-naphthyl)-methanone (**5**) with $NaBH_4$ (high yield) and by a non-enantioselective yeast reduction with *Saccharomyces cereviciae* (low yield). (Scheme 3) The racemic acetate **7** was synthesized by esterification of the alcohol **6** with acetic anhydride.



Scheme 2



Scheme 3

Conclusions

Kinetic resolutions of saphenic acid (**2**) catalyzed by CALB were very efficient. Saphenic acid has one small group (methyl) and one large group (tricyclic phenazine ring) connected to the stereocenter. We have found earlier that n-propyl is the largest group that can fit into the stereospecificity pocket of CALB, leading to high enantioselectivity in kinetic resolutions of secondary alcohols.[19] The enantioselectivity is not to the same extent dependent on the size of the large group. CALA shows high enantioselectivity for phenyl-(2-naphthyl)-methanol (**6**) (one phenyl and one naphthyl group connected to the stereocenter) which is in accordance with previous reported results.[11-16]

Experimental Section

General Procedures. Table 1 (See Results and Discussion part) shows the enzymes used in screening experiments. Novozym 735 (immobilized lipase A from *Candida antarctica*, CALA), Novozym 435 (immobilized lipase B from *Candida antarctica*, CALB), Lipozyme RM IM (immobilized lipase from *Rhizomucor miehei*, RM IM) and Lipozyme TL IM (immobilized lipase from *Thermomyces lanuginosa*, TL IM) were from Novozymes. Other enzymes were purchased from Sigma-Aldrich. Immobilized lipase B from *Candida antarctica* (CALB,

Novozym 435) had an activity of 7000 PLU/g, and a water content of 2 % w/w. Enzyme catalysts were rinsed with hexane and re-used several times with retention of enantioselectivity and activity according to previous results.[20] Chemicals were purchased from Sigma-Aldrich. Solvents were dried over molecular sieve from Union Carbide (3 Å) and analyzed by a Metrohm 756 Karl Fisher Coulometer.

Saphenic acid (**2**) was a gift from Professor John Nielsen, University of Århus, Denmark. The transesterification reactions and hydrolyses were performed at 30°C and 200 rpm in an Infors MINITRON Shaker Incubator. (A pH stat was not used due to decomposition of saphenic acid in day light.) All transesterification reactions were performed in toluene:hexane (2:1) and hydrolyses in phosphate buffer (pH 7.0). Column and flash chromatography were performed using silica gel 60 from Sigma-Aldrich, with pore size 0.0663-0.2000 mm and 0.035-0.070 mm, respectively.

Analyses

Optical rotations were determined using a Perkin Elmer 243 B automatic polarimeter, concentrations are given in g/100 mL. Chiral HPLC analyses gave the ee_s- and ee_p-values from which the degree of conversion was determined according to $c = ee_s / (ee_s + ee_p)$. Internal standards were not used because of the possibility of affecting the physical and chemical nature of the reaction medium. In control experiments under the reaction conditions without enzyme, no acylation was observed.

Chiral analyses were performed using a HPLC system (Varian 9010 pump, Varian 2550 UV detector, Rheodyne 7125 manual injector and Varian 9095 autosampler) equipped with Daicel Chiralpack AD (no 19025, 25 cm, 4.6 mm i.d., particle size 10 μm packed with amylose tris-(3,5-dimethyl phenyl carbamate) and Daicel Chiralpack OD-H (no 14325, 25 cm, 4.6 mm i.d., particle size 5 μm packed with cellulose tris-(3,5-dimethyl phenyl carbamate). Star Chromatography Workstation 4.5.1 was used for data processing.

Chromatographic parameters

(*R*)-**2** R_t 49.5 min, (*S*)-**2** R_t 58.5, R_S 3.46, separated by AD column with hexane:EtOH (85:15), flow 0.5 mL/min, 254 nm.

(*R*)-**6** R_t 74.6 min, (*S*)-**6** R_t 60.3, R_S 5.41, separated by OD-H column with hexane:isopropanol (95:5), flow 0.5 mL/min, 254 nm.

(*R*)-**7** R_t 13.0 min, (*S*)-**7** R_t 13.9, R_S 2.08, separated by OD-H column with hexane:isopropanol (95:5), flow 0.5 mL/min, 254 nm.

NMR spectroscopy was performed in $CDCl_3$ solutions, using Bruker DPX 300 and 400, operating at 300 and 400 MHz for ^1H and 75 and 100 MHz for ^{13}C, respectively. Enantiomeric ratios, *E*, were calculated based on ping-pong, bi-bi kinetics using the computer program E & K Calculator version 2.1b PCC.[21,22] Mass spectra were performed on a Mat 95 XL Thermo Quest Finnigan with EI as ionizing source.

