The Utility of Modified Cyclodextrin, Sol-Gel Procedure and Gas Chromatography in Lipase-mediated Enantioselective Catalysis: Kinetic Resolution of Secondary Alcohols

Die Nützlichkeit von modifizierten Cyclodextrinen, des Sol-Gel Prozesses und der Gas-Chromatographie in Lipase-unterstützter enantioselektiver Katalyse: Kinetische Racematspaltung von sekundären Alkoholen

DISSERTATION

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If we wish to catch with Nature, we shall need to use the same methods as she does, and I can foresee a time in which physiological chemistry will not only make greater use of natural enzymes but will actually resort to creating synthetic ones.

Emil Fischer, 1902

Acknowledgements

In my opinion, doing research is like running an off-road track. One can know in which direction wants to go, however, one can not foresee what will be coming along the way. The varying nature of challenges one meets stimulate ones mind and the passage of each obstacle gives a feeling of satisfaction and adds to the excitement of what is to come. Now, close to the finishing line of this run, I would like to address my sincere gratitude to the persons who have accompanied me along the course and those who have been there by the side to support me.

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Content

Abbreviations

Å	Ångström(0.1 nm)
Ac	Acetyl
ANL	*Aspergillus niger* Lipase
AY 30	Lipase type II, crude, from *Porcine pancreas*
AYS	*Candida rugosa* Lipase
BINAP	2,2´-bis(diphenylphosphino)-1,1´-binaphthyl
CAL-B	*Candida antarctica* lipase B(Novozyme 435)
CCL	*Candida cylindracea* lipase
CD	Cyclodextrin
Chirasil-β-Dex	Octamethylene-permethyl-*b*-cyclodextrin bounded to dimethyl polysiloxane
Conv.	Conversion
CRL	*Candida rugosa* typeVII
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleoside triphosphate
E	Enantiomeric ratio
EDX	Energy dispersive X-ray
ee	Enantiomeric excess
ee_p	Enantiomeric excess of product
ee_s	Enantiomeric excess of substrate
EM	Electron microscopy
EP100(MP1000)	Microporous polypropylene powder
Eq.	Equation
ES	Enzyme-substrate complex
EtOH	Ethanol
GC	Gas chromatography
GC/MS	Gas chromatography connected to mass spectrometer
GI	Gastro-intestinal
HPLC	High performance liquid chromatography
hr	Hours
L	Liter
LDPE (MP4000)	Microporous low density polyethylene

Lipozyme IM	Lipozyme, immobilized lipase from *Rhizomucor miehei*
Log P	Partition coefficient
mp	Melting point
Min	Minute(s)
MTMS	Methyltrimethoxysilane
n.d.	Not determined
NMR	Nuclear magnetic resonance
Novozyme 525 L	Liquid version of novozyme 435
P	Product
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PEG	Polyethyleneglycol
PFL	*Pseudomonas fluorescens* lipase
PPL	*Porcine pancreas* lipase
PS-30	*Pseudomonas cepacia* lipase
PSL	*Pseudomonas cepacia* lipase
PSL-C	*Pseudomonas cepacia* immobilized on Ceramic(s)
PSL-D	*Pseudomonas cepacia* immobilized on diatomaceous earth
rac.	Racemic
rec.	Recombinant
rpm	Rotation per minute
R_s	Resolution
S	Substrate
SDS	Sodium *n*-dodecyl sulphate
SEM	Scanning electron microscopy
SP 525	Free lipase from *Candida antarctica*
t	Time
TBME	tert. butyl methyl ether
TEMED	N,N,N',N'-Tetramethyl-ethylenediamine
THF	Tetrahydrofuran
TMOS	Tetramethoxysilane
TOF	Turnover frequency
Tris	Tris-(hydroxymethyl)-aminomethane
U	unit
Vol.	Volume

1. General Introduction

1.1. Chirality

Molecular chirality is an important aspect in chemical science. The word chiral originates from the Greek word *cheir* meaning ´hand` and was initially introduced in physics vocabulary by Lord Kelvin at the end of the 19 th century.[1]

The importance of chirality has constantly increased in the last decades and it is today a central issue of organic and pharmaceutical chemistry especially in the development of new drugs.

Chirality is a geometrical attribute; an object that is not superimposable upon its mirror image is said to be chiral while an achiral object is the one that is superimposable on its mirror image. A couple of such mirror images constitute an enantiomeric pair (Greek *enantios*, opposite). One of the most common used examples to describe chirality are the right and left hands which form an enantiomeric pair (Fig. 1.1). It is obvious that the left hand is the mirror image of the right one and both are not superimposable. Chirality can be made up around a point (central chirality), like an atom tetrahedrally coordinated to four different substituents; an axis (axial chirality), e.g. in allenes or ortho-substituted biaryls; or a plane (planar chirality), e.g. in cyclophanes or 1,2-disubstituted ferrocenes (Fig. 1.2).

The axial chirality in ortho-substitued biaryls is an example of atropisomerism[2] and is a consequence of a restricted rotation between the aromatic rings, permitting isolation of two enantiomeric species. A chiral molecule may contain several (n) stereogenic centers giving rise to a maximum of 2^n possible stereoisomers, which will be pairwise mirror images of each other, just like our hands. Two enantiomers possess equal physical properties, such as density, boiling point, refractive index, etc.., except for their ability to rotate the plane of linearly polarized light. Enantiomers rotate polarized light in opposite directions, dextrorotatory (*d* or +) causing a clockwise rotation or levorotatory (*l* or -) causing an equal rotation in the opposite direction.

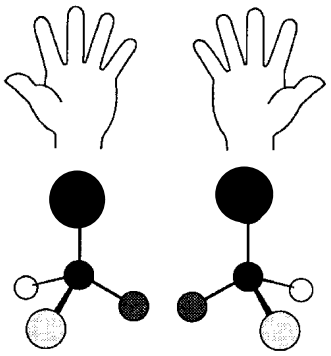


Fig. 1.1: Example of chirality, objects and their non-superimposable mirror image.

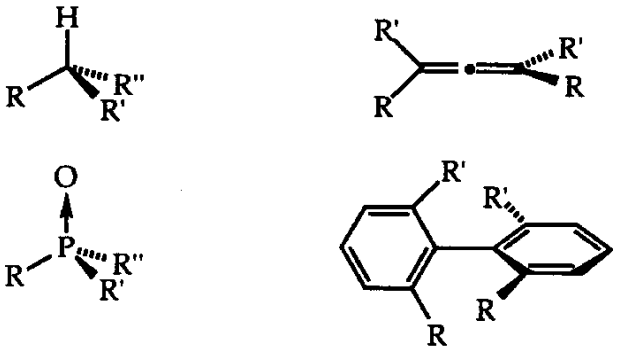
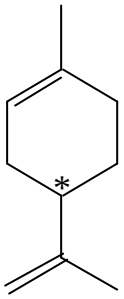


Fig. 1.2: Examples of central, axial and planar chirality (from left to right) in organic molecules.

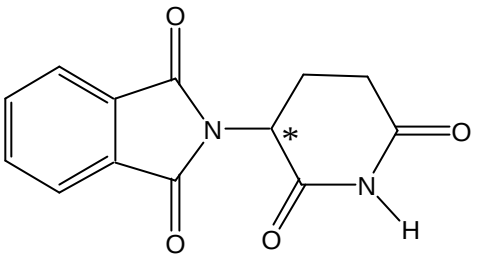
1.2. Chirality in biological systems

A wide range of chiral building blocks has been found in nature. Many of these predominate only in one enantiomeric form, e.g. L-amino acids and D-carbohydrates. As a consequence, all proteins and genetic materials, DNA and RNA, are chiral and the mechanisms behind several important processes in living organisms, such as metabolism and regulatory processes, are based on enantioselectivity.[3] This should be relevant to drug development as two enantiomers quite commonly have different pharmacological effects. In an achiral environment, enantiomers possess identical chemical properties. However, in a chiral environment such as the human body, which to a large extent consists of proteins made from (L)-*α*-amino acids, they often act as two different types of molecules with different chemical properties and consequently also different biological activity. In fact, the human body is structurally chiral (enantiomorphous), with the heart lying to the left of the center, and the liver to the right. Helical seashells are chiral, and most spirals resemble a right-handed screw. Also, many plants show chirality in the way they wind around supporting structures, e.g. the

honeysuckle, *Lonicera sempervirens*, winds as a left-handed helix while bindweed, *Convolvulus arvensi*, winds in a right-handed way.
The molecules of natural sugars are almost all classified as being D-configurated, including the sugar that occurs in DNA. DNA itself possesses a helical structure and all naturally occurring DNA turns to the right.
Chiral molecules can elicit their different behavior in many ways, including the way they affect the human beings. One enantiomeric form of limonene has the odor of oranges while the other enantiomer has the odor of lemons.



Limonene



Thalidomide

Indeed, the difference in chirality may have much more tragic effects on human beings. A well-known example is the thalidomide catastrophe in the sixties. A 1:1 mixture of both enantiomers (racemic mixture of 3-phthalimidoglutarimide) was marketed as a sedative and used to alleviate the symptoms of morning sickness in pregnant women. Unfortunately, it was not realized until too late that the (+)-(R)-form is responsible for the desired sedative effect while the (-)-(S)-form which is also present in the drug (in equal amount) causes serious fetal malformations of arms and legs in many children born subsequent to the use of the drug. The evidence regarding the effect of the two enantiomers is complicated by the fact that under physiological conditions, the two enantiomers are interconverted.[3]
Furthermore, (S)-ibuprofen inhibits platelet aggregation whereas the (R)-ibuprofen is totally inactive.[4,5] There are numerous additional examples of different activities of two enantiomers which put a demand on the pharmaceutical industry to treat the two enantiomers of a chiral drug as two different compounds. As an example, only 3% of the synthetic drugs marketed in 1990 were sold as pure enantiomers, whereas the corresponding number is increased to be

>75% in 2000. Recently, chiral drug sales surge toward $100 billion per year.[6] To account for the need of pure single enantiomers, research for new enantioselective analytical and synthetic methods has accelerated.

1.3. Primary sources of pure enantiomers

Four primary sources of pure enantiomers are used in the access to enantiomerically pure drugs and agrochemicals.[7]

The interconversion of naturally occurring chiral molecules that exist in nature in enantiomerically pure form (e.g., carbohydrates, terpenes, aminoacids and alkaloids). In many cases, pure enantiomers can directly be recovered from plant or animal materials by extraction techniques or protein hydrolysis.

De novo fermentation (microbial synthesis) of the inexpensive and abundantly available carbohydrate feedstock, such as sucrose or molasses. This is an important source of relatively simple molecules such as lactic, tartaric and L-amino acids and other complex substances such as antibiotics, hormones and vitamins.

The access to pure enantiomers from either chiral or prochiral starting materials. This is one of the most fascinating topics in organic synthesis.
The separation of enantiomers by crystallization or chromatography.

1.4. Synthetic methodology towards enantiomerically enriched substances

Synthetic routes to enantiomeric compounds can be divided into three categories depending on the type of raw material used (Fig. 1.3).

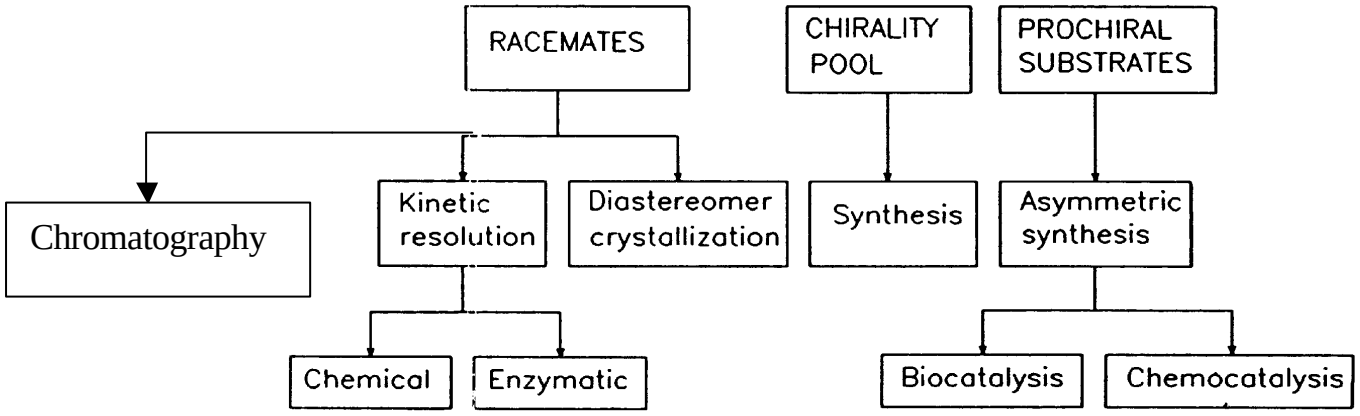
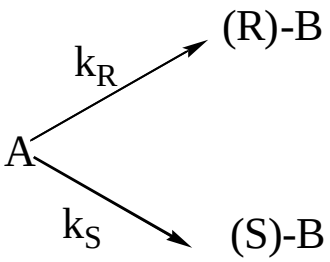
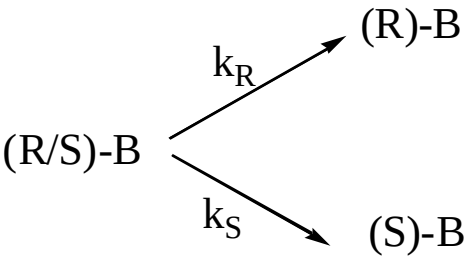


Fig. 1.3: Methods for enantiomeric preparation.

In principle, two ways are desired in the preparation of enantiomers[2,8], (a) Stereoselective synthesis (b) Resolution of the racemate.



(a) Stereoselective synthesis



(b) Resolution of the racemate

1.4.1. Stereoselective synthesis

In enantioselective synthesis, a chiral compound is synthesized from an achiral precursor in such a way that the formation of one enantiomer predominates over the other.[2] The enantioselectivity of the reaction is induced by the presence of a diastereomeric complex and is a result of the formation of two distinct diastereomeric transition states separated in energy by the amount $\Delta\Delta G^{++} > 0$. The ratio of the rate constants for the formation of the two enantiomers, k_R and k_S , is related to $\Delta\Delta G^{++}$ according to equation 1.1.[9] Assuming a kinetically controlled reaction, the k_R/k_S ratio will be reflected in the relative amount of each enantiomer formed.

$$\Delta\Delta G^{++} = \left| RT \ln (k_R/k_S) \right| \qquad \text{(Eq. 1.1)}$$

The diastereomeric complex may be required either in a stoichiometric amount with respect to the reactant, or in a catalytic amount. However, complicated preparation procedures or expensive reagents can sometimes limit the availability of certain diastereomeric complexes. For large-scale syntheses, processes are often developed in which the chiral catalysts are recycled.[8] An example of diastereomeric complexes used in enantioselective hydrogenation of allylic alcohols is given in chapter 6.

1.4.2. Resolution of racemates

Despite the impressive new progress in enantioselective synthesis, the dominant production method to obtain single enantiomer in industrial synthesis consists of the resolution of racemates.[6] The resolution of enantiomers can be divided into four categories (Fig. 1.4) direct preferential crystallization, crystallization of diastereisomeric salts, chromatography and kinetic resolution.

a) Preferential crystallization (also referred to as resolution by entrainment) is widely used on the industrial scale, e.g. in the manufacture of chloramphenicol[11] and α-methy-L-dopa.[12]
Haarmann & Reimer, the market leader in synthetic (-)-menthol, utilizes the preferential crystallization of menthyl benzoate enantiomers. This can be induced by seeding the bulk with one of the

pure enantiomers. This process is used in the production of (-)-menthol. [13]

This process is technically feasible only with racemates that form conglomerates (ones that consist of mechanical mixtures of crystals of the two enantiomers in equal amounts). Unfortunately, less than 20% of all racemates are conglomerates, the rest comprising true racemic compounds that can not be separated by preferential crystallization. The success of the preferential crystallization is depending on the fact that for conglomerate, the racemic mixture is more soluble than either of the enantiomers.[7]

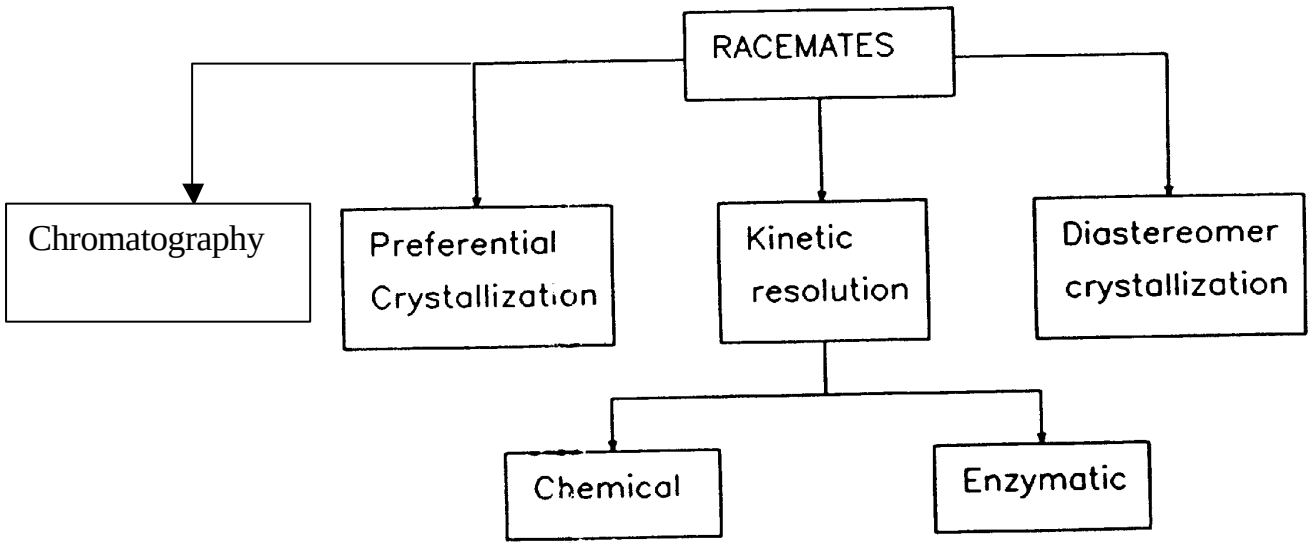


Figure 1.4: Methods used in the resolution of racemates.

b) Diastereomer Crystallization. In case of the racemate is a true racemic mixture, this can not be separated by preferential crystallization, but can be resolved using diastereomer crystallization developed by Pasteur in 1848. A solution of the racemic mixture in water or methanol is allowed to react with a pure enantiomer (resolving agent), thereby forming a mixture of diastereomers that can be separated by crystallization.

c) Catalytic kinetic resolution. The third method used in the resolution of racemates is the kinetic resolution. The success of this

method is depending on the fact that the two enantiomers react at different rates with a chiral entity. From the economic point of view, the chiral entity should be present in catalytic amounts; it may be a biocatalyst (enzyme or a microorganism) or a chemocatalyst (chiral acid or base or a chiral metal complex). Examples of the kinetic resolution of secondary alcohols using either enzyme or a metal catalyst are discussed in details in the following chapters.
A very well known example of the kinetic resolution is the Pasteur's fermentation of an aqueous solution of racemic ammonium tartrate by *Penicillium glaucum mold.*

As a simple definition, the kinetic resolution is a process in which one enantiomer of a racemic mixture is transferred faster than the other into a product.

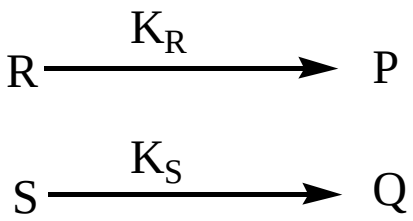


Fig. 1.5: Catalytic kinetic resolution.

The kinetic resolution occurs when $K_R \# K_S$ and the reaction is stopped somewhere between 0 and 100% conversion. Ideally one enantiomer reacts much faster than the other, for example, if (R) (reactant) is the faster reacting enantiomer ($K_R > K_S$). In this case, and at 50% conversion of the initial 50/50 mixture leads to a final mixture of 50% (S) (reactant) and 50% product (P) (Fig. 1.5).

1.5. Enzymatic biocatalytic kinetic resolution

Enzymes are biological catalysts of extraordinary selectivity and efficiency.[3] They catalyze and regulate reactions of important biochemical pathways. In common with all catalysts, they accelerate the attainment of chemical equilibrium but can not mediate a thermodynamically unfavorable reaction. Since they are chiral, they are able to assert a dramatic chiral influence on the reaction due to the chiral active site they owe. The reactant molecules are bound

instantaneously to the active site while the reaction takes place. Only one enantiomer of a chiral reactant fits it properly and is able to undergo the reaction while the second enantiomer is left unreacted and in enantiomerically pure form.

In fine chemical industry, biocatalysis is generally used to produce enantiomeric molecules that are either pharmaceutical products or used as chiral building blocks for numerous pharmaceutical compounds. Also, antibiotics and therapeutic proteins are produced biologically.
Some well known examples of enzymatic kinetic resolution in pharmaceutical industry include Andeno´s production of intermediates for the heart drugs diltiazem (calcium channel blocker) and captopril (antihypertensive), the production of single isomer form of the analgesic *S*-ibuprofen and (-) lactam for the HIV drug abacavir launched by ChiroTech and the BASF´s production of chiral amines using a lipase in organic solvents.

1.5.1. Historical development of the use of enzymes[14]

In fact, the history of enzymes returns back thousands of years to ancient Greece. Since then scientific research has turned our life into the best for human being.
Enzymes from microorganisms were used in ancient cooking, baking, brewing, alcohol production and cheese-making. These enzymatic processes were known since prehistoric times. In 1833, Payen and Persoz (from France) isolated an enzymatic complex from malt, naming it "diastase", and the Swedish chemist Jöns Jacob Berzelius described the first enzymatic hydrolysis of starch. In 1836, Theodor Schwann (from Germany) isolated a substance responsible for albuminous digestion in the stomach and named it “pepsin”, the first enzyme prepared from animal tissue. The fermentation of sugar to ethanol by yeast is a process that has been known for very long time. In 1839 the German chemist Justus von Liebig developed a mechanistic explanation for the role of yeast in the fermentation process. He viewed the yeast present in the fermentation mixture as a decomposing matter that emitted certain vibrations [the sugar atoms suffer a displacement; they rearrange themselves in such a way as to form alcohol and carbon dioxide (spontaneous reaction)].

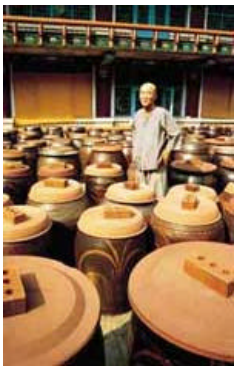
In 1858, the French chemist and biologist Louis Pasteur proved that fermentation occurs only in the presence of living cells. This divergence in the understanding of the role of yeast in the fermentation process caused heated debate between Liebig and Pasteur. In 1897 the German chemists Eduard Buchner and Hans Buchner discovered that a cell-free extract of yeast could cause alcoholic fermentation. The yeast cell produces the enzyme, and the enzyme brings about fermentation. In 1883, Johan Kjeldahl from Copenhagen developed a method for detecting nitrogen. This method was extensively used in the determination of protein in food. This was based upon the determination of nitrogen existing in amino acids, which constitute proteins. The method was the basis for the development of quantitative enzymology and general biotechnology.

L. Pasteur

1858

J. Kjeldahl

1883



J. Takamine

1891

E. Fischer

1894

Röhm's family

1914

In 1891, the Japanese biochemist Jokichi Takamine, developed in the USA, the commercial production of Koji from the fungus *Aspergillus oryzae* and called it "takadiastase". Koji is prepared from steamed rice into which a mixture of mould fungi is inoculated. In 1894, the German chemist Emil Fischer developed the lock-and-key theory based on the properties of glycolytic enzymes. He recognized that a vital function of enzymes also depends on the stereochemical configuration of the molecules (e.g. the position of the atoms relative to one another). Fischer was the first to determine the molecular structures of glucose (or grape sugar) and fructose and to synthesize them from glycerol in 1890.

In 1903, Victor Henri concluded in Paris that an enzyme combines with its substrate to form an enzyme-substrate complex as an essential step in enzymatic catalysis.
In 1913, based on this idea, the general theory of enzyme action was expressed mathematically by Leonor Michaelis of Germany and Maud Lenora Menten of Canada. They postulated that the enzyme E first combines with its substrate S to form an enzyme-substrate complex ES in a relatively fast reversible reaction: $E + S \longrightarrow ES$. The latter complex then breaks down in a second, slower reversible reaction to yield the reaction product P and the free enzyme:
$ES \longrightarrow P + E$.
In 1914, Burnus, the first new detergent product launched in the market by Röhm and his wife from Germany. The enzyme was so effective that only a small quantity was required. Burnus was originally sold in tablet form as a stain remover, one tablet being mixed with 10 liters of water. Afterwards, it was modified and sold as a washing powder in 50 g boxes. In 1926, James B. Sumner of the USA demonstrated that enzymes are proteins and performed the first crystallization of an enzyme.
At the same time, the Danish scientist K. Lindestroem-Lang investigated the ionization of proteins and laid down a basic formalism for the purification of enzymes. In 1953, James Dewey Watson of Indiana, USA, and Francis Harry Compton Crick of Cambridge, UK, proposed the double-helix structure of DNA. They shared the Nobel Prize in 1962 with Maurice Hugh Frederick Wilkins, whose diffractograms were used for their proposal.
George Wells Beadle, Edward Lawrie Tatum and Joshua Lederberg (USA) received the Nobel Prize in 1958, with Beadle and Tatum for concluding that the characteristic function of the gene was to control the synthesis of a particular enzyme and Lederberg for his discoveries concerned with the genetic recombination and the organization of the genetic material of bacteria.
K. Linderstrøm -Lang proposed the following definitions for the structural hierarchy of proteins like enzymes:[7]
Primary structure:
It refers to the chemical structure of the polypeptide chain or chains in a given protein, i.e. the number and sequence of amino acid residues linked together by peptide bonds
Secondary structure:
It refers to any such folding which is brought about by linking together

the carbonyl and imide groups of the backbone by means of hydrogen bonds

Tertiary structure:

An organization of secondary structures linked by looser segments of the polypeptide chain stabilized (primarily) by side-chain interactions. Disulphide bonds are included in this level (essential for the catalytic activity of the enzyme).

Quaternary structure:

The aggregation of separate polypeptide chains into the functional protein (several polypeptide chains bind together to form larger oligomers).

1963-1974, start for the commercial utilization of enzymes e.g. alcalase protease in detergents. Launching of glucoamylase free of transglucosidase. It allowed starch to be broken down into glucose. Since this time, all glucose production has switched from traditional acid hydrolysis to enzymatic hydrolysis. From 1984- present recombinant DNA technology brought revolution to the field of the development of new enzymes. Molecular screening and protein engineering start to be efficient processes in finding many more of nature's own enzyme variant.

1.5.2. Enzymes in organic solvents

Enzymatic catalysis in non-aqueous media significantly extents conventional aqueous-based biocatalysis.[15] Water is a poor solvent for nearly all applications in industrial chemistry since most organic compounds of commercial interest are very sparingly soluble and are often unstable in aqueous solutions. Furthermore, the removal of water is tedious and expensive due to its high boiling point and high heat of vaporization. Side reactions like hydrolysis, racemization, polymerization and decomposition are often accompanying such processes. Chemists realized these limitations due to the use of enzymes in aqueous media and started long time ago to develop enzymatic procedures in organic solvents. Biocatalytic transformations in organic solvent offer the following advantages:

1. Better overall yield and the recovery of the product is facilitated by the use of low-boiling point organic solvents.

2. Non-polar substrates are converted at a faster rate due to their increased solubility.[16]
3. Microbial contamination is negligible in case of using living cells in biotransformation.
4. Deactivation and/or substrate or product inhibition is minimized.
5. Side reactions such as unfavorable hydrolysis are largely suppressed.
6. Immobilization of enzymes is not required, the enzyme can be recovered by simple filtration.
7. Denaturation of enzymes is minimized in organic solvents.
8. Shifting thermodynamic equilibria to favor synthesis over hydrolysis.

However, the use of enzymes in organic solvents has some drawbacks like:

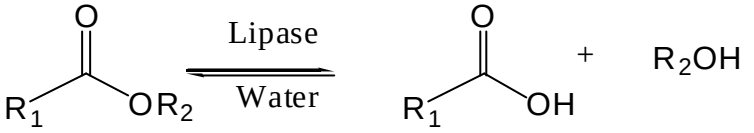
1. Their decreased catalytic activities (due to the heterogeneous system), which are generally several orders of magnitude, lower than in aqueous solution.

2. Many enzymatic reactions are prone to substrate or product inhibition, which deactivates the enzymes at higher substrate or product concentration leading to a decrease in the reaction rate and enantioselectivity.

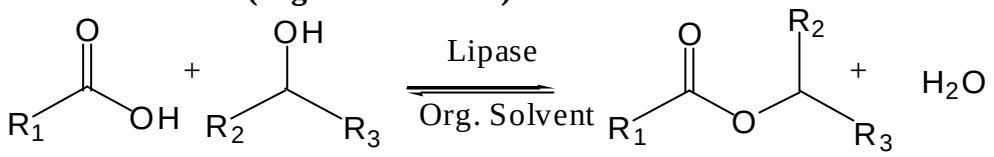
1.5.3. Lipases

Lipases (E.C.3.1.1.3) belong to the family of the serine hydrolases and can be found in animals, plants and microorganisms, where their function is to catalyze the hydrolysis of triglycerides to glycerol and fatty acids. They catalyze hydrolysis as well as the reverse reaction, esterification, transesterification and interesterification (Fig. 1.6.).

1. Hydrolysis (aqueous medium)

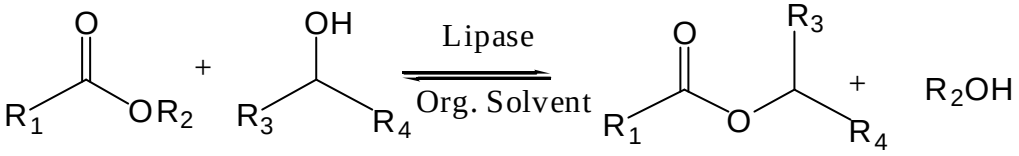


2. Esterification (organic medium)

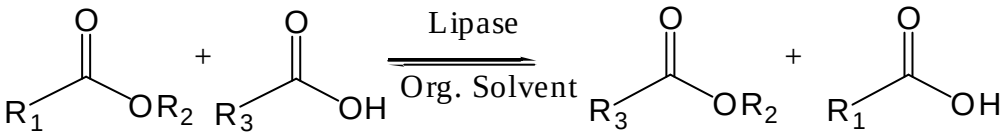


3. Transesterification

a) Alcoholysis



b) Acidolysis



4. Interesterification

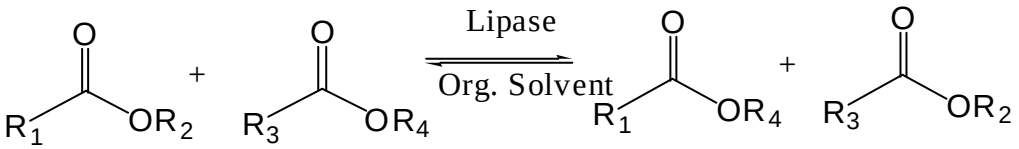


Fig. 1.6: Reactions catalyzed by lipases**.**

The direction of the reaction is depending on the adequate solvent medium used, e.g. aqueous or organic solvents. (Fig. 1.6)
The kinetic resolution of racemic secondary alcohols in organic solvent systems is a well-known example of the application of lipase in organic synthesis.

1.5.4. Kinetic resolution of alcohols

A variety of enantiomerically pure secondary alcohols can be obtained via lipase-catalyzed transesterification using ethyl acetate as acylating agent in organic solvents[17] or via the hydrolysis of the corresponding acetate in aqueous solution.[18,19]

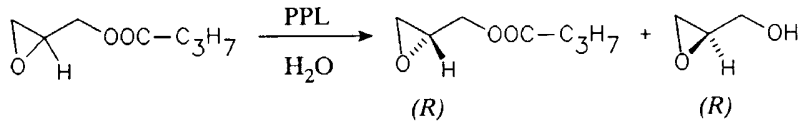


Fig. 1.7: (PPL)-catalyzed hydrolysis of racemic glycidyl butyrate.

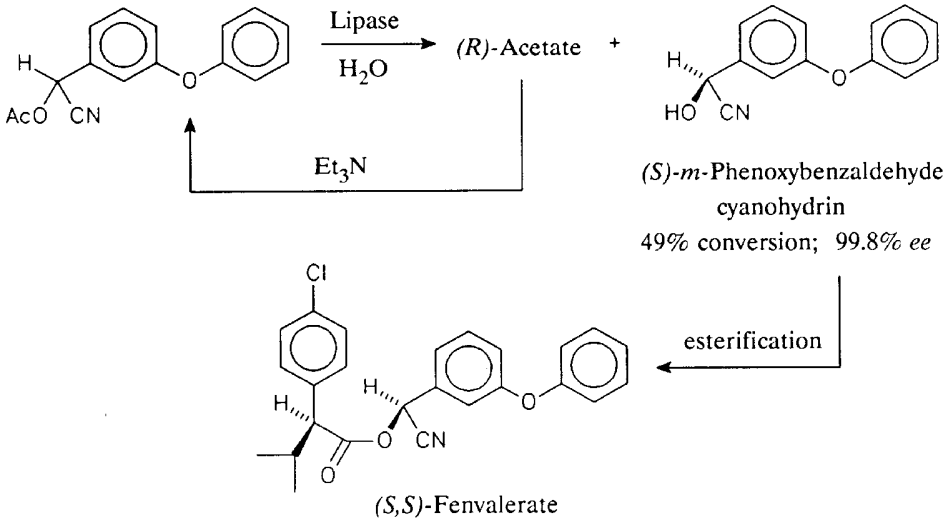


Fig. 1.8: Enantioselective hydrolysis of the acetate of racemic *m*-phenoxybenzaldehyde cyanohydrin.

Enantiomeric glycidyl derivatives, which are of interest as intermediates to enantiomeric beta blockers, have been synthesized by the *Porcine pancreas* lipase (Fig. 1.7). Another reaction of considerable interest is the enantioselective hydrolysis of the acetate of racemic *m*-phenoxybenzaldehyde cyanohydrin[20] to yield a key

intermediate for the enantiomerically pure pyrethroid insecticide (S,S)-fenvalerate (Fig. 1.8).

1.5.5. Mechanism of lipase-catalyzed reactions

Lipases differ widely in the number of amino acids in their primary sequences. For example, the *Candida rugosa* lipase contains more than 500 amino acids whereas, *Candida antarctica* lipase B contains less than 200 amino acids. According to their positional selectivity, lipases are often divided into three classes, namely, sn-1,3-specific, sn-1(3)specific, or nonspecific lipases.[21] The common feature of all lipases is that their active site is built up of the three amino acids serine, aspartate or glutamate and histidine, which are referred to as the catalytic triad. The three-dimensional structure of all lipases follows a common motif, the α/β-fold of hydrolase, where lipase is consisting of eight mostly parallel β-sheets surrounded on both sides by α-helices[22] (Fig. 1.9).

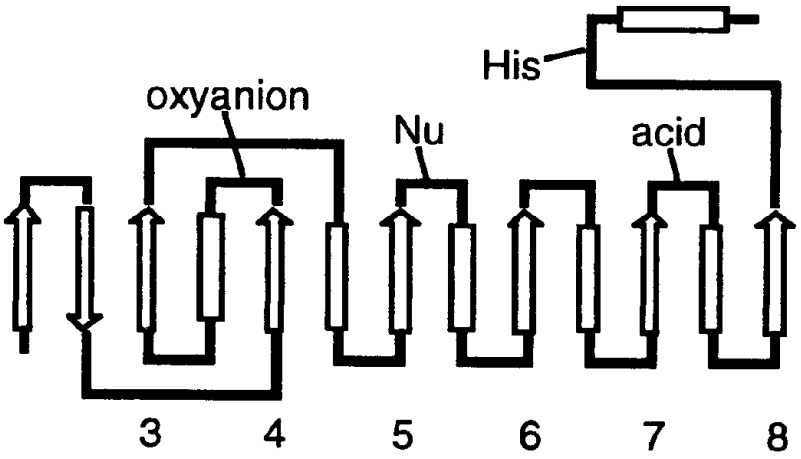


Fig. 1.9: Schematic representation of α/β-fold of hydrolase.[22]

The triad and several oxyanion-stabilizing residues are thought to compose the active center of lipases. A lid or flap (a helical segment), which blocks the active center, is responsible for the important characteristics of lipase-interfacial activation which let them to be distinguished from other hydrolases such as serine protoases or esterases. Based on the x-ray structure of lipase before and after binding to the substrate, researchers believe that lipases are

activated before they take part in biochemical transformation reactions.[24]

The water-soluble lipases take part in the catabolism of lipids. Thus, lipase reaction takes place at a water-oil interface.
A freely dissolved lipase in the absence of an aqueous/lipid interface resides in its inactive state and a part of the enzyme molecule covers the active side. However, when the enzyme contacts the interface of a biphasic water-oil system, a short α-helix (the lid) is folded back leading to activation of the lipase. In other words, with no substrate present, the lid is closed and the enzyme is inactive, whereas in presence of substrate, the water-oil interface exists, and the lid is opened then lipase is active.[15] In contrary, cutinase and acetylcholine esterase lack a lid and show no interfacial activation.[25] The catalytic triad (Ser, His and Asp), the actual chemical operator in the active side, is shown in the mechanism of a serine-hydrolase-catalyzing hydrolysis of an ester (Fig. 1.10). The special arrangement of these three groups perform a decrease of the pK-value of the serine hydroxy group thus enabling it to perform a nucleophilic attack on the carbonyl group of the substrate R^1COOR^2 (step I). Thus, the acyl moiety of the substrate is covalently linked onto the enzyme, forming the acyl enzyme intermediate and
liberating the alcohol R^2OH. In the second step, water (regarded as the nucleophile, Nu.) attacks the acyl-enzyme intermediate, regenerating the enzyme and releasing the carboxylic acid R^1COOH (step II). Depending on the medium used (aqueous or organic medium) any other nucleophile can compete with water for the acyl-enzyme intermediate thus leading to a number of synthetically useful transformations:

1. If the alcohol R^4OH attack the acyl-enzyme intermediate, the ester R^1COOR^4 is formed (interesterification reaction).[26]

2. Attack of the amine R^3NH_2 results in the formation of an amide R^1CONHR^3 (enzymatic aminolysis of esters).[27]

3. Attack of hydrogen peroxide yields the peracids R^1COOOH.[28]

4. Hydrazinolysis yields hydrazides[29] and the action of hydroxylamine results in the formation of hydroxamic acid derivatives.[30]

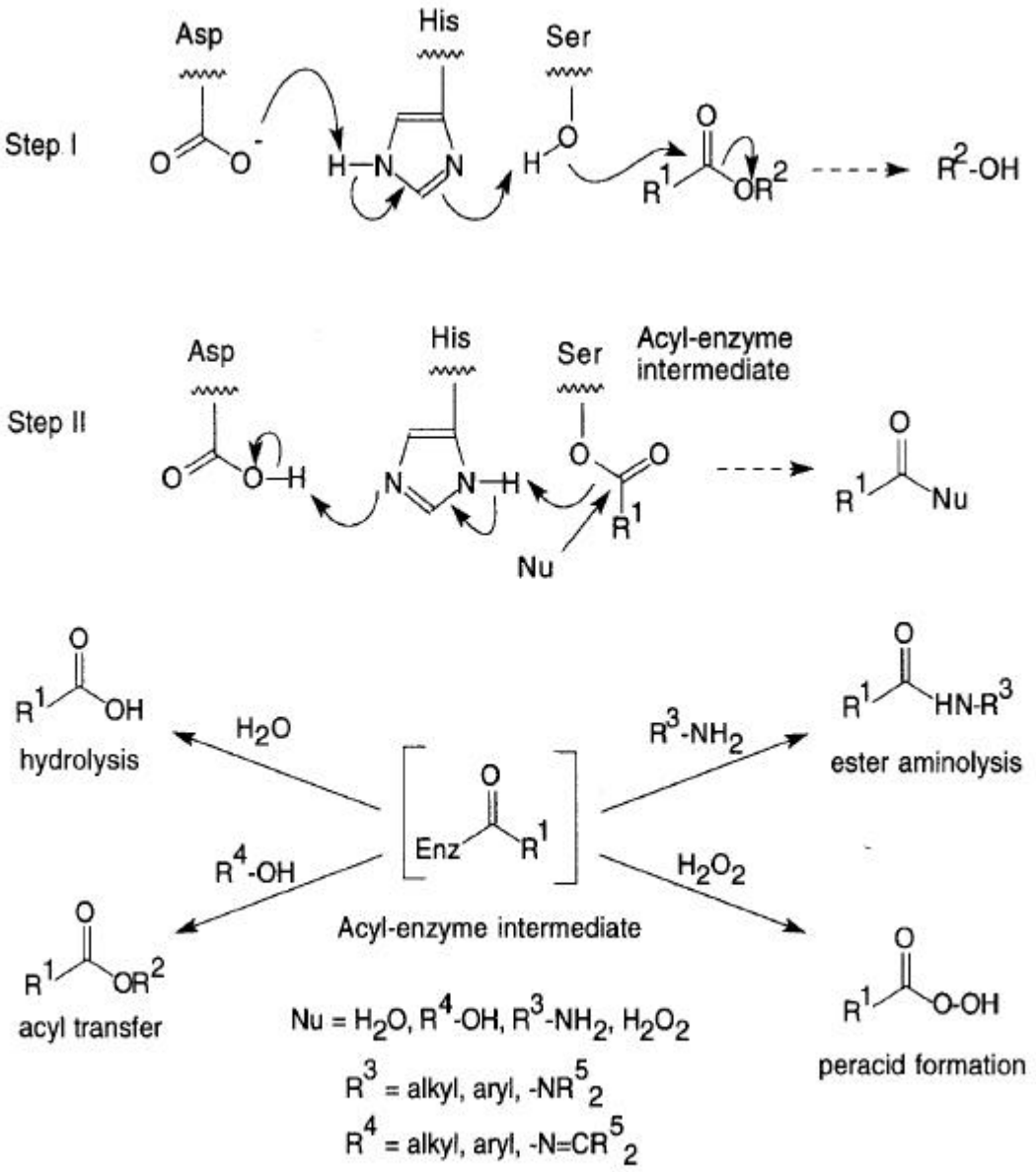


Fig. 1.10: The serine hydrolase mechanism.