Compound characterization

Saphenic acid butanoate (**4**)**.** Saphenic acid (**2**) (0.46 g, 1.72 mmol) was dissolved in a solution of toluene:hexane (2:1, 75 mL) with vinyl butanoate (1.09 mL, 8.6 mmol). The reaction vessel was covered with aluminium foil. Molecular sieve (2.0 g) and lipase A from *Candida antarctica* (CALA – not stereoselective) (0.5 g) was added. The reaction was incubated at 30°C and 200 rpm for 12 days before concentration under pressure and column chromatography. The butanoate of saphenic acid, **4**, was isolated in 73 % yield and the unconverted saphenic acid (**1**) was isolated in 12 % yield.

Phenyl-(2-naphthyl)-methanol (6). Method 1. Biocatalytic reduction. Phenyl-(2-naphthyl)-methanone (**5**) was reduced by a cell culture of *Saccharomyces cereviciae* (not enantioselective) by the following procedure: Sucrose (16.0 g) and dry yeast (5.0 g) were added to water (100 mL) in a 500 mL flask. The medium was incubated by 30°C 1/2 h before 5 (50 mg, 0.215 mmol in 3 mL EtOH) was transferred every half hour (250 μL) to the reaction flask until all of the substrate was added. The reaction was incubated at 30°C and 200 rpm for 14 days before work-up. The reaction mixture was saturated with NaCl and the solvent phase was removed by suction (2 x) under reduced pressure. The reaction product was extracted with toluene (4 x 50 mL) and the organic phase dried over $MgSO_4$. Toluene was evaporated under reduced pressure. Phenyl-(2-naphthyl)-methanol (**6**) was isolated as a slightly yellow solid in 14.4 % yield and 99 % purity (HPLC).

Method 2. Non-enzymatic reduction. Phenyl-(2-naphthyl)-methanone (**5**) (1.0 g, 4.303 mmol) was dissolved in MeOH (50 mL) before addition of $NaBH_4$ (0.326 g, 8.618 mmol). The reaction was stirred for 2 h. HCl (1 M) was then added dropwise until evolution of gas stopped. The solvent was removed under reduced pressure. The white solid was dissolved in toluene (25 mL) and washed with H_2O (3 x 20 mL) before drying of the organic phase over $MgSO_4$. Phenyl-(2-naphthyl)-methanol (**6**) was isolated as a yellow solid in 95 % yield and 99 % purity after removal of the solvent under reduced pressure. Melting point 79.4-80.4°C, EI/MS, (*m/z*) M^+ 234, 215, 155, 129, 105, 77. ^{1}H NMR: 2.81 (1H, d, *J* 3.1 Hz), 5.78 (1H, d, *J* 2.9 Hz), 7.16-7.32 (6H, m), 7.36-7.43 (2H, m), 7.66-7.75 (4H, m). ^{13}C NMR: 76.70, 125.34, 126.47, 126.69, 127.24, 128.20, 128.20, 128.60, 128.80, 129.00, 133.30, 133.70, 141.60, 144.10.

Acetate of phenyl-(2-naphthyl)-methanol (7). Phenyl-(2-naphthyl)-methanol (**6**) (0.5 g, 2.136 mmol) was dissolved with Ac_2O (0.436 g, 4.272 mmol) and dimethyl amino pyridine (DMAP) (0.287 g, 0.235 mmol) in CH_2Cl_2 (20 mL). The reaction mixture was cooled at 0°C for 30 min before addition of dicylohexyl carbodiimide (DCC) (0.458 g, 2.221 mmol). The reaction was stirred at 0°C over night. Dicyclohexyl urea (DCU) was removed by filtration and the reaction mixture was washed with HCl (0.5 M, 3 x 5 mL), $NaHCO_3$ (5.0 %, 2 x 5 mL) and distilled H_2O (2 x 10 mL). The organic phase was dried over $MgSO_4$ and the solvent removed under reduced pressure. The crude product was purified by column chromatography with toluene:methanol (10:1). The acetate **7** was isolated as a yellow oil in 63 % yield and 99 % purity (HPLC).