1.5.6. Kinetics of the lipase-catalyzed esterification reaction

Lipases do not follow the Michaelis Menten kinetics, but rather show a sigmoidal increase in reaction velocity with increasing the substrate concentration. However, many enzymatic reactions follow a pattern of

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \overset{k_2}{\rightleftharpoons} E + P$$

k_1 $(L\ mmol^{-1}\ min^{-1})$

k_{-1} (min^{-1})

k_2 (min^{-1})

Eq.: 1.2

$$v = \frac{v_{max} [S]}{K_M + [S]} \qquad K_M = \frac{k_{-1} + k_2}{k_1}$$

K_M $(mmol^{-1})$ Michaelis constant

the basic model presented by Michaelis-Menten (Eq. 1.2). From the steady state assumption, they postulated the existence of an enzyme-substrate complex (ES), which is formed by the reversible reaction of substrate S and the enzyme E. The dissociation of the complex (ES) to E and P (product) is assumed to be the rate determining step.

k_1 = rate constant for the association constant of the ES-complex

k_{-1}= The dissociation constant of ES-complex into E and S

k_2 = ´turnover number` (also referred to k_{cat})

Quite often enzymatic reactions involve two or more substrates, e.g. lipase-catalyzed esterification (Fig. 1.11). In this case, the standard state assumption is also valid. Two substrate enzymatic reactions often follow three mechanisms, rapid equilibrium random bi bi, ordered bi bi and Ping-Pong bi bi mechansim.[23]

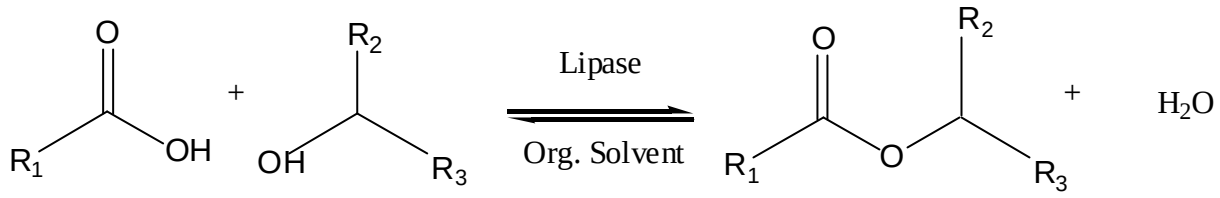


Fig. 1.11: Lipase-catalyzed esterification reaction.

The lipase-catalyzed esterification follows the Ping-Pong Bi Bi kinetics. If a fatty acid (A) is bound to the lipase in the first step, followed by releasing water (P), then, the alcohol (B) is bound to the acyl-enzyme (F). Finally, the new ester (Q) is formed, regenerating the enzyme. The lipase oscillates between the free enzyme form (E) and the acyl enzyme intermediate (F) (Fig. 1.12)

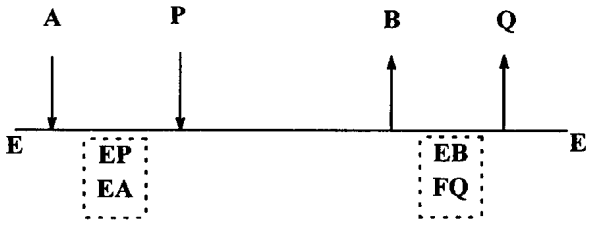


Fig. 1.12: Ping-Pong Bi Bi mechanism.

The reaction equation corresponds to the following expression:

$$\nu = \frac{V_{max}\,[A]\,[B]}{K_m B[A] + K_m A[B] + [A]\,[B]}$$

In the case of competitive substrate inhibition, the reaction rate can be expressed as:

$$\nu = \frac{\nu_{max}\,[A]\,[B]}{K_m B[A] + K_m A[B](1+ [B]/k_i + [A]\,[B])}$$

If the concentration of alcohol B ([B]) in the reaction phase is constant, this equation can be written as Michaelis Menten equation:

$$\nu = \frac{\nu_{max}\,[A]}{K_m + [A]}$$

1.6. Analytical methods: determination of the enantiomeric excesses (ee)

The development of accurate non-chiroptic methods for the determination of enantiomeric purity, which began in the late 1960´s, has been critical for the development of enantioselective synthesis. Thus a prerequisite in the enzyme-catalyzed kinetic resolution of secondary alcohols is a precise and reliable assessment of the degree of enantioselectivity, enantiomeric purity and conversion. Among these methods are: 1) polarimetric methods, 2) gas chromatographic methods, 3) liquid chromatographic methods and 4) NMR spectroscopy. The modern and most sensitive methods used in the determination of enantiomeric purity of the outcome of kinetic resolution reactions, allowing a detection as little as 0.1% of one enantiomer in the presence of another, are chiral GC and HPLC methods. In this work, the utility of gas chromatography in the determination of enantiomeric excesses of enantiomers resulting from the kinetic resolution of secondary alcohols will be demonstrated.

$$\%\ ee = \frac{R - S}{R + S}.\ 100 \quad \text{For } R > S$$

Enantiomeric ratio (E): The ratio of the rate constants for the reaction of pair of enantiomers with an optically active reagent or catalyst (e.g., ER= KR/KS). E is a measure of the efficiency of a kinetic resolution and varies with the extent of the conversion[7].

Polarimetric and gas chromatographic methods (old and modern methods, respectively) used in the determination of enantiomeric excess are compared below.

1.6.1. Polarimetric methods

The classical method of determining the enantiomeric excess of a sample is to measure its enantiomeric purity using a polarimeter. The optical enantiomeric is derived by expressing the measured specific rotation $[\alpha]$ as a percentage of the specific rotation of the pure enantiomer $[\alpha]_{max}$. Measurements are taken under standardized conditions indicating the wavelength of the incident plane polarized light, the temperature of the measurement, the solvent and the concentration of the analyte in grams per 100 cm^3 (also possible as neat liquid).
The measurement is fast and straightforward and requires a relatively inexpensive polarimeter. However, it possesses some disadvantages. Among these disadvantages are the sample under analysis must be homogenous, devoid of traces of chiral impurities and should be isolated from a reaction mixture without accidental enantiomeric enrichment. For compounds with low rotatory power, large samples may be required to produce measurable optical rotations. The maximum specific rotation $[\alpha]_{max}$ of the pure enantiomer [32] should be known with certainty adding to that, specific rotations are sensitive to temperature and concentration and estimated errors from these combined effects are at least +/-4%. The specific rotation is defined to be:

$$[\alpha]^t_\lambda = \frac{100 \cdot \alpha}{l.c}$$

α = observed optical rotation
l = cell path length in dm (usually *l* = 1)
c = concentration in g per 100 cm^3 of solvent
t = temperature (Celsius)
λ = Wavelength of incident light (nm)

The enantiomeric purity is $P = \frac{[\alpha]}{[\alpha]_{max}}$

1.6.2. Gas chromatographic methods

An attractive method for the determination of the enantiomeric purity of substrates and products resulting from the enzyme-catalyzed kinetic resolution of secondary alcohols is chiral gas chromatography (GC).[31] This sensitive method is unaffected by the presence of impurities in the analyzed sample, is quick and simple to carry out. This method is based on the fact that molecular association may lead to an efficient chiral recognition leading to enantiomeric separation when a chiral stationary phase (e.g. cyclodextrins) is used in GC. The gas (mobile phase, e.g. hydrogen, helium, nitrogen) is carrying the chiral analyte through the stationary phase. The enantiomers to be analyzed undergo rapid and reversible diastereomeric interactions with the chiral stationary phase and hence may be eluted at different times. One of the limitations associated with this method is that the sample should be sufficiently volatile, thermally stable and resolvable on the chiral stationary phase used. The measurement of the enantiomeric excess using GC is linked with a high degree of precision (+/-0.05%) so that reliable data may be obtained.[32] It is to be noted that high enantiomeric excesses (ee) up to 99% may be detected.

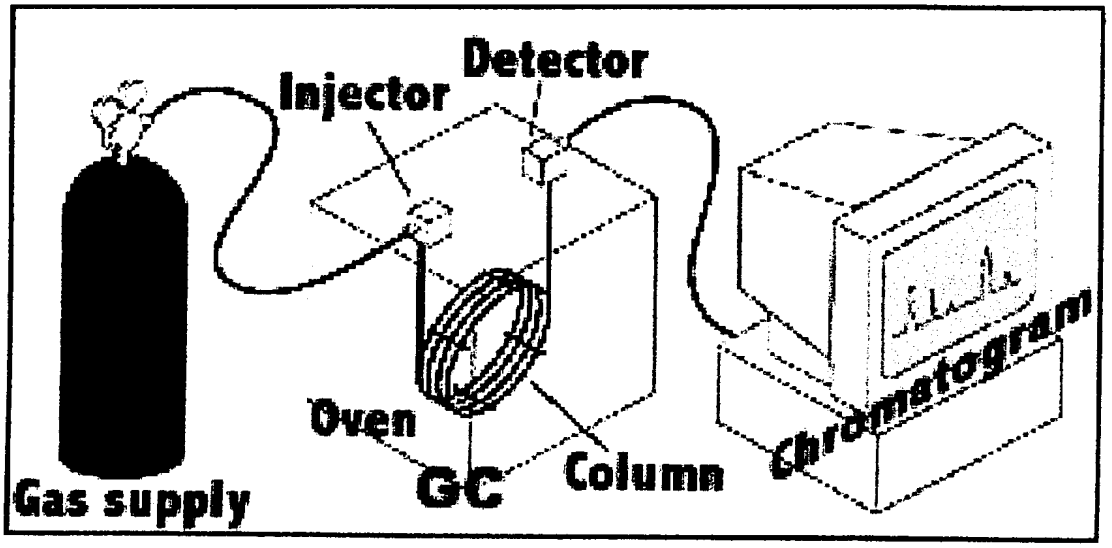


Fig. 1.13: A schematic representation of gas chromatography

2. Scope

Biocatalytic transformations in organic solvents (e.g. esterification or transesterification) has several advantages, one of the most important advantage is the shifting of thermodynamic equilibria to favor synthesis over hydrolysis allowing several important reactions to take place. However, the use of enzymes in organic solvents has some drawbacks like their decreased catalytic activity and substrate or product inhibitions (the advantages and disadvantages of using enzymes in organic solvents are discussed in details in section 1.5.2).

The scope of the investigations performed in this thesis is a trial to find a suitable way to overcome the disadvantages accompanied to the use of lipases in organic solvents. The idea is to use macrocyclic additives (e.g. modified and free cyclodextrins) and immobilized methods (e.g. sol-gel procedure) to enhance the conversion and enantiomeric ratio in lipase-catalyzed transesterification in organic solvent. The modified methods will be used in the production of enantiomerically pure compounds used as chiral building blocks.

Attempts to find a suitable innocuous acyl donor and to produce enantiomers in gram scale and in high enantiomeric excess using lipase-catalyzed kinetic resolution of the racemic substarte will be also considered.

The lipase-catalyzed kinetic resolution of secondary alcohols can be an efficient method in the production of enantiomerically pure compounds if a reliable method is used in the determination of the enantiomeric excesses of substrates and products resulting from the enzymatic resolution. Gas-chromatography will be used as an efficient method in the determination of the enantiomeric excesses of substrates and products resulting from the enzymatic reactions.

3. Gram-scale preparation of (R)- and (S)-1-(2-Furyl)ethanol via lipase-catalyzed irreversible transesterification using isopropenyl acetate in organic solvents

3.1. Introduction

Despite the impressive new progress in enantioselective synthesis, the dominant production method to obtain single enantiomers consists of the resolution of racemates.[6] Enantiomerically pure secondary alcohols are useful chiral auxiliaries in organic synthesis and they are preferably synthesized by enzymatic kinetic resolution of the racemates. This biochemical transformation process has become a standard reaction protocol in organic synthesis.[24] In the presence of a suitable acyl donor, an enzyme as well as a solvent, and at the optimum temperature, one enantiomer of the racemic mixture is selectively transferred to the corresponding ester leaving the second unreacted enantiomer in enantiomerically pure form. The reversible enzymatic process usually requires a long reaction time and a large excess of the ester as acyl donor to achieve a reasonable degree of conversion.[33] In order to render the process irreversible, the use of various activated esters like vinyl acetate,[34] trifluoroethyl esters, chloroethyl esters,[35] cyanomethyl esters,[36] and acid anhydrides[37] have been reported. However, these methods have some drawbacks, like the generation of toxic side products thereby deactivating the enzyme and inhibiting the formation of the required products,[38] or the generation of water which causes the undesired hydrolysis of the enantiomerically pure ester, leading to a decrease in the conversion and in the enantiomeric excess. Therefore, vinyl acetate has been used because the elusive vinyl alcohol tautomerizes to acetaldehyde, thereby shifting the equilibrium to the required product.

Yet, the generation of acetaldehyde resulting from the tautomerization of vinylalcohol when using vinylacetate as acyl donor has the following disadvantages:
1) Acetaldehyde leads to a deactivation of various lipases especially *Geotrichum candidum* and *Candida rugosa*, the later being one of the most important lipases having a broad applicability to substrates in organic synthesis.[39]
2) Acetaldehyde undergoes dimerization via aldolisation-crotonisation liberating water (unfavored in enzyme-catalyzed reactions in organic

solvents) which may hydrolyze the enantiomerically pure ester formed and hence decreases the conversion and the enantioselectivity.
3) Acetaldehyde can react with the enantiomerically pure alcohol generated yielding hemiacetals.

1-(2-furyl)ethanol and its derivatives are chiral building blocks of numerous natural products such as carbohydrates, macrolides, pheromones and alkaloids.[40] These chiral starting materials should be available as both enantiomers in high enantiomeric excess. Several accesses to chiral 1-(2-furyl)ethanol have been reported using titanium(IV)tartrate chemistry,[41] enantioselective reduction of 2-acetylfuran,[42] microbial[43] or enzymatic reduction[44] of its ketone, and enzymatic hydrolysis[45] and esterification.[46]

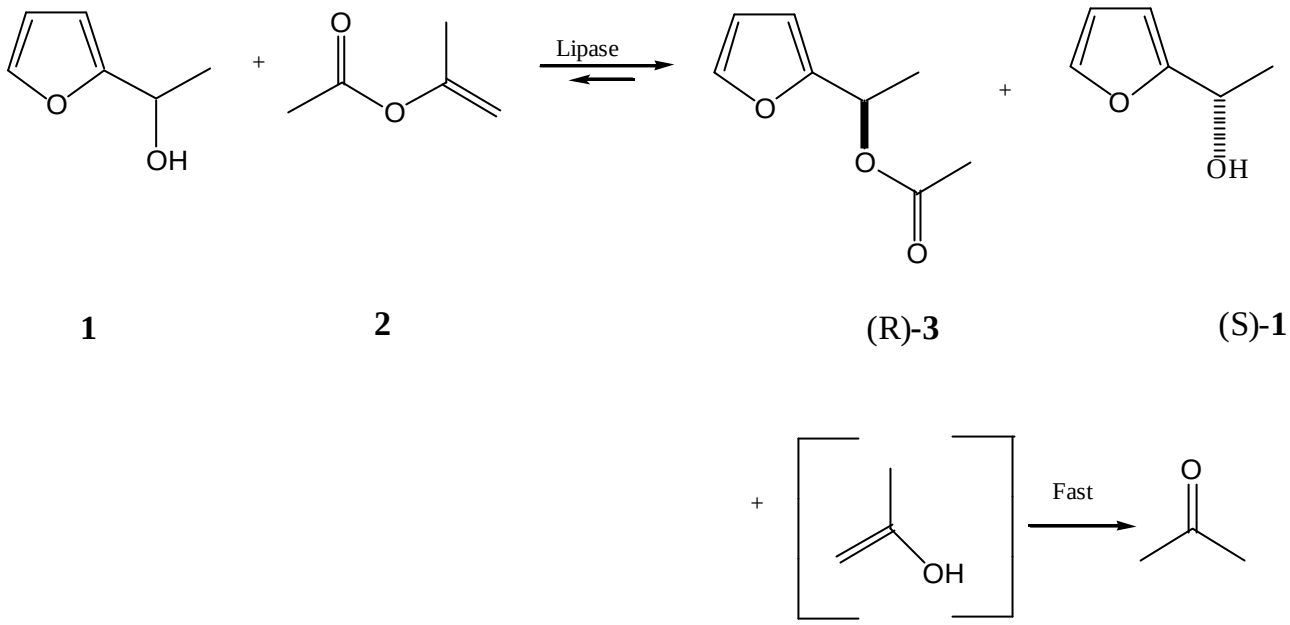
The lipase catalyzed transesterification of 1-(2-furyl)ethanol using vinyl acetate as acyl donor has been reported,[34] however the conversion was too low when using *Candida rugosa* lipase despite its broad applicability to a wide range of substrates. This is due to the sensitivity of the *Candida rugosa* lipase to acetaldehyde,[39] the unavoidable by-product in lipase-catalyzed acyl transfer reactions with vinyl esters.

Taking the disadvantage of vinyl acetate as acyl donor in enzymatic reactions into consideration, it was decided to develop an effective preparative method for the production of enantiomerically pure (R)- and (S)-1-(2-furyl)ethanol using isopropenyl acetate as an innocuous acyl donor. An efficient method for the preparation of highly enantiomerically pure (R)- and (S)-1-(2-furyl)ethanol through lipase-catalyzed transesterification of racemic 1-(2-furyl)ethanol in organic solvents has been found.

3.2. Results and discussion

3.2.1. Lipase-catalyzed enantioselective transesterification of 1-(2-furyl)ethanol

Different lipases were screened in catalyzing the transesterification of racemic 1-(2-furyl)ethanol using isopropenyl acetate as acyl donor in the organic solvent isooctane (2,2,4-trimethyl-pentane).



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

Fig. 3.1: Lipase-catalyzed transesterification of (**1**) using isopropenyl acetate as acyl donor in organic solvents.

Among the lipases tested were lipase from *Pseudomonas cepacia* (PSL), *Candida rugosa* (AYS), lipase AY 30, lipase type II, crude, from *Porcine pancreas* (PPL) and type VII from *Candida rugosa* (CRL), lipase from *Candida cylindracea* (CCL), *Hog pancreas*, lipase immobilized in sol-gel-AK from *Aspergillus niger* and lipase immobilized in sol-gel-AK on sintered glass from *Mucor miehei*. Transesterification was carried out at 40^{o}C in isooctane at a molar ratio of isopropenyl acetate to racemic (**1**) of 2:1 to ensure the irreversibility of the reaction.

A simultaneous base line separation of both substrate and product enantiomers was achieved using heptakis-(2,3-di-*O*-methyl-6-*O*-tert-butyldimethylsilyl)-*b*-cyclodextrin as a stationary phase by GC[47] with a separation factor α = 1.32 and resolution R_s = 17 for the ester (**3**) and α = 1.08 and R_s = 3.5 for the alcohol (**1**). The unusual elution order (S)-**3**, < (R)-**1**, < (S)-**1**, < (R)-**3** should be noted (Fig. 3.2).

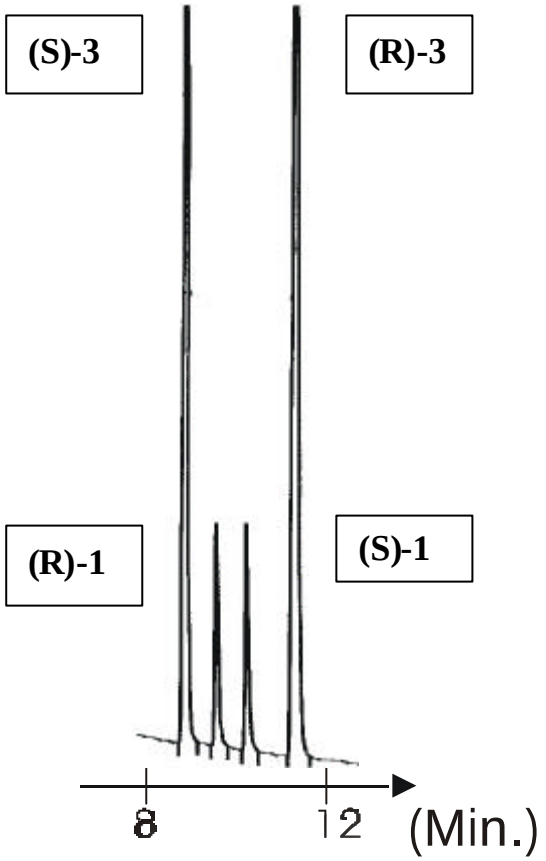


Fig. 3.2: Gas-chromatographic separation of racemic mixture of (**1**) and (**3**) on heptakis-(2,3-di-*O*-methyl-6-*O*-tert-butyldimethylsilyl)-*b*-cyclodextrin as stationary phase in GC.
Oven temperature was 65°C isothermal for 15 min.

The results are summarized in table 3.1.

Table 3.1: Lipase catalyzed transesterification of (R,S)-**1** in isooctane.

Lipase	Time (hr)	[%]ee_s (S)-**1**	[%]ee_p (R)-**3**	% conversion	E
PSL	21	75	99.9	43	>300
AYS	161	20	75	26	8.2
AY30	161	8	35	18	2.3
PPL	161	11	43	21	3.0
CCL	21	25	24	51	2.0
Hog pancreas lipase	161	24	48	35	4.0
Aspergillus niger lipase	161	<1	7.7	<1	1.2
Mucor miehei	161	31	99.9	24	n.d

ee_s: enantiomeric excess of substrate (alcohol). ee_p: enantiomeric excess of product (ester). n.d: Not determined. E: enantiomeric ratio[48].

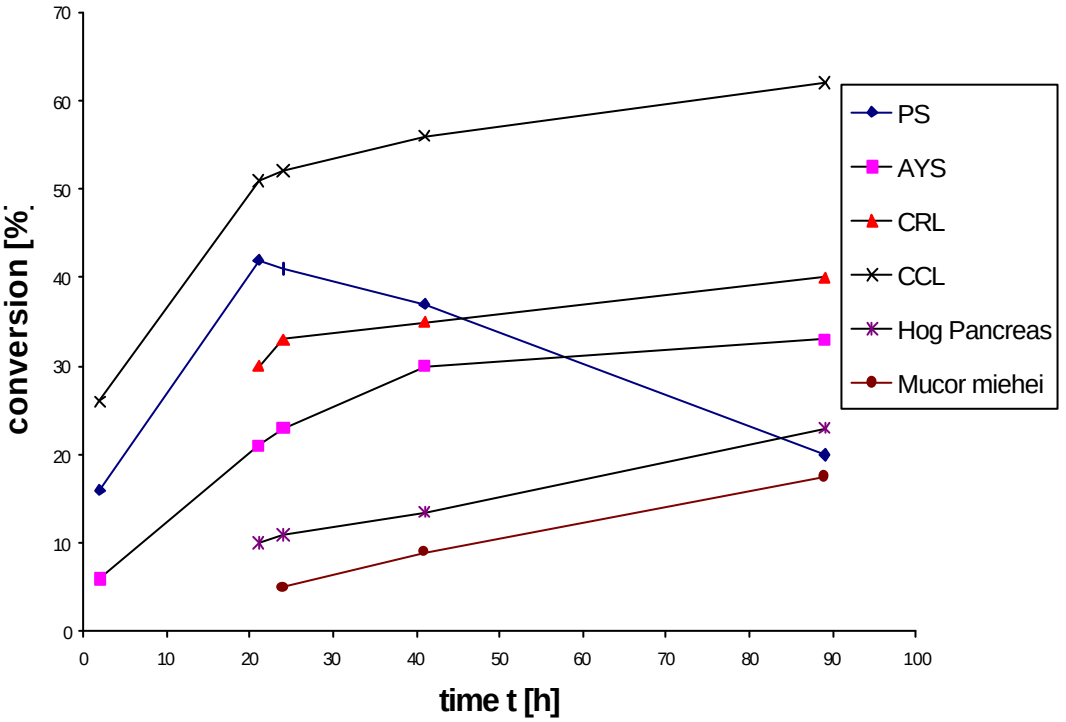


Fig. 3.3: Plot of the conversion against the time in the lipase-catalyzed transesterification of (**1**).

In all cases, (R)-**1** is the faster reacting enantiomer yielding (R)-**3** in high enantiomeric excess. In case of Lipase PSL and *Mucor miehei*, the esters produced are isolated in high enantiomeric excess (ee >99 %). PS lipase was the best lipase employed in the transesterification reaction of (**1**) in isooctane in regard to the enantiomeric excess of the remaining substrate (S)-**1** and that of the product (R)-**3** as well as the rate of conversion (43 % in 21 hrs) and enantiomeric ratio E > 300. However, a drop in conversion was noticed after 21 hrs (Fig. 3.3). It is assumed that this drop in conversion was due to: i) product inhibition which might occur due to the accumulation of the product, when the conversion is increased, thus competing with the active site of the enzyme. ii) acetone, the unavoidable by-product released from the tautomerization of vinyl alcohol resulting from the transesterification of (**1**), is playing an unknown role in decreasing the conversion and the enantiomeric excess of both substrate and product. Using molecular sieves, to scavenge the liberated acetone, enhanced the reaction rate and enantiomeric excesses of both substrate and products.
These results were a stimulus to find out the role of acetone in decreasing the conversion and the enantiomeric excess of both substrate and product.

3.2.2. Acetone: The undesired by-product in lipase-catalyzed transesterification of 1-(2-furyl)ethanol using isopropenyl acetate in organic solvents

In fact, using isopropenyl acetate as an innocuous acyl donor leads to the formation of isopropenyl alcohol (**4**) (resulting as a by-product in lipase-catalyzed transesterification reaction) which tautomerizes to acetone (Fig. 3.4). Acetone has been thought not to disturb the reaction rate and not to affect the enantiomeric excess of both substrate and product. Unexpectedly, it was found that acetone is an undesired by-product in lipase-catalyzed transesterification of 1-(2-furyl)ethanol.

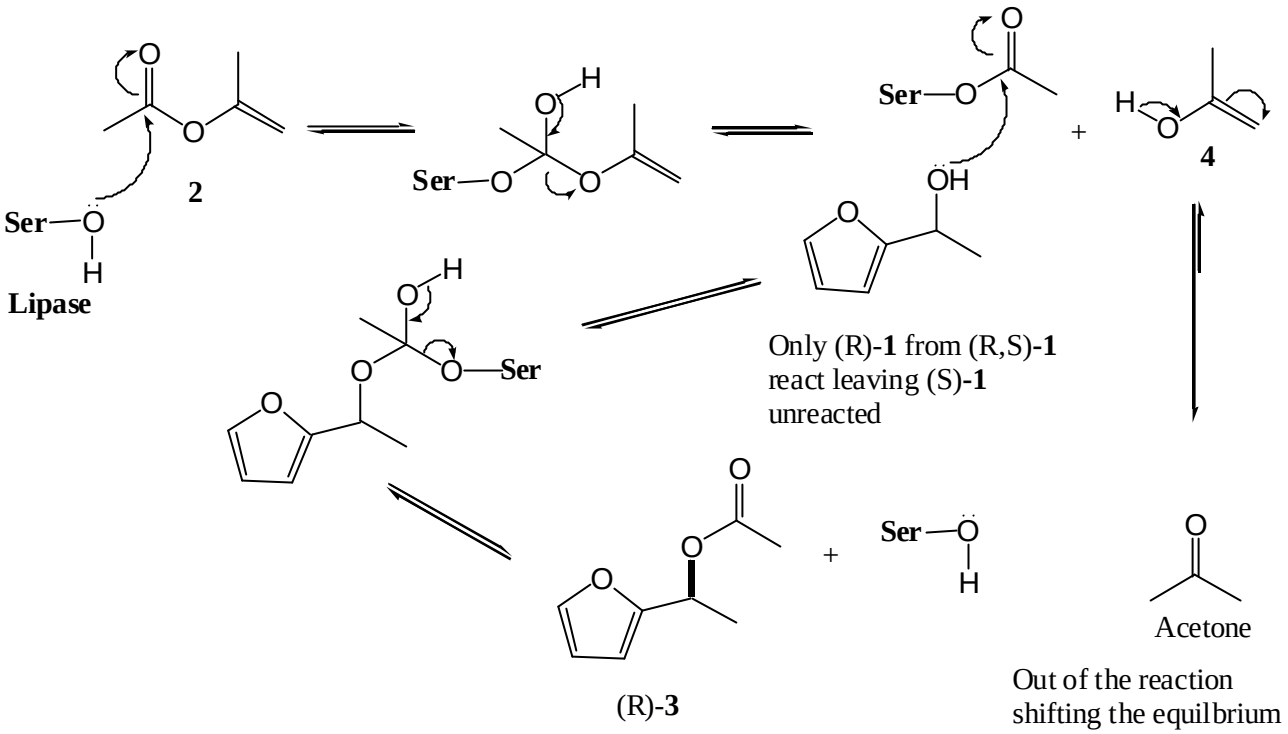


Fig. 3.4: Expected mechanism of the lipase-catalyzed transesterification of (**1**) using isopropenyl acetate as acyl donor in organic solvents.

Acetone is reacting in an undesired side reaction with the alcohol to form the acetal liberating water that affect the reaction rate as well as the enantiomeric excess of both substrate and product.

In fact, acetone was known to react with alcohol under acidic condition to form acetals. This procedure was used to synthesize the acetal (**4**) by reacting the alcohol (**1**) with acetone using CaI_2 as a catalyst. The yield was 65% of acetal (**4**). A base line separation of this compound was achieved by analysis on GC using the same column used for the resolution of 1-(2-furyl)-ethanol (Fig. 3.6). The chromatogram shows three peaks consisting of the racemic form (1:1) and the meso form. The same reaction of (**1**) with acetone was now repeated using lipase PS (as catalyst) in isooctane instead of the acid catalyst. Surprisingly, it was found that ketal (**4**) was indeed formed although very slowly (Fig. 3.7). This has been also observed when using other lipases (e.g. Lipase AYS, Novozyme 435, Lipozyme RM IM). Therefore, it is concluded that the acetone

released as a by-product from lipase-catalyzed transesterification of (**1**) can react with the alcohol (**1**) to form the acetal (**4**). Since ketal formation is an acid-catalyzed reaction, the lipase is working as an acid catalyst without any stereoselectivity.

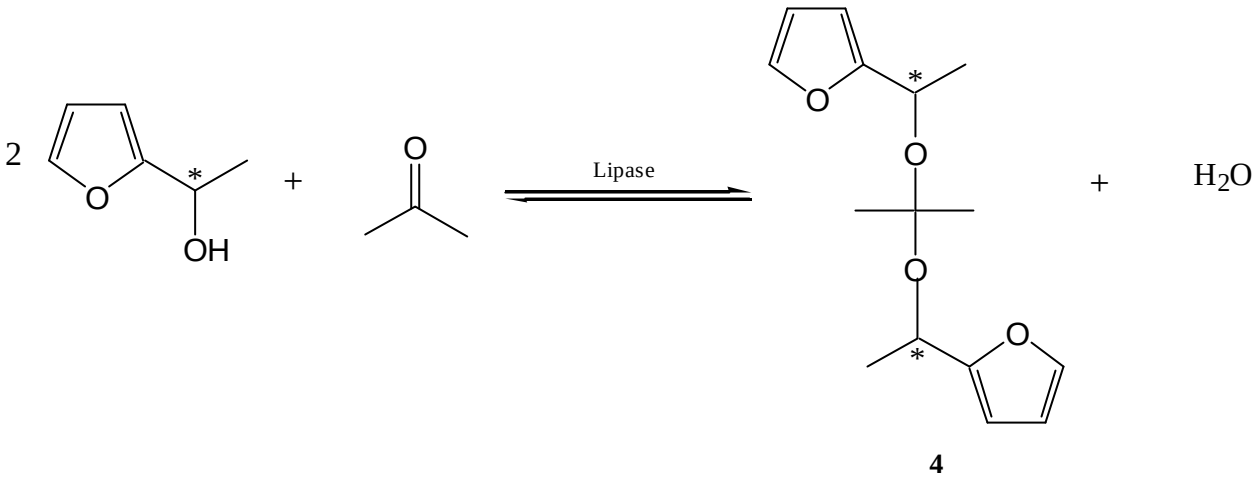


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Fig. 3.5: Undesired ketal formation in lipase-catalyzed transesterification of 1-(2-furyl)ethanol when using isopropenyl acetate as acyl donor.

To confirm the formation of acetal (**4**) during the lipase-catalyzed reaction, the PS lipase-catalyzed transesterification of 1-(2-furyl) ethanol (**1**) using isopropenyl acetate was repeated. A temperature program by which the peaks of acetal (**4**) can be detected at higher retention time was developed. The three characteristic peaks of acetal (**4**) were again observed during the kinetic resolution of 1-(2-furyl) ethanol. The structure of (**4**) was confirmed by GC/MS and by comparison with the synthesized sample using CaI_2.

The only difference between the synthesized (**4**) and that formed in lipase-catalyzed transesterification reaction was in the ratio of peak areas as it was expected. For the synthesized (**4**) using lipase, and racemic alcohol (1:1, peak area of enantiomers) the acetal obtained showed a 1:1:2 ratio of peak areas, exactly as that obtained by acid-catalyzed reaction (the reference used in this investigation). However, for the acetal (**4**) obtained during the kinetic resolution of (**1**) the ratio of peak area was changed (1:1.1:1.5). In fact, this was expected since during the lipase-catalyzed kinetic resolution of (**1**) the alcohol present is not racemic (both enantiomers will not be equal during the reaction since one is selectively esterified faster than the

other by the lipase leaving the other enantiomer in pure form). Since PS lipase select (R)-**1** from racemic (**1**) to be esterified forming (R)-**3** and leaving (S)-**1** in the pure form behind, it can be expected in the acetal formed more (S,S) than (R,R) and for the meso form [the third peak, (R,S) and (S,R)] which was before 2.5, will be less in the acetal (**4**) formed as an undesired by-product during the lipase-catalyzed transesterification of (**1**) using isopropenyl acetate as acyl donor. To the best of our knowledge this is the first observation of such by-product in lipase-catalyzed transesterification of secondary alcohol using isopropenylacetate as acyl donor.

In addition to the consumption of a part of alcohol (**1**), involved in the transesterification reaction, to form the undesired acetal (**4**), water is released as a by-product from ketal formation, which is unfavorable for the transesterification reaction as well as the enzyme stability.

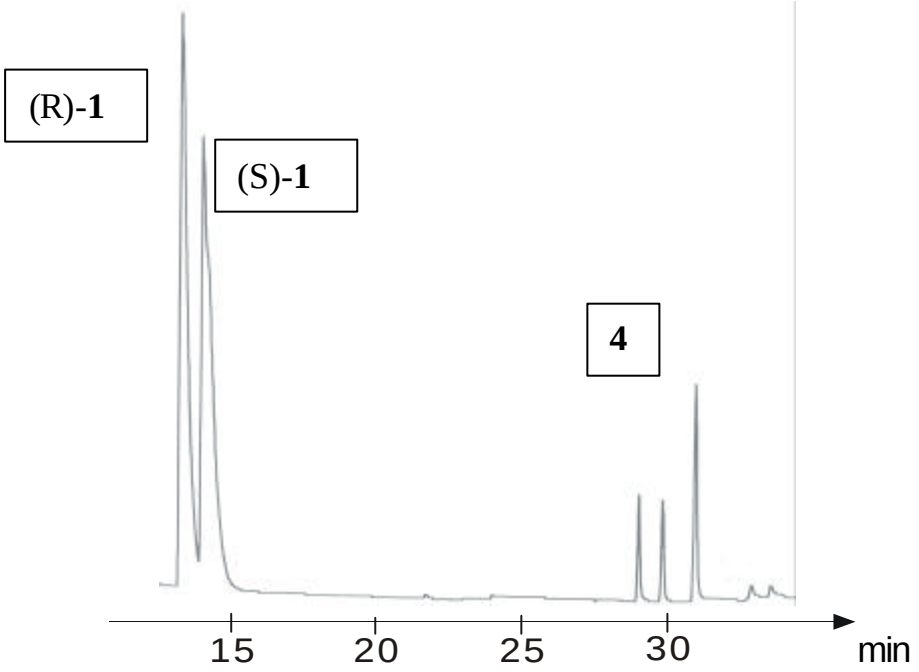


Fig. 3.6: Gas-chromatographic separation of the reaction mixture of the acid-catalyzed synthesis of acetal (**4**) resulting from the reaction of (**1**) and acetone on heptakis-(2,3-di-*O*-methyl-6-*O*-tert-butyldimethylsilyl)-*b*-cyclodextrin as stationary phase in GC. Oven temperature was 65°C (isothermal) for 15 min, then 5°C /min. until 110°C for 35 min.

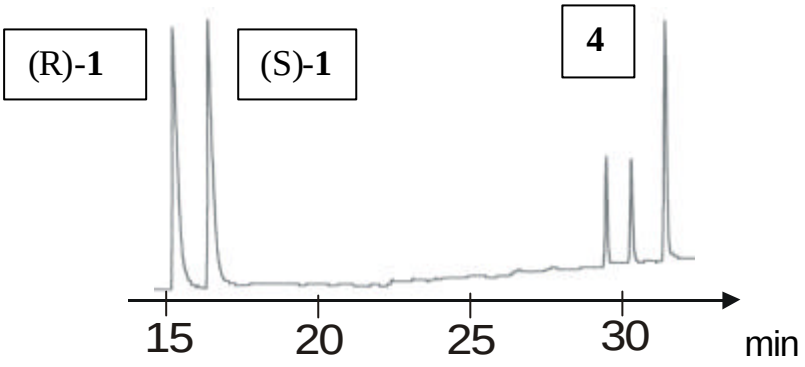


Fig. 3.7: Gas-chromatographic separation of the reaction mixture of lipase-catalyzed synthesis of acetal (**4**) resulting from the reaction of (**1**) and acetone after 3 days on heptakis-(2,3-di-*O*-methyl-6-*O*-tert-butyldimethylsilyl)-*b*-cyclodextrin as stationary phase in GC. Oven temperature was 65°C (isothermal) for 15 min, then 5°C /min. until 110°C for 35 min.