Enzyme screening in transesterification reactions. Saphenic acid (**2**) (0.1 g, 0.34 mmol) and vinyl butanoate (0.237 mL, 1.872 mmol) were dissolved in a solution of toluene:hexane (2:1, 20

mL). The solution was divided into 10 reaction vessels, and molecular sieve (2 beads) was added to each vessel (one blind sample without enzyme). The reactions were started by addition of enzyme (50 mg) and incubated (30°C, 200 rpm) for 7 days. TLC and HPLC showed that lipase A and B from *Candida antarctica* and lipases from *Rhizomucor miehei* (Lipozyme RM IM) and *Thermomyces lanuginosus* (Lipozyme TL IM) gave products. However, Lipozyme RM IM showed low stereoselectivity and CALA and Lipozyme TL IM showed no stereoselectivity. These reactions were of that reason not scaled up. Lipase B from *Candida antarctica* showed the highest conversion of product ester with high enantiomeric excess. Use of vinyl acetate showed similar results. The reaction of **2** with CALB and vinyl butanoate was scaled up.

The same procedure was used in screening of enzymes for phenyl-(2-naphthyl)-methanol (**6**) with use of the enzymes listed in Table 1, except for the lipase from papaya and Acylase A from *Aspergillus melleus*. Lipase A from *Candida antarctica* showed the highest conversion of product ester and this reaction was scaled up.

Transesterification reaction of 2. Saphenic acid (**2**), (0.44 g, 1.65 mmol) and vinyl butanoate (1.05 mL, 8.3 mmol) were added to toluene:hexane (2:1, 75 mL) and the reaction was started by addition of CALB (0.818 g). Molecular sieve (2.0 g) was added, and the vessels were covered with aluminium foil. The reactions were incubated for 3 days. Enzyme and molecular sieve were removed by filtration, and silica (1.0 g) was added the filtrate before concentration under reduced pressure. Separation on silica with $CHCl_3$:isopropanol (95:5) gave the yellow solid (*S*)-saphenic acid, (*S*)-**2**, in 35 % yield and 97.2 % *ee*. After recrystallization the yield was 25 % and *ee* 100 %, $[\alpha]_D^{20}$ = + 16.82 (c 0.41, $CHCl_3$), $[\alpha]_D^{20}$ = – 30.43 (c 0.23, DMSO), mp 218.4-219.2°C. (*R*)-saphenic acid butanoate ((*R*)-6-[1-(1-oxobutoxy)ethyl]-phenazine-1-carboxylic acid), (*R*)-**4**, was obtained in 41 % yield and 96.4 % *ee*, $[\alpha]_D^{20}$ = – 48.33 (c 0.41, $CHCl_3$) $[\alpha]_D^{20}$ = – 52.83 (c 2.29, DMSO), mp 162.3-163.5°C. Recrystallization of (*R*)-**4** by the same procedure as in (*S*)-**2** did not increase *ee*. (*S*)-**2**: EI/MS (*m/z*) M^+ 268, 253, 225, 205, 181. ^{1}H NMR (ppm):1.82 (3H, d, *J* 6.6 Hz), 4.12 (1 H, d, *J* 5.8 Hz), 5.84 (1H, p, *J* 6.3), 7.94-8.01 (2H, m), 8.06 (1H, dd, *J* 8.9 Hz and J 7.1 Hz), 8.2 (1H, dd, *J* 8.4 Hz and *J* 1.6 Hz), 8.53 (1H, dd, *J* 8.7 and *J* 1.4 Hz), 8.99 (1H, dd, *J* 7.1 Hz and J 1.4 Hz) 15.47 (1H, br, s).

(*R*)-**4**: EI/MS (*m/z*) M^+ 337, 293, 267, 250, 221, 204, 179. ^{1}H NMR (ppm): 0.99 (3 H, t, *J* 7.4 Hz), 1.76 (3H, d, *J* 6.6 Hz), 1.67-1.8 (2H, m) 2.44 (2H, dt, *J* 7.4 Hz), 7.22 (1H, q, *J* 6.5 Hz), 7.98-8.06 (3H, m), 8.19 (1H, m), 8.55 (1H, dd, *J* 8.7 Hz and *J* 1.4 Hz), 8.96 (1H, dd, *J* 7.1 Hz and *J* 1.4), 15.52 (1H, br, s). ^{13}C NMR: 13.72, 18.54, 22.33, 36.50, 67.50, 124.67, 126.91, 127.16, 130.18, 133.00, 135.50, 137.50, 139.60, 139.80, 141.30, 142.15, 142.50, 165.92, 172.73.

Recrystallization. (*S*)-Saphenic acid ((*S*)-**2**) (0.154 g, 0.577 mmol) was dissolved in hot MeOH (2 mL). The reaction was cooled overnight. The remaining solvent was removed by filtration and the crystals were isolated and dried. The *ee* increased from 97.2 % to 100 % measured by HPLC.