3.2.3. Effect of molecular sieves

In order to overcome the drop in conversion observed for lipase PSL by scavenging the liberated acetone thereby shifting the equilibrium in the desired forward direction, 100 mg molecular sieves 4 Å were added to the reaction mixture. Indeed the use of molecular sieves enhanced the rate of the reaction, the enantiomeric excess of both substrate and product as well as the enantiomeric ratio E (Fig. 3.8,3.9).

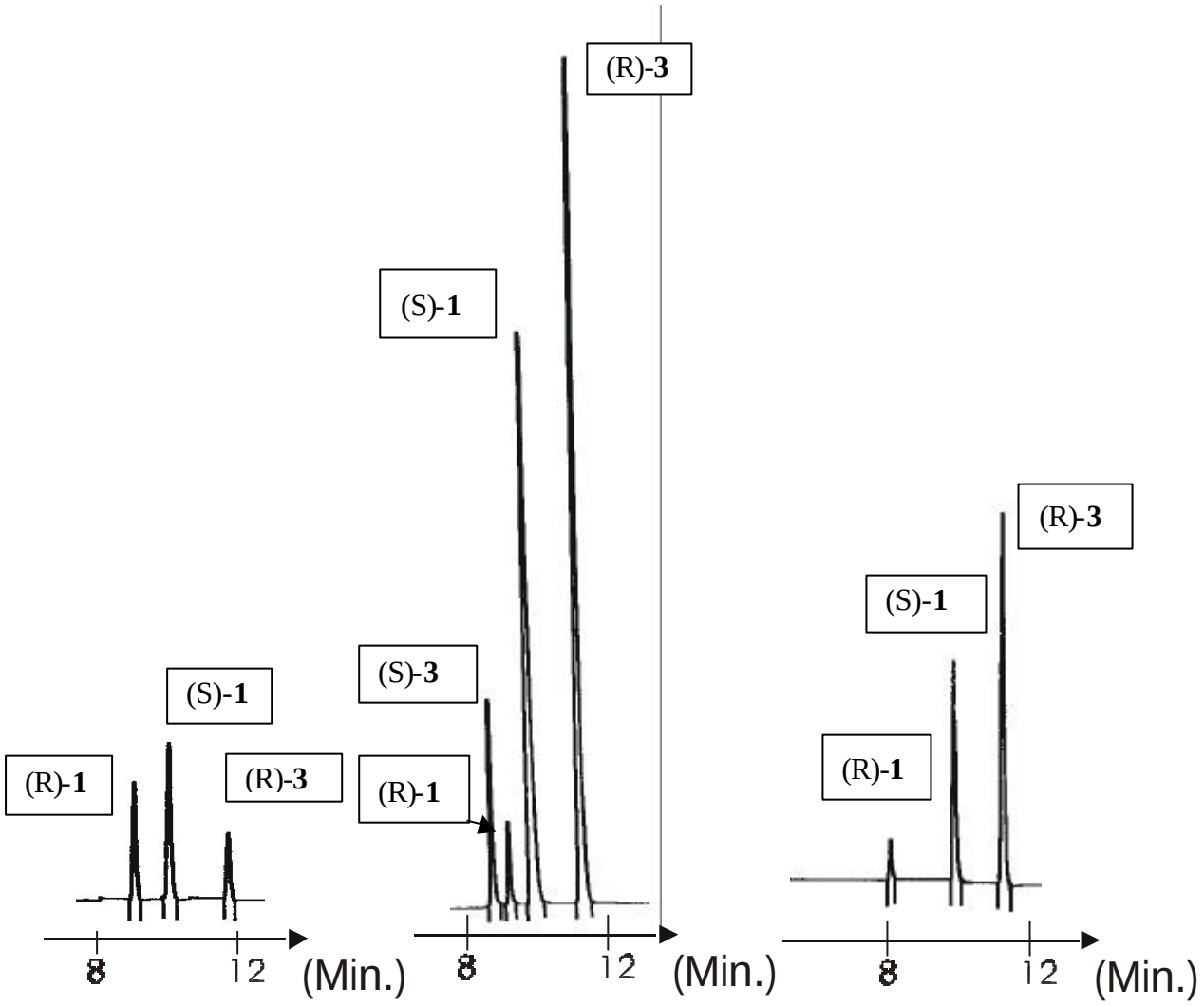


Fig. 3.8a **Fig. 3.8b** **Fig. 3.8c**

Fig. 3.8. Gas-chromatographic separation of the enantiomers of both substrate (**1**) and product (**3**) on heptakis-(2,3-di-*O*-methyl-6-*O*-tert-butyldimethylsilyl)-*b*-cyclodextrin of the PS-catalyzed transesterification of (**1**) in isooctane (**Fig. 3.8a:** at t=2 hrs without molecular sieves, ee_s =19%, ee_p=99.9%, conv. =16%, E >300; **Fig. 3.8b:** at t=2 hrs with molecular sieves, ee_s=83%, ee_p =69%, conv. =55%, E= 14; **Fig. 3.8c:** at t=21 hrs without molecular sieves, ee_s =75, ee_p=99.9, conv. =43, E>300.

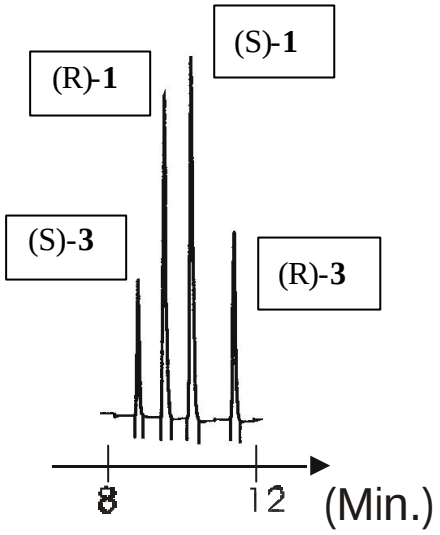


Fig. 3.9a

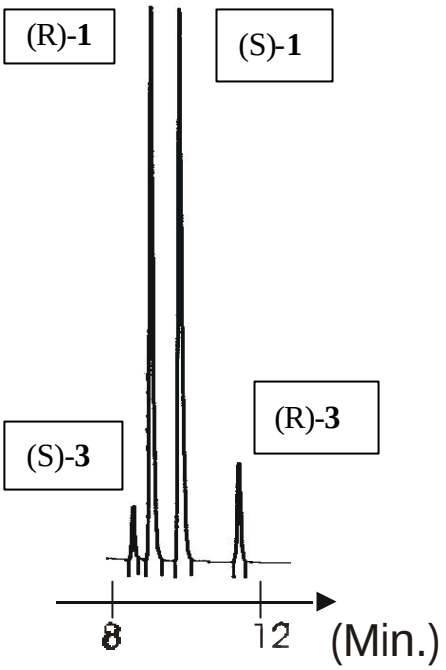


Fig. 3.9b

Fig. 3.9a: Gas-chromatographic separation of the enantiomers of both substrate (**1**) and product (**3**) on heptakis-(2,3-di-*O*-methyl-6-*O*-tert-butyldimethylsilyl)-*b*-cyclodextrin of the CRL-catalyzed transesterification of (**1**) in isooctane at t=2 hrs without molecular sieves, ee_s=10, ee_p=27, conv. =26, E= 2; **Fig. 3.9b:** CCL-catalyzed transesterification of (**1**) in isooctane at t=2 hrs with molecular sieves. The retention time of (S)-**3**, (R)-**1**, (S)-**1**, (R)-**3**, were 9.0, 9.4, 10.2, 11.6 min, respectively.

Three enzymes (PSL, CRL, and CCL) were chosen for further investigations in order to study the effect of organic solvents on the enantiomeric ratio (E) of the transesterification of (**1**) in the presence of molecular sieves (Table 3.2).

Table 3.2: Lipase-catalyzed transesterification of (**1**) in organic solvents using molecular sieves.

Lipase	Solvent	Time (hr)	ee_s % (S)-**1**	ee_p % (R)-**3**	conv. %	E	Time (hr)	ee_s % (S)-**1**	ee_p % (R)-**3**	conv. %	E
PSL	Isooctane	2	82	69	54	13	21	96	99.9	48	>300
	TBME	2	67	74	48	14	21	98	3	97	2.6
	n-Hexane	2	79	79	50	30	21	7	6	54.5	1.2
	n-Heptane	2	91	73	55	19	21	99.7	2	98	3.0
CRL	Isooctane	2	5	56	7	4	21	15	63.5	18.7	5.2
	TBME	2	1	55	2	3.5	21	6	46	12	3.0
	n-Hexane	2	2.5	55	4.3	3.5	21	5.6	99.8	5.3	>300
	n-Heptane	2	4	48	8.0	3	21	14	68.2	17	6.0
	Toluene	2	1.5	46	3.2	3	21	6.2	61	9.3	4.4
CCL	Isooctane	2	3.5	43	8	3	21	8.3	69	11	6.0
	TBME	2	1.5	>99	1.5	n.d	21	9.4	65	13	5.2
	n-Hexane	2	3.2	41	7.2	2.4	21	14	64.4	18.1	5.3
	n-Heptane	2	8	34	19	2.2	21	16	46.5	26.2	3.2
	Toluene	2	<1	33	<1	2	21	13	53.6	20.1	4.0

TBME: tert. butyl methyl ether

3.2.4. Effect of organic solvents on the transesterification of 1-(2-furyl)ethanol

Lipases have attracted much attention to organic chemists because of their high stability in organic solvents. The choice of the organic solvent for a lipase catalyzed reaction is known to be very important in determining the enantioselectivity, which critically depends on the reaction medium employed.[49] It has been reported that solvents with log P values more than two exhibit high enantioselectivity, whereas solvents with log P less than two show detrimental effects on the enzymes.[50] P is employed as an index for the solvent hydrophobicity in biocatalytic reactions[51] and is defined as the ratio of the concentration of a substance in two immiscible phases at equilibrium (octanol and water). Several solvents (isooctane: log P =4.5, TBME: log P =1.35, n-hexane: log P= 3.5, *n*-heptane: log P =4, toluene: log P=2.5) were investigated for the transesterification of (**1**) at 40°C in the presence of molecular sieves 4 Å (Table 3.2). When using the PS lipase in the transesterification of (**1**), the best solvent was isooctane (log P= 4.5) in regard to the enantiomeric excess of substrate (S)-**1** and product (R)-**3**, conversion and enantiomeric ratio (ee_s=96%,

ee_p=99.9%, conv. =48%, E>300) after 21 hrs. Surprisingly, a high reaction rate (conv. =91.3% and 97% in 21 hrs) and ee_s>99 and 98% were observed when using toluene (log P=2.5) and TBME (log P=1.35), respectively, however the enantiomeric excess of product (R)-**3** (ee_p=9.4% and 3% respectively) as well as the enantiomeric ratio were too low.

For CRL, high enantiomeric excess of product (R)-**3** (99.8%) and enantiomeric ratio E>300 were observed in *n*-hexane (log P=3.5), however, a low enantiomeric excess of substrate (S)-**1** (5.6%) as well as conversion (5.3%) were obtained after 21 hrs. The use of isooctane (log P= 4.5) increases the conversion to 18.7 % and enantiomeric excess of substrate (S)-**1** to 15% but decreases the enantiomeric excess of product (R)-**3** to 63.5% and the enantiomeric ratio E to 5.2. For CCL, the highest reaction rate (conv. = 26.2% in 21 hrs) and enantiomeric excess of the substrate (S)-**1** (16%) were obtained when using *n*-heptane (log P=4), however, the enantiomeric excess of product (R)-**3** and the enantiomeric ratio E were the lowest in comparison with the CCL-catalyzed transesterification of (**1**) in other solvents. High enantiomeric excess of the product (R)-**3** (69%) as well as enantiomeric ratio E=6 were obtained when using isooctane as solvent for the enzymatic transesterification reaction.

As described in the experimental part, the gram-scale enzymatic transesterification of 1-(2-furyl)ethanol using PSL has been performed. (S)-**1** was isolated in 75% yield with an enantiomeric excess ee > 99%**.** The high ee of (S)-**1** was obtained at the expense of the ee of (R)-**3** (53%).

3.2.5. Effect of reaction time

Prolonging the reaction time or increasing the temperature should cause the faster reacting enantiomer to reach its state of equilibrium more rapidly. Prolonging the reaction time however leads to some further conversion of the slower reacting enantiomer and, along with this, in less satisfactory enantiomeric excess of the products.

It is concluded that the enzyme of choice for the lipase-catalyzed transesterification of (**1**) using isopropenyl acetate as acyl donor is lipase from *Pseudomonas cepacia* (PSL) in isooctane in the presence of molecular sieves. The optimized kinetic resolution of (**1**) is characterized by the following data: time = 21 hrs, ee_s 96%, ee_p >99%, conv. = 48%, E >300. The procedure was applied for a gram-scale production of essentially enantiomerically pure 1-(2-furyl) ethanol (75% yield).

4. The utility of modified cyclodextrin in enzymatic reactions: Enhanced reaction rate and enantioselectivity in lipase-catalyzed transesterification in organic solvents

4.1. Introduction

4.1.1. The use of enzymes in organic solvents and their limitations

Enzymes, especially lipases, have been well established as a valuable biocatalysts in organic synthesis.[24] These natural catalysts have been shown to be a very useful tool for the synthesis of chiral molecules under non-aqueous conditions. Their efficiency in conducting transformations in organic solvents, safety, ease of handling and the mild conditions under which they operate (typically room temperature and around neutral pH) render them a useful tool in organic synthesis. Compared to the aqueous solution, the use of an organic reaction medium can have some interesting advantages. Among these advantages are:

1. Enhancement of thermal stability of the enzyme.
2. Easy separations of the suspended enzyme from the reaction medium for further use.
3. Increased solubility of the substrate.
4. Favorable equilibrium shift to synthesis over hydrolysis.
5. Elimination of undesired reactions caused by water.
6. The generation of a possibly new selectivity of the enzyme.[52]

However, as previously mentioned (section 1.5.2), the use of enzymes in organic solvents has some drawbacks. In fact, keeping the substrate concentration at a low level through continuous addition can circumvent substrate inhibition, however, product inhibition is still difficult to control even by gradual removal of the product by physical means.[15]

a) Substrate inhibition

Substrate inhibition is linked with the decrease of enzyme activity at higher concentrations of the substrate. Physiologically, this prevents the wasteful digestion of an abundant substrate and the accumulation of the corresponding product. Substrate inhibition is described in Figure 4.1.

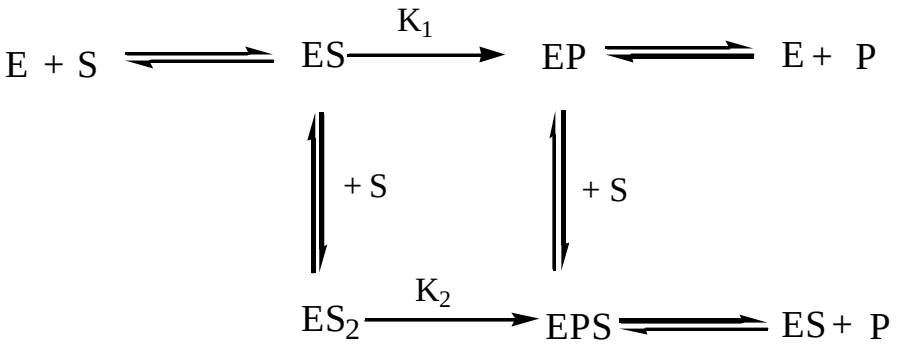


Fig. 4.1: Equilibria associated with enzymes demonstrating substrate inhibition (K_1>K_2).

At low concentration of the substrate, the enzyme-substrate complex (ES) regarded as binary complex dominates, thus the rate of the reaction increases with increasing substrate concentration. As the substrate concentration increases further and further, the ternary complex, ES_2 start to appear and the rate of the reaction decreases. If enzymes, which demonstrate substrate inhibition, are used on industrial scale, low substrate concentration and therefore a large-scale reaction is required.

b) Product inhibition

Product inhibition is linked with the decrease of enzyme activity at higher concentrations of the product. It can result from the binding of the product to the binary enzyme-substrate complex reducing its catalytic activity or through a decreased extent of product dissociation (Fig. 4.2). The later represents the product inhibition in its simplest form. The result of product inhibition is a decrease in the concentration of the reactive Michaelis complex leading to the decrease in the reaction rate as more products are formed.

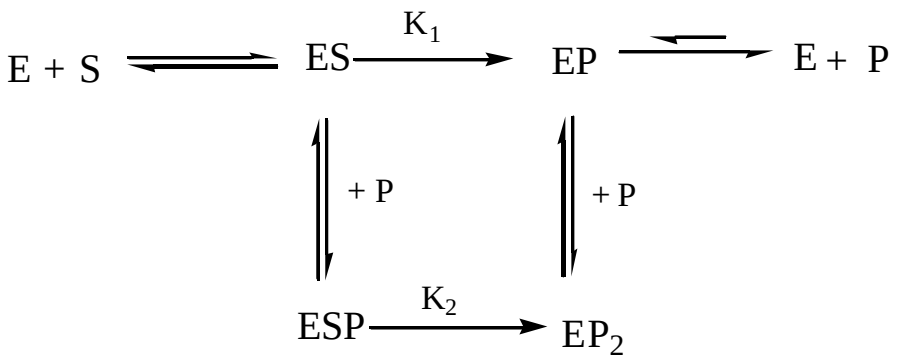


Fig. 4.2: Equilibria associated with enzymes demonstrating product inhibition ($K_1 > K_2$).

Several strategies have been explored to overcome the lower activity of enzymes in organic solvents to make them more appealing to organic chemists. These include the methods of enzyme preparation,[53] protein engineering,[54] chemical modification of the enzyme,[55] the control of the pH value,[56] co-lyophilization with lyoprotectants [57] and salts,[58] addition of water-mimicking compounds like formamide, glycol or DMF,[59] immobilization techniques,[60] imprinting with substrates and substrate analogues,[61] formation of substrate salts,[62] and cross-link crystallization.[63]

It has been reported that an additional improvement of the reaction condition can be achieved by using certain additives, which might have a beneficial influence on the microenvironment of the enzymes and hence improve their catalytic activity and selectivity in organic solvents.[64]

Among these additives, crown ethers and specially thiacrown ethers have been successfully used to enhance the activity and the selectivity of *Pseudomonas cepacia* lipase (PSL) in the enantioselective formation and hydrolyses of carboxylic esters.[65] In fact, co-lyophilization of various enzymes with crown ether[66] leads to enhancement of the activity of the enzyme and also increases the enantioselectivity in organic solvents. It is conceivable to assume that the crown ether can bind to the enzyme in the co-lyophilization process leading to a possible solubilization of enzymes in organic solvents, and as a consequence, to accelerate the rate of transesterification reaction.

Also, such binding can increase the flexibility of the enzymes in solvents leading to enhancement of their activity and enantioselectivity.[67] The enhancement of the activity of enzymes by

crown ether could also be attributed to its capability to facilitate the removal of water molecules from the active site upon substrate binding.[66] Cyclodextrins, another class of a macrocyclic structure, have been successfully used to improve enzymes activity and to increase the reaction rate and enantioselectivity in enzyme-catalyzed reactions in organic solvents.[68]

In this work, the versatility of cyclodextrins as regulators for the *Pseudomonas cepacia* lipase and additives to enhance the reaction rate and enantioselectivity in lipase-catalyzed enantioselective transesterification of 1-(2-furyl)ethanol in an organic solvent, will be demonstrated.

c) An introduction to cyclodextrins

Naturally occurring cyclodextrins (CDs) are a family of cyclic α-(1,4)-linked glucose oligomers with 6, 7, or 8 glucose units, corresponding to α-, β-, and γ-cyclodextrin. They were discovered 100 years ago and produced from amylose by a highly selective enzymatic cyclization containing six, seven, or eight glucose monomers. They possess homogenous toroidal structures. The internal hydrophobic cavity and the external hydrophilic rim of the chemically modified CDs render them ideal for modeling enzyme-substrate binding,[69] drug delivery,[70] catalysis,[31] host-guest interaction,[71] chiral separation[42] and molecular recognition in self-assembled monolayers.[72]

In the pharmaceutical industry, CDs have mainly been used as complexing agents to increase the aqueous solubility of poorly water-soluble drugs, and to increase their bioavailability and stability. In addition, they can be used to reduce or prevent gastro-intestinal (GI) or ocular irritation, reduce or eliminate unpleasant smells or tastes, prevent drug-drug or drug-additive interactions, or even to convert oils and liquid drugs into micro crystalline or amorphous powders.

The ability of CDs to form complexes with a variety of organic compounds, helps to alter the apparent solubility of the molecule, to increase stability in the presence of light, heat and oxidizing conditions and to decrease the volatility of compounds.

CDs can also be used as processing aids to isolate compounds from natural sources and to remove undesired compounds such as cholesterol from food products.

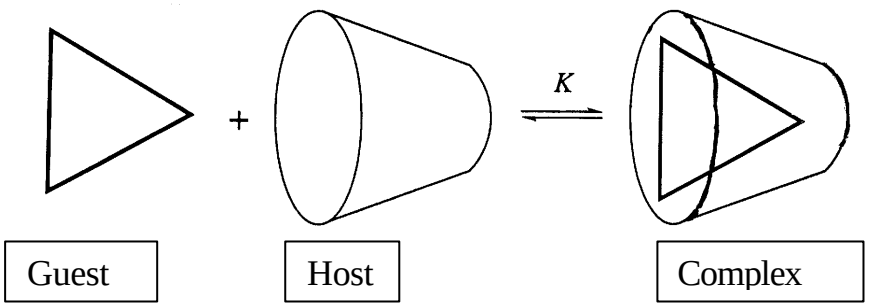


Fig. 4.3: Inclusion of a guest within a cyclodextrin host to form a complex.

These diverse applications of CDs promoted us to study their effects when they are used as additives in lipase-catalyzed transesterification of 1-(2-furyl)ethanol in organic solvent.

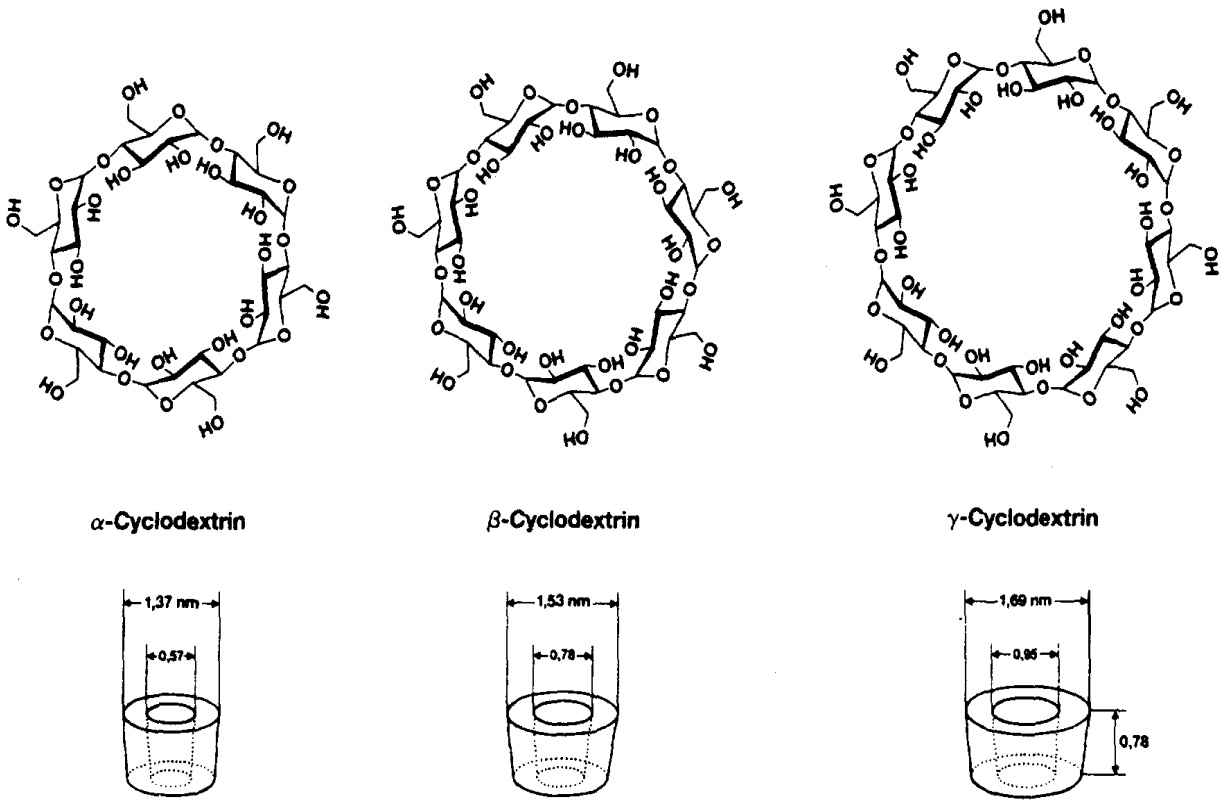
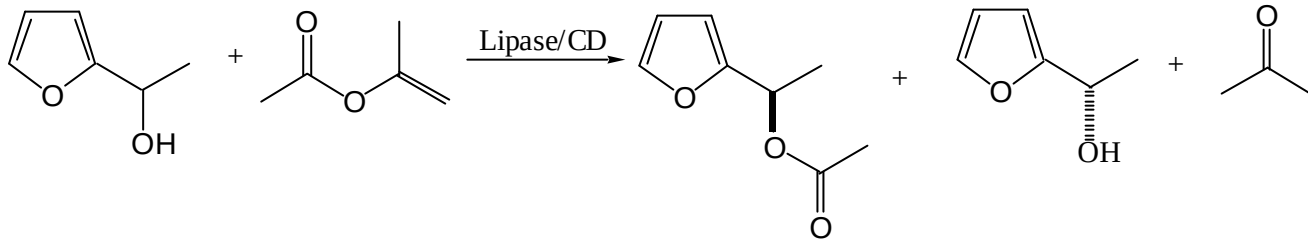


Fig. 4.3: Structures of cyclodextrins.

4.2. Results and discussion

4.2.1. Co-lyophilized lipase with cyclodextrin-catalyzed transesterification of 1-(2-furyl)ethanol

Two methods of enzyme preparation have been investigated. The first consists of a lyophilization of *Pseudomonas cepacia* lipase (PSL), our model enzyme, from phosphate buffer (pH 6) (lyophilized enzyme only, without additives). The second enzyme preparation was a co-lyophilization of PSL with various CDs using the same phosphate buffer (co-lyophilized enzyme with CD). The effect of the addition of CDs on the activity and the enantioselectivity E of PSL-catalyzed transesterification of (**1**) in toluene was studied on a model example of lipase-catalyzed transesterification of 1-(2-furyl)ethanol using isopropenylacetate as acyl donor (Fig. 4.4).



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

Fig. 4.4: Co-lyophilized lipase with cyclodextrin-catalyzed enantioselective transesterification of 1-(2-furyl)ethanol (**1**) using isopropenyl acetate (**2**) in toluene.

Compared to the commercially available PSL (used as purchased), a slight decrease in activity has been observed for the PSL lyophilized from phosphate buffer alone (pH 6). This is probably due to partial denaturation of the enzyme during lyophilization. This slight decrease in activity was not observed when the enzyme was co-lyophilized with peracetylated cyclodextrin using the same phosphate buffer.
Compared to the lyophilized PSL-catalyzed transesterification of (**1**) in toluene, no enhancement effect on the enzyme enantioselectivity or the reaction rate was observed when the PSL was co-lyophilized

with *b*-cyclodextrin. However, an increase in the enzyme enantioselectivity and catalytic rate has been observed when the lipase was prepared in the presence of *a*- and *g*-cyclodextrin at a 1:1 weight ratio of enzyme to cyclodextrin (Fig. 4.5).

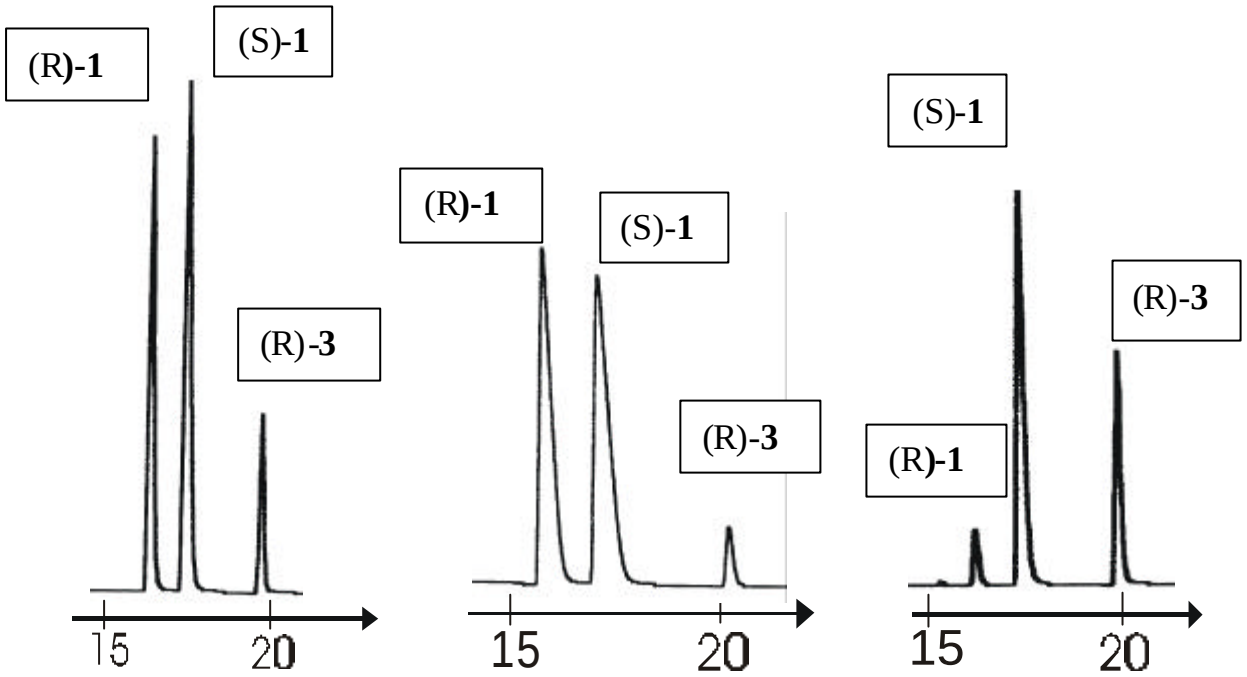


Fig. 4.5: Co-lyophilized PSL with native *a*-, *b*- and *g*-cyclodextrin, respectively, at a 1:1 mol ratio of enzyme to cyclodextrin catalyzed enantioselective transesterification of (**1**) in toluene.

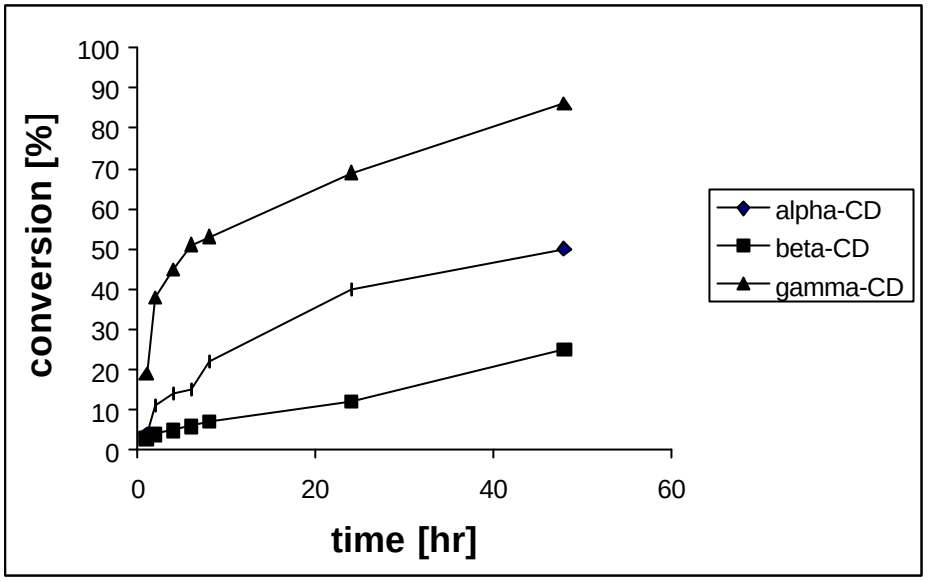


Fig. 4.6: Plot of the conversion against the time in the co-lyophilized lipase with native cyclodextrins catalyzed transesterification of (**1**) in toluene.

Table 4.1: Comparison between the lyophilized enzyme without additive (none) and the co-lyophilized PSL with native cyclodextrin (1:1) in catalyzing the enantioselective transesterification of (**1**) in toluene.

Additives	Time (hr)	ee_s [%]	ee_p [%]	Conv. [%]	E
None (PSL lyophilized)	24	80	99	30	28
α-CD	24	65	97	45	>100
β-CD	24	12	94	12	33
γ-CD	24	98	44	68	>100

It was concluded from this preliminary screening of native CD additives that there might be a relation between the solubility of CD in

water and their ability to bind to the enzyme in the co-lyophilization process. In fact the solubility of native CDs in water at 25°C is varying from 14.5; 1.85; 23.2 [g/100ml] for *a*-, *b*- and *g*-cyclodextrin, respectively. The differences in their cavity diameter, which are ranging from 4.7-5.3, 6.0-6.5, 7.5-8.3 Å,[73] respectively, suggest specific macrocycle-enzyme interactions. The poor solubility of the unmodified *b*-cyclodextrin in water is resulting from the intramolecular hydrogen bonding of the secondary hydroxyl groups. Attempts to break this hydrogen-bond network by persubstitution using methyl, acetyl and hydroxypropyl groups improve their water solubility[74] and their physicochemical properties.[75] In fact hydroxyl groups in CD could play an important role in the activation of lipase. To examine the effect of hydroxyl groups, several derivatized CD have been used in the present study. Among the CD derivatives tested were permethylated *a*- and *b*-cyclodextrin, (3-hydroxypropyl)-*g*-cyclodextrin, (2-hydroxypropyl)-*a*-, *b*-, and *g*-cyclodextrin and peracetylated *b*-cyclodextrin.

In general, hydrophilic CD derivatives such as permethylated, hydroxyalkylated, and branched CDs have been used for the improvement of low solubility, dissolution rate, and bioavailability of poorly water soluble drugs.[76] Hydrophobic CDs like the ethylated and acylated derivatives have been used as sustained release carriers for water-soluble drugs.[77]

Permethylated *b*-cyclodextrin has been used to enhance the enantioselectivity and reaction rate of the enzyme subtilisin Carlsberg suspended in organic solvent.[38] However, the introduction of an acetyl group to the free *b*-cyclodextrin is considered to be totally different from the methyl group from the point of view of bulkiness and hydrogen-bonding ability. Therefore peracetylated *b*-cyclodextrin is expected to exhibit different behavior which can influence the enzymes character if it is added prior to lyophilization of PSL in buffer. Also hydroxypropylated *a*-, *b*-, and *g*-cyclodextrin have been used to increase the catalytic activity of *a*-chymotrypsin in organic solvent.[36]

In this investigation, it was found that permethylated *a*- and *b*-cyclodextrin as well as (2-hydroxypropyl) *a*-cyclodextrin were efficient (reaction terminated after 24 hrs) in enhancing the catalytic activity of PSL when they were added prior to lyophilization process in phosphate buffer (Table 4.1). Peracetylated *b*-cyclodextrin (reaction

terminated after 8 hrs) was the most effective among the CDs used as additives prior to lyophilization. The enhancement of the catalytic activity of PSL using these kinds of CD derivatives leads to an increase in the enantioselectivity and reaction rate of lipase-catalyzed transesterification of (**1**) in organic solvent.

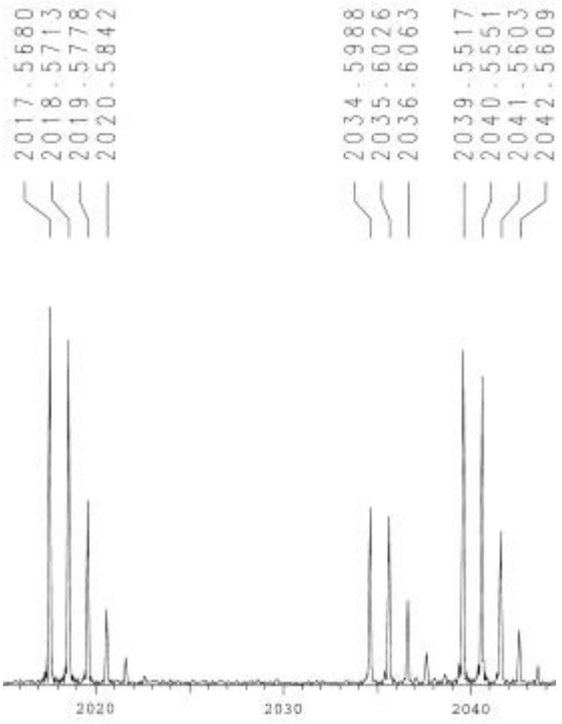


Fig. 4.7: Positive Ion FAB Mass Spectrum of peracetylated-*b*-cyclodextrin. Expected masses $[M+H]^+$ =2017.59; $[M+Na]^+$= 2039.58; $[M+NH_4]^+$= 2034.62.

4.2.2. Effect of concentration additives on the PSL-catalyzed transesterification of (1)

Comparing with other CDs used as additives in the PSL-catalyzed transesterification of (**1**), the enhancement of the enantioselectivity and reaction rate was effective when using peracetylated *b*-cyclodextrin in co-lyophilized form with lipase.

Since the magnitude of the enzyme enhancement in organic solvents by macrocyclic compounds is depending on the ratio of additive to enzyme,[65] three different concentrations have been used, 1:1; 1:2; 1:6 enzyme to cyclodextrin weight ratio.
When the peracetylated *b*-cyclodextrin was employed at a 1:6 weight ratio of enzyme to cyclodextrin, a significant activation of PSL has been observed when compared to the data of the lyophilized PSL from buffer alone (Fig. 4.8). The reaction was terminated in 8 hrs with 56% conv., >99% ee_s, 80% ee_p, E=52.

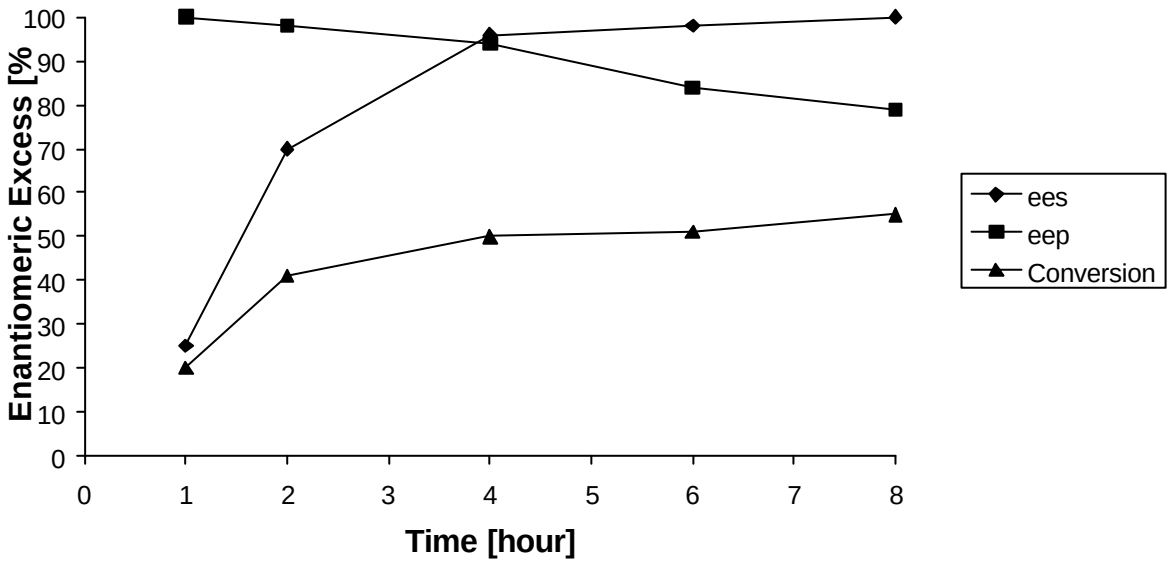


Fig. 4.8: Co-lyophilized lipase with peracetylated *b*-cyclodextrin (1:6 weight ratio) catalyzed enantioselective transesterification of 1(2-furyl)ethanol in toluene.