Hydrolysis of the butanoate of saphenic acid (4). Butanoate **4** (0.423 g, 1.256 mmol) was dissolved in MeOH (10 mL) and transferred to phosphate buffer (pH 7.0, 50 mL) The reaction vessel was covered by aluminium foil and CALB (0.5 g) was added. The reaction was incubated

for 7 days. The enzyme was removed by filtration and the filtrate was extracted with $CHCl_3$. The organic phase was dried over $MgSO_4$, filtrated and evaporated with silica (1.0 g) under reduced pressure. The silica sample was transferred to a packed silica column for purification. (*R*)-saphenic acid, (*R*)-**2**, was isolated in 29 % yield with 96.6 % *ee*. After recrystallization by the same method as for (*S*)-**2**, the yield was 19.3 % and *ee* 100 % analyzed by HPLC. $[\alpha]_D^{20}$ = – 15.91 (c 0.41, $CHCl_3$), $[\alpha]_D^{20}$ = + 29.78 (c 0.23, DMSO). (*S*)-**4** was isolated in 36 % yield with 99 % purity and 81 % *ee* (HPLC). $[\alpha]_D^{20}$ = + 33.90 (c 0.41, $CHCl_3$). The *ee* of (*S*)-**4** was not improved by crystallization.

Transesterification reaction of phenyl-(2-naphthyl)-methanol (6). Phenyl-(2-naphthyl)-methanol (**6**) (0.813 g, 3.468 mmol) and vinyl acetate (1.6 mL, 17.34 mmol) were dissolved in a mixture of toluene:hexane (2:1, 50 mL). Molecular sieve (2.0 g) was added and the reaction was started by addition of CALA (1.0 g). The reaction mixture was incubated for 48 h. Enzyme and molecular sieve were removed by filtration and the solvent was removed under reduced pressure. The crude product was purified by column chromatography with toluene:MeOH (10:1). The acetate (*R*)-**7** was isolated as a slightly yellow viscous oil in 47.3 % yield, 99 % purity (HPLC) and 95 % enantiomeric excess. $[\alpha]_D^{20}$ = +17.83 (c 0.83, $CHCl_3$), $[\alpha]_D^{20}$ = + 30.36 (0.83, Benzene). EI/MS (*m/z*) M^+ 276, 234, 215, 202, 156, 127, 107 and 77. ^{1}H NMR: 2.17, (3H, s), 7.05 (1H, s), 7.2-7.47 (8H, m), 7.77-7.82 (4H, m). ^{13}C NMR: 21.30, 76.95, 124.94, 126.01, 126.22, 126.28, 127.18, 127.64, 127.95, 128.09, 128.38, 128.51, 132.91, 133.07, 137.50, 140.06, 170.02.

The alcohol (*S*)-**6** was isolated as a solid in 35.8 % yield and 99 % purity (HPLC) and 94.5 % *ee*. $[\alpha]_D^{20}$ = + 11.21 (c 0.83, $CHCl_3$), $[\alpha]_D^{20}$ = – 6.51 (0.83, Benzene), mp 79.4-80.4°C. Recrystallization of (*S*)-**6** from Et_2O did not increase *ee*.

Hydrolysis of the acetate of phenyl-(2-naphthyl)-methanol (7) by CALA. The acetate of phenyl-(2-naphthyl)-methanol (**7**) (0.371 g, 1.345 mmol) was dissolved in MeOH (10 mL) before it was added to a phosphate buffer solution (pH 7.0, 50 mL). CALA (1.0 g) was added and the reaction mixture was incubated for 10 days. The enzyme was removed by filtration and the reaction mixture was extracted with toluene (3 x 10 mL). The organic phase was dried over $MgSO_4$ before the solvent was removed under reduced pressure. The crude product was purified by column chromatography (toluene:MeOH, 10:1). (*R*)-phenyl-(2-naphthyl)-methanol ((*R*)-**6**) was isolated in 25 % yield, 99 % purity and 98.5 % *ee* (HPLC). $[\alpha]_D^{20}$ = – 11.61 (c 0.83, $CHCl_3$), $[\alpha]_D^{20}$ = + 6.89 (c 0.83, Benzene). The (*S*)-acetate of phenyl-(2-naphthyl)-methanol ((*S*)-**7**) was isolated in 23 % yield, 99 % purity and 94.5 % *ee* (HPLC). $[\alpha]_D^{20}$ = – 17.01 (c 0.83, $CHCl_3$), $[\alpha]_D^{20}$ = – 29.73 (c 0.83, Benzene).

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