When permethylated *a*- and *b*- cyclodextrin as well as (2-hydroxypropyl) *a*-cyclodextrin were used prior to lyophilization (1:6 weight ratio enzyme to CD), the activity of PSL was increased and the enhancement of the reaction rate and enantioselectivity were significant, compared to the lyophilized enzyme without CD, but not as the peracetylated *b*-cyclodextrin.

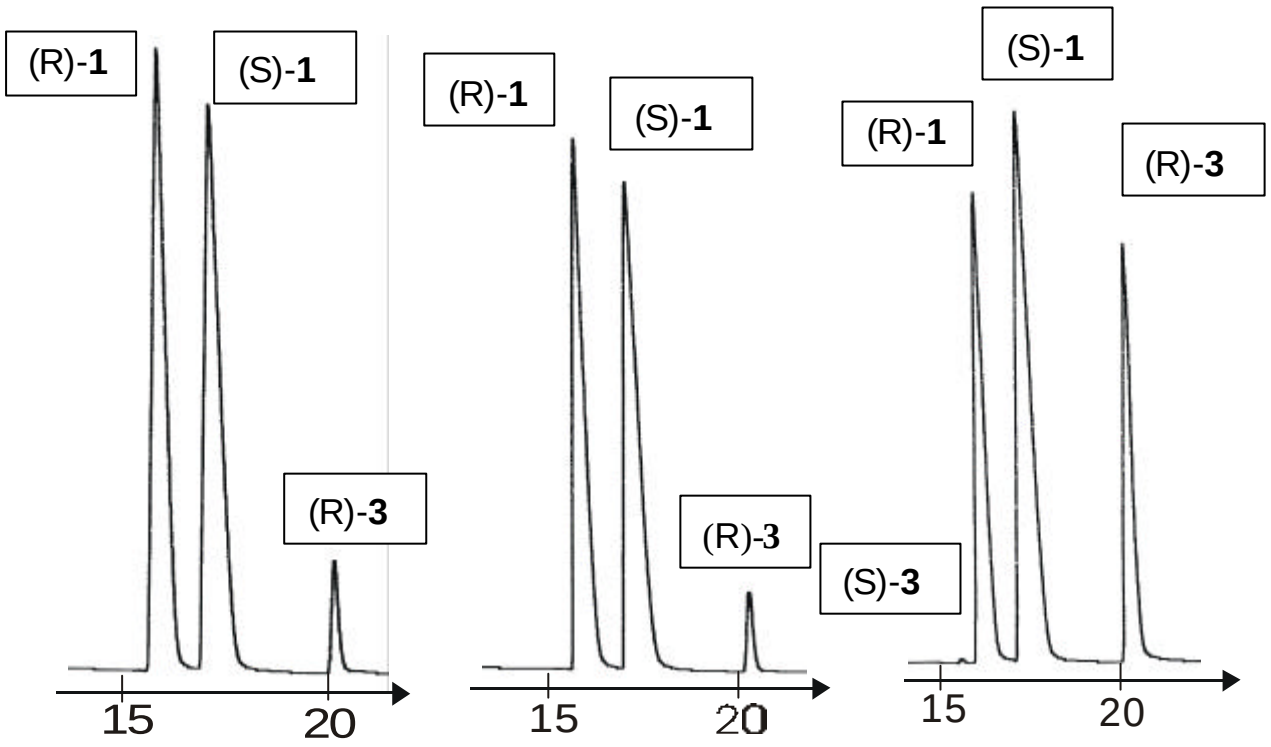


Fig. 4.9a **Fig. 4.9b** **Fig. 4.9c**

Fig. 4.9: Gas-chromatographic separation of the enantiomers of both substrate (**1**) and product (**3**) on heptakis-(2,3-di-*O*-methyl-6-*O*-tert-butyldimethylsilyl)-*b*-cyclodextrin of the co-lyophilized PSL with peracetylated-*b*-cyclodextrin-catalyzed transesterification of (**1**) in toluene at t=1 hr. **Fig. 4.9a**: 1:1 enzyme/Ac-*b*-cyclodextrin weight ratio, ee_s =4.5%, ee_p=99%, conv. =4.5%, E >100; **Fig. 4.9b**: 1:2 enzyme/Ac-*b*-cyclodextrin weight ratio, ee_s=5%, ee_p =99%, conv. =5%, E>100; **Fig. 4.9c**: 1:6 enzyme/Ac-*b*-cyclodextrin weight ratio, ee_s =25%, ee_p=99%, conv. =20%, E>100.
The retention time of (S)-**3**, (R)-**1**, (S)-**1**, (R)-**3**, were 14.7, 15.5, 16.8, 20.2 min, respectively.

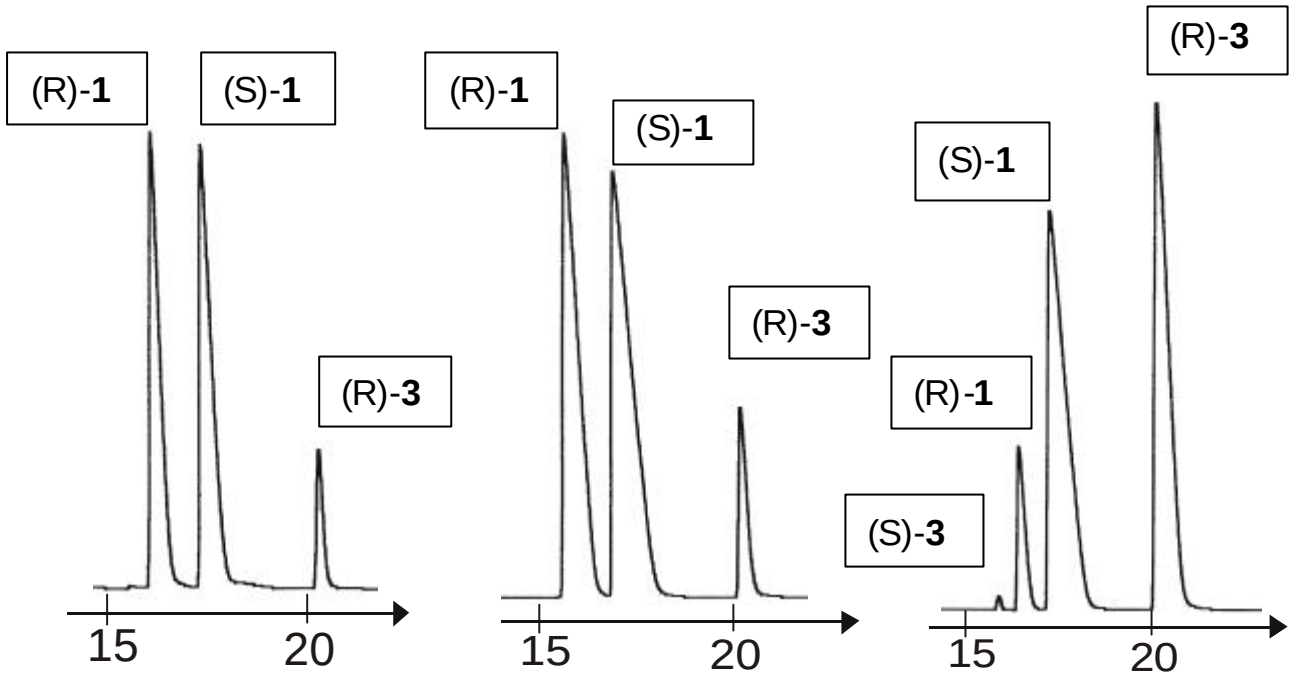


Fig. 4.10a **Fig. 4.10b** **Fig. 4.10c**

Fig. 4.10: Gas-chromatographic separation of the enantiomers of both substrate (**1**) and product (**3**) on heptakis-(2,3-di-*O*-methyl-6-*O*-tert-butyldimethylsilyl)-*b*-cyclodextrin of the co-lyophilized PSL with peracetylated-*b*-cyclodextrin-catalyzed transesterification of (**1**) in toluene at t=2 hrs. **Fig. 4.10a:** 1:1 enzyme/Ac-*b*-cyclodextrin weight ratio, ee_s =7.3%, ee_p=96%, conv. =7%, E =54; **Fig. 4.10b:** 1:2 enzyme/Ac-*b*-cyclodextrin weight ratio, ee_s=9%, ee_p =99%, conv. =8%, E>100; **Fig. 4.10c**: 1:6 enzyme/Ac-*b*-cyclodextrin weight ratio, ee_s =70%, ee_p=98%, conv. =42%, E>100.
The retention time of (S)-**3**, (R)-**1**, (S)-**1**, (R)-**3**, were 14.7, 15.5, 16.8, 20.2 min, respectively.

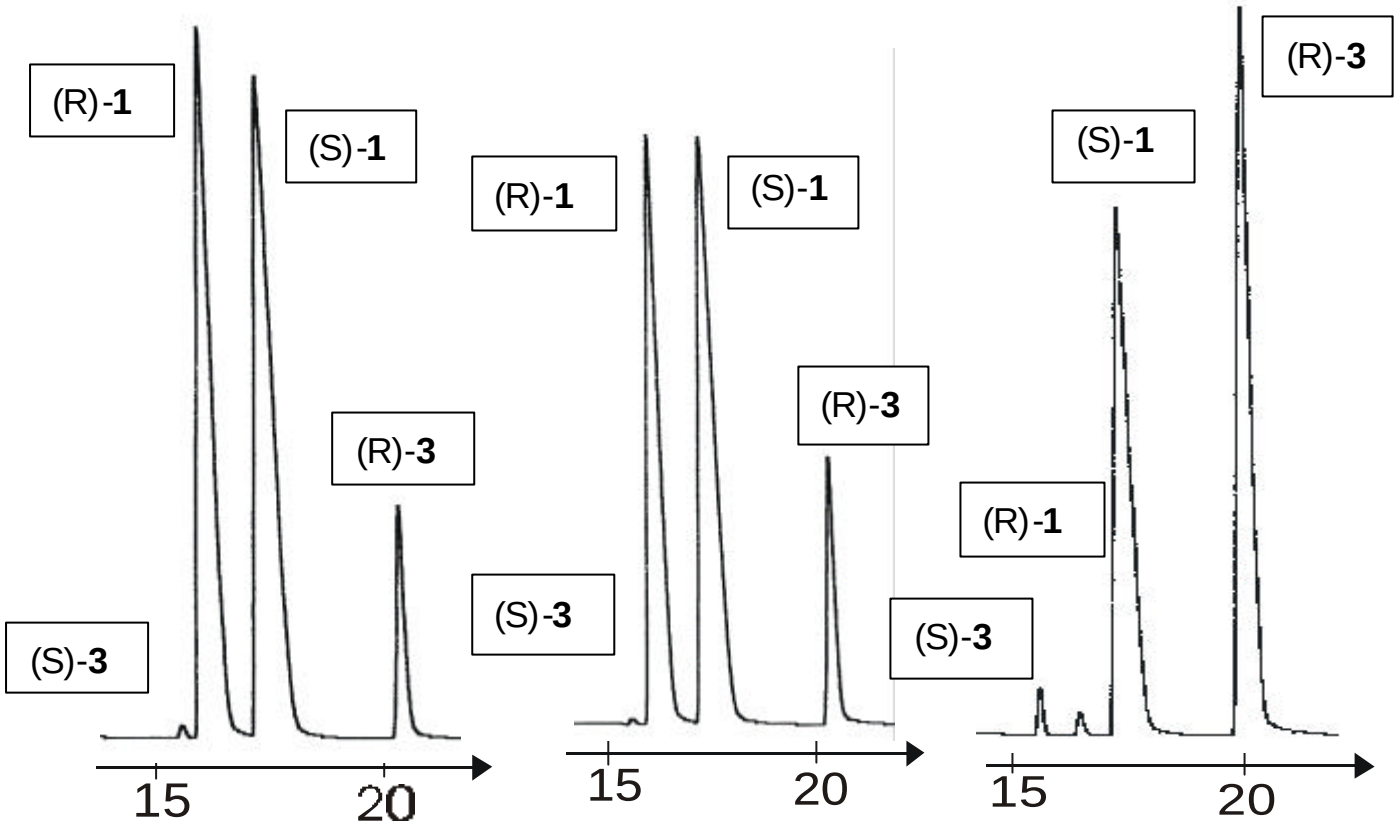


Fig. 4.11a **Fig. 4.11b** **Fig. 4.11c**

Fig. 4.11: Gas-chromatographic separation of the enantiomers of both substrate (**1**) and product (**3**) on heptakis-(2,3-di-*O*-methyl-6-*O*-tert-butyldimethylsilyl)-*b*-cyclodextrin of the co-lyophilized PSL with acetylated-*b*-cyclodextrin-catalyzed transesterification of (**1**) in toluene at t=6 hrs. **Fig. 4.11a**: 1:1 enzyme/Ac-*b*-cyclodextrin weight ratio, ee_s =8%, ee_p= 86%, conv. =9%, E =15; **Fig. 4.11b**: 1:2 enzyme/Ac-*b*-cyclodextrin weight ratio, ee_s=14%, ee_p = 84%, conv. =14%, E =13; **Fig. 4.11c**: 1:6 enzyme/Ac-*b*-cyclodextrin weight ratio, ee_s = 91.5%, ee_p=84.5%, conv. = 50%, E = 38
The retention time of (S)-**3**, (R)-**1**, (S)-**1**, (R)-**3**, were 14.7, 15.5, 16.8, 20.2 min, respectively.

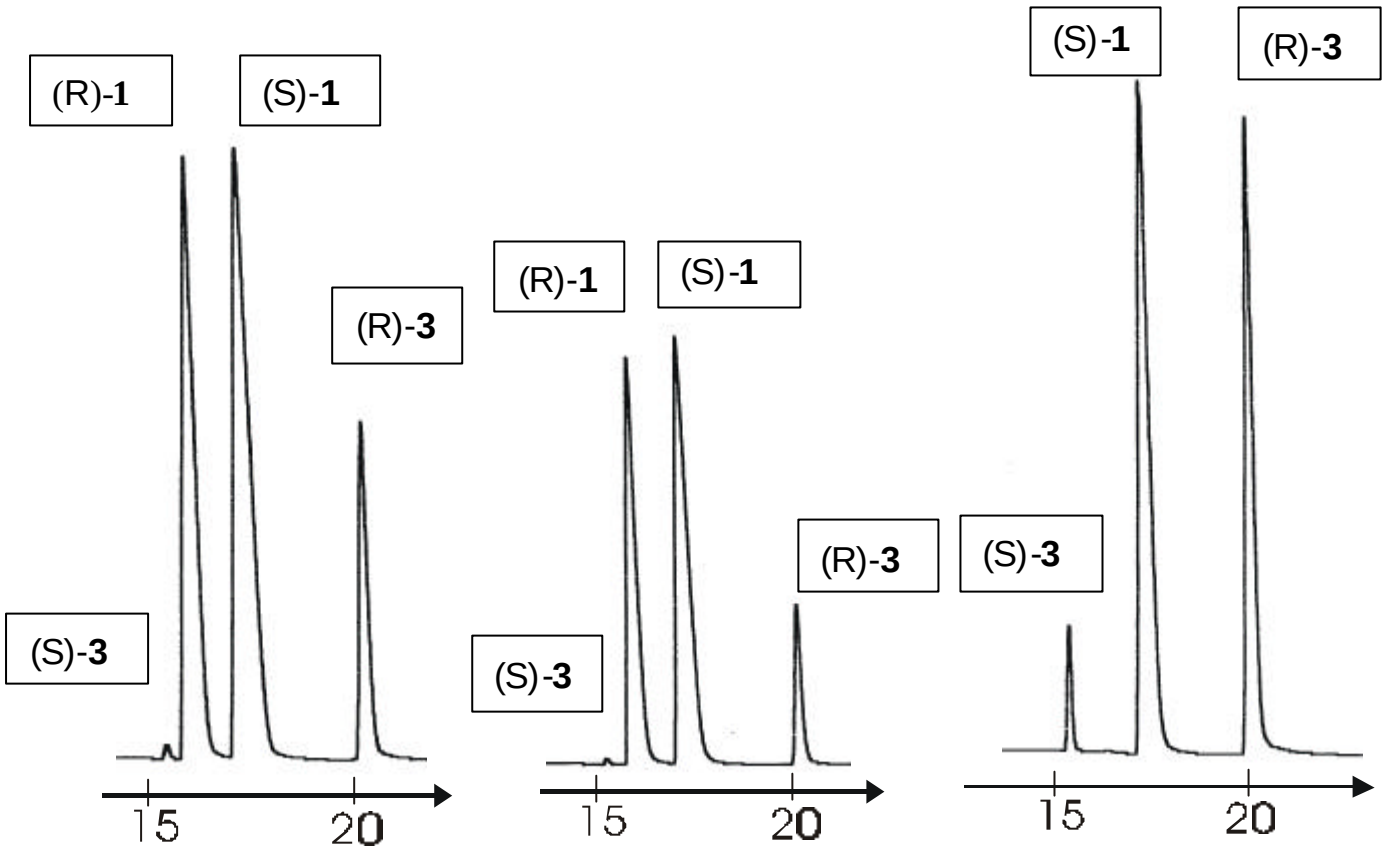


Fig. 4.12a **Fig. 4.12b** **Fig. 4.12c**

Fig. 4.12: Gas-chromatographic separation of the enantiomers of both substrate (**1**) and product (**3**) on heptakis-(2,3-di-*O*-methyl-6-*O*-tert-butyldimethylsilyl)-*b*-cyclodextrin of the co-lyophilized PSL with acetylated-*b*-cyclodextrin-catalyzed transesterification of (**1**) in toluene at t=8 hrs. **Fig. 4.12a**: 1:1 enzyme/Ac-*b*-cyclodextrin weight ratio, ee_s =14%, ee_p=94%, conv. =13%, E =39; **Fig. 4.12b**: 1:2 enzyme/Ac-*b*-cyclodextrin weight ratio, ee_s=14%, ee_p =94%, conv. =13%, E=38; **Fig. 4.12c**: 1:6 enzyme/Ac-*b*-cyclodextrin weight ratio, ee_s =99.9%, ee_p=79.1%, conv. =56%, E=88.
The retention time of (S)-**3**, (R)-**1**, (S)-**1**, (R)-**3**, were 14.7, 15.5, 16.8, 20.2 min, respectively.

Table 4.2: Co-lyophilized lipase with cyclodextrin-catalyzed enantioselective transesterification of (**1**) in toluene: Effect of the concentration ratio of enzyme to CD on the reaction improvement

Cyclodextrin (Weight ratio enzyme to CD)	ee_s[%] (S)-1	ee_p[%] (R)-3	Conv. [%]	E	Time [hr]
Mα-CD					
1:1	27.1	97	22	104	24
1:2	32	96	25	85	
1:6	>99	45	68	20	
Mβ-CD					
1:1	13	98	12	154	24
1:2	16	97	14	113	
1:6	86	95	48	130	
2HPα-CD					
1:1	13	99	11	>100	24
1:2	51	98	34	168	
1:6	94	92	50	81	
2HPβ-CD					
1:1	4	>99	3.5	>100	24
1:2	5	>99	4.5	>100	
1:6	15	99	13	>100	
2HPγ-CD					
1:1	4	99	3.5	>100	24
1:2	8	99	7	>100	
1:6	33	>99	25	>100	
3HPγ-CD					
1:1	2	99	2	>100	24
1:2	4	>99	3.7	>100	
1:6	31	>99	24	>100	

When using a 1:6 enzyme ratio to CD, in the presence of permethylated α-cyclodextrin, the reaction was terminated after 24 hrs with 68% conv., >99% ee_s, 45% ee_p and E=20 . In case of permethylated β-cyclodextrin, the reaction was terminated with 48% conv., 86% ee_s, 95% ee_p, and E=130. In case of (2-hydroxypropyl) α-

cyclodextrin the reaction was terminated with 50% conv., 94% ee_s, 92% ee_p and E=81. Other CD derivatives showed only poor enhancement of the catalytic activity of PSL in organic solvent. Results are summarized in table 4.2.

These results were a stimulus to study in details the effect of peracetylated *b*-cyclodextrin additives upon the enzyme behavior.

4.2.3. Influence of the organic solvent on the co-lyophilized enzyme behavior

As discussed in chapter 2, the choice of the organic solvent for a lipase-catalyzed reaction is known to be very crucial in determining the enantioselectivity which critically depends on the reaction medium employed. A trial to correlate the observed enantioselectivity with various solvents, depending on their log P (partition coefficient) has been performed. However, for this kind of enzyme preparation (co-lyophilized lipase with peracetylated *b*-cyclodextrin) and (lyophilized enzyme only), no correlation between the enzyme prepared, solvents and the enantioselectivity has been found (Table 4.3). For example, (compared with the lyophilized lipase only), the enhancement of the enantioselectivity using co-lyophilized lipase with peracetylated *b*-cyclodextrin was 6.7 fold higher in magnitude in isooctane (log P= 4.5) while in THF (log P= 0.49) the enhancement was 6.17 fold higher in magnitude. Also in 1,4-dioxane (log P= -1.1) the enhancement in enantioselectivity was 5.79 fold higher in magnitude while in toluene (log P= 2.5) the enhancement was 1.78 fold higher in magnitude. In regard to the enantioselectivity (E), isooctane was the best solvent employed in the transesterification of (**1**) using the co-lyophilized lipase with peracetylated *b*-cyclodextrin. However, in regard to the reaction rate and enantiomeric excess of both substrate and product, toluene was the best solvent employed in this kind of enzyme preparation.

Table 4.3: Enantioselectivity enhancement of co-lyophilized lipase with peracetylated *b*-cyclodextrin-catalyzed transesterification of (**1**) in different organic solvents.

Solvent	Log P[a]	Lyophilized[b] lipase	Peracetylated *b*-cyclodextrin co-lyophilisate[c]	Enhancement
isooctane	4.5	E[d]= 46.22	E= 311.47	6.74
THF	0.49	E= 93.63	E= 580.90	6.17
1,4-dioxane	-1.1	E= 27.91	E= 161.62	5.79
n-hexane	3.5	E= 11.35	E= 40.18	3.54
acetonitrile	-0.33	E= 99.70	E= 347.97	3.49
toluene	2.5	E= 28.89	E= 51.45	1.78

[a] P= partition coefficient; defined as the ratio of the concentration of a substance in two immiscible phases at equilibrium (octanol and water). [b] PSL lyophilized from phosphate buffer alone, pH 6.
[c] Lyophilized from aqueous phosphate buffer containing peracetylated *b*-cyclodextrin at a 1:6 weight ratio of lipase to cyclodextrin.[d] E= enantiomeric ratio.

In this study, reaction acceleration by CD without lipase was not observed. Only in presence of lipase, an influence on the reaction was noticeable. It is concluded from this reaction that CDs do not catalyze the enantioselective transesterification of (**1**) by themselves, but they influence the enzyme activity, probably by changing the conformation of the enzymatic catalytic site. In an additional experiment, PSL was lyophilized from buffer and suspended in toluene, then CD was added next to the reaction mixture at the 1:6 weight ratio as before, a slight enhancement of the reaction rate and enantioselectivity has been observed. This enhancement was much

smaller than that observed when CD was used prior to lyophilization with PSL in phosphate buffer using the same weight ratio. The enhancement of the reaction rate of PSL-catalyzed transesterification of (**1**) was in the order 1:6 > 1:2 > 1:1.

Some conclusions can be derived from this experiment. First, CD must interact with the enzyme in a specific unknown way, changing its conformation and hence influences its behavior. This behavior is the result of the co-lyophilization of the enzyme with peracetylated *b*-cyclodextrin. Second, the excess of CD that did not bind to the enzyme remains in solution and acts as a host-guest complex. This can explain why the reaction rate was much increased when using 1:6 weight ratio rather than using 1:1 weight ratio enzyme to CD. In fact, the ability of CDs to form host-guest complex limits substrate as well as product inhibition.[66] The encapsulation of product within the cavity of CD reduces the amount free in solution, therefore, enhance the reaction rate. The ability of 1-(2-furyl)ethanol (**1**) as well as its ester (**3**) to be included in the peracetylated CD cavity was proved by complexation-induced shifts in ^{1}H as well as ^{13}C NMR (shown later)

A preferential or enantioselective binding of substrate (**1**) with CD making one enantiomer more accessible by the enzyme, could be also contributing to the enhancement caused by CDs, allowing an enantioselective reaction with the acyl donor. When performing the esterification of (**1**) using pyridine as a catalyst (other than lipase), acetyl chloride and using peracetylated *b*-cyclodextrin (600 mg) as additive, surprisingly, a poorly enantioselective reaction has been observed and the ester formed did not show the expected 1:1 peak area ratio. It is concluded that, the inclusion of (**1**) in peracetylated-*b*-cyclodextrin cavity has occurred in a preferential way. This preferential complexation of (**1**) by peracetylated *b*-cyclodextrin cavity allows one enantiomer of (**1**) to be tightly attached by the CD while the other leaves the cavity faster to react with the acyl donor (acetyl chloride in this case) forming the corresponding ester. It was observed that (R)-**1** was the faster enantiomer in leaving the cavity while the (S)-**1** was the slower one. The reaction has the following data: time = 7 hrs, ee_s = 0.26%, ee_p = 1.94%, Conv. = 12.02% and E = 1.04 and in 2 days, ee_s= 5.2%, ee_p=31%, Conv. = 15% and E = 2.03. The same effect has been observed when using permethylated *b*-cyclodextrin (600 mg) as additive, the reaction has the following data: time = 7 hours, ee_s = 0.31%, ee_p = 16.7%, Conv. = 2.0% and E = 1.4 and in 2 days, ee_s = 0.6%, ee_p = 7.0%, Conv. = 8.0% and E =

1.16. However, the yield was very poor compared to the normal esterification reaction performed without CD additives. This supports the idea that the improvement of the reaction condition of co-lyophilized PSL with CD catalyzed transesterification of (1) in organic solvent was based on a combined effect of CDs and enzyme, both of them contributing in the improvement of the reaction parameters.

Trying to find a proof for the binding of CD with PSL forming one block leading to a change in the enzyme behavior, SEM (scanning electron microscopy) and EDX (energy dispersive X-ray) of the co-lyophilized lipase with peracetylated *b*-cyclodextrin have been investigated. It is to be noted that other modified CDs (perbutyrated CD) did not show any enhancement when used in a co-lyophilized form with lipase.

4.2.4. Scanning electron microscopy (SEM) and energy dispersive X- ray (EDX) of co-lyophilized lipase with peracetylated *b*-cyclodextrin

In general, electron microscopy (EM) is one of the most useful tools in chemical science. This is particularly important in the field of heterogeneous catalysis by which one can define, describe and characterize catalytic materials.[29] A large improving in the field of electron microscope as analytical tool has been observed within the last few years. Scanning instruments now permit the imagining and the identification of nanoclusters consisting of only few atoms. In order to spot some light on what has happened physically during the co-lyophilization of lipase with peracetylated *b*-cyclodextrin, scanning electron microscopy (SEM) has been used to compare the morphology of peracetylated *b*-cyclodextrin alone (Fig 4.13a, b), lipase alone (Fig. 4.13c), co-lyophilized lipase with peracetylated *b* -cyclodextrin 1:1, 1:2 and 1:6 weight ratio (Fig. 4.13d, e, f, respectively). Comparing all micrographs in figure 4.13, it was concluded that the morphology of lipase (Fig. 4.13c) has been totally changed upon mixing with peracetylated *b*-cyclodextrin in the co-lyophilized process.

Because of difficulties in classical elemental analysis, EDX-measurements (Fig. 4.14) were carried out. A typical EDX spectra of enzyme alone is shown in figure 4.14a, peracetylated *b*-cyclodextrin

alone (Fig. 4.14b) and co-lyophilized lipase with peracetylated *b*-cyclodextrin 1:6 weight ratio (Fig. 4.14c). Qualitative analysis confirmed the presence of carbon and oxygen in peracetylated *b*-cyclodextrin. Surprisingly, silicon (largest peak), oxygen, sodium, aluminum, calcium and iron were found in lipase only. After co-lyophilization of lipase with peracetylated *b*-cyclodextrin, EDX analysis confirmed the presence of carbon, oxygen, sodium, silicon and calcium in attempts that lipase and peracetylated *b* -cyclodextrin are together in one particle and not separated. It was concluded from the SEM and EDX analysis that the addition of CD to the enzyme changes its morphology. This change in morphology of lipase may contribute in the enhancement of enantioselectivity and enantiomeric ratio in lipase-catalyzed transesterification reactions.

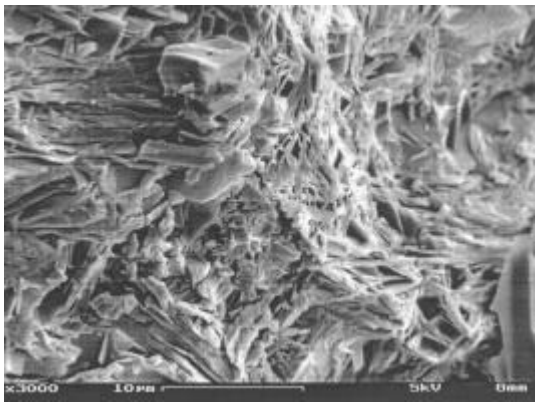


Fig. 4.13 a

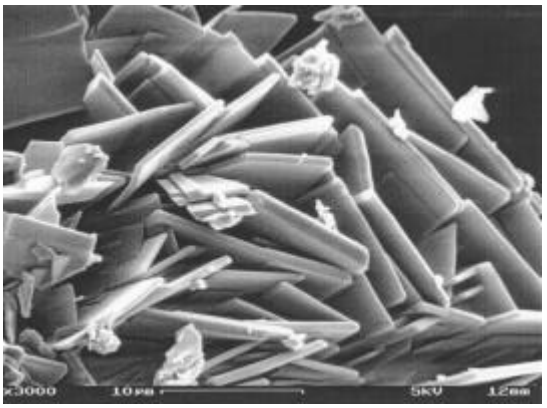


Fig. 4.13 b

Fig. 4.13 a: A typical SEM-micrograph of pure peracetylated *b*-cyclodextrin; **Fig. 4.13 b:** An SEM-micrograph of magnified part of fig. 4.13 a.

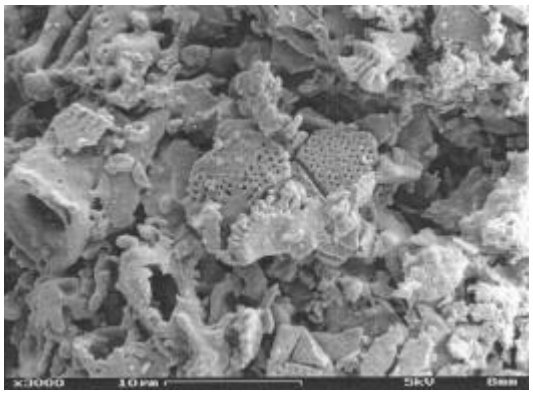


Fig. 4.13 c

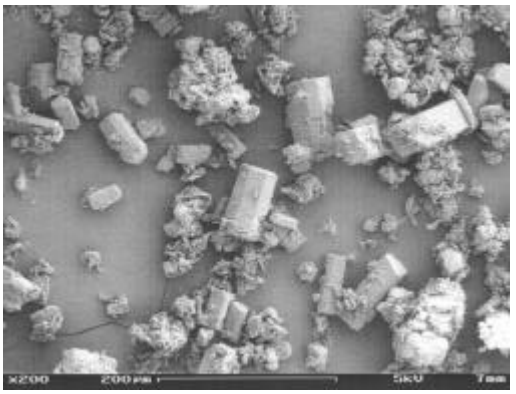


Fig. 4.13 d

Fig. 4.13 c: A typical SEM-micrograph of the commercially available *Pseudomonas cepacia* lipase; **Fig. 4.13 d:** An SEM-micrograph of co-lyophilized lipase with peracetylated *b*-cyclodextrin (1:1 weight ratio).

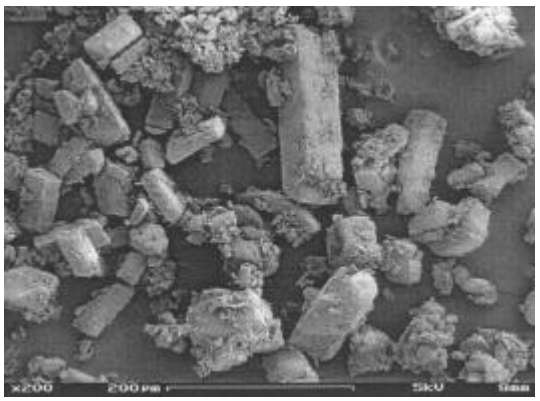


Fig. 4.13 e

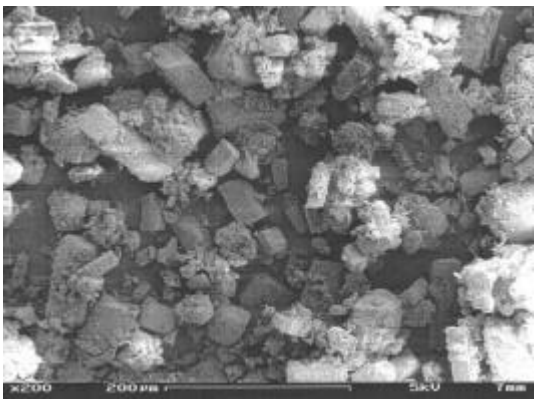


Fig. 4.13 f

Fig. 4.13 e: A typical SEM-micrograph of the co-lyophilized lipase with peracetylated *b*-cyclodextrin (1:2 weight ratio); **Fig. 4.13 f:** An SEM-micrograph of co-lyophilized lipase with peracetylated *b*-cyclodextrin (1:6 weight ratio).

Fig. 4.13: An SEM study showing the difference between enzyme before and after co-lyophilization with peracetylated *b*-cyclodextrin.

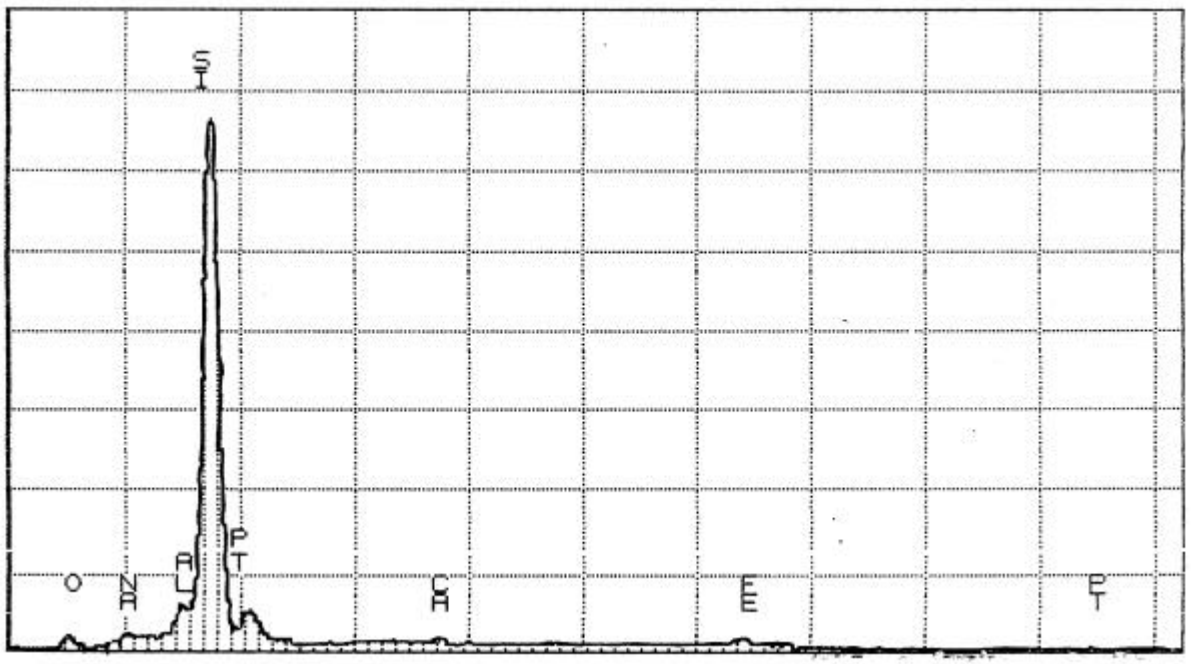


Fig. 4.14a: A typical EDX spectrum of the commercially available *Pseudomonas cepacia* lipase.

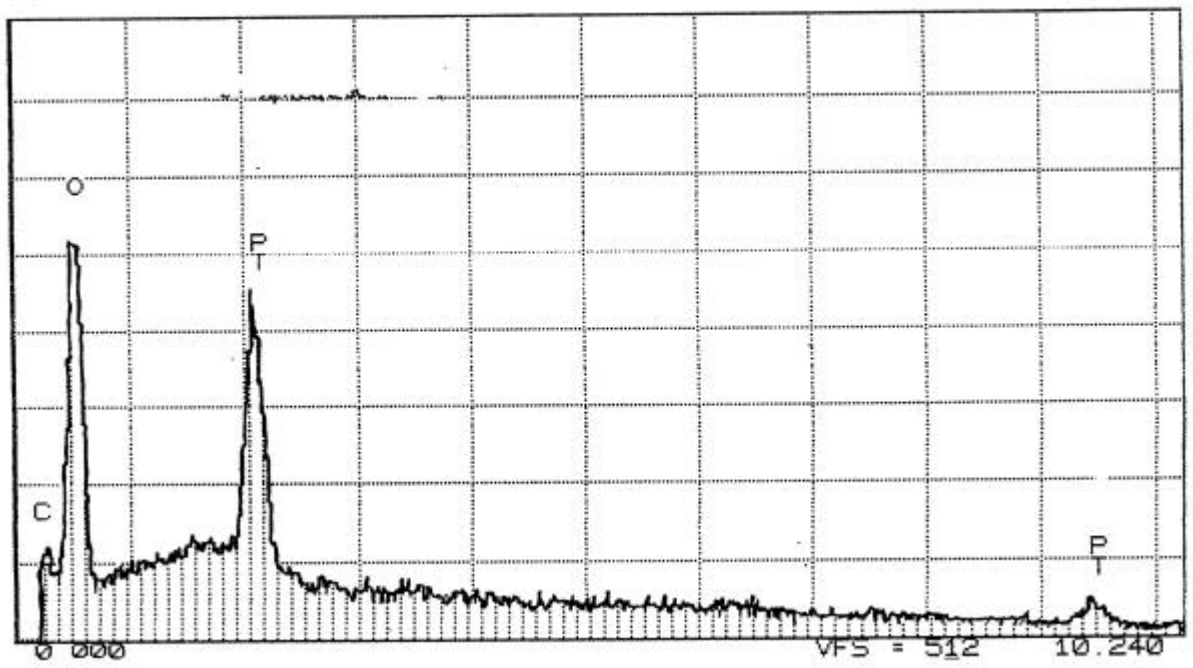


Fig. 4.14b: A typical EDX spectrum of the synthesized peracetylated *b*-cyclodextrin.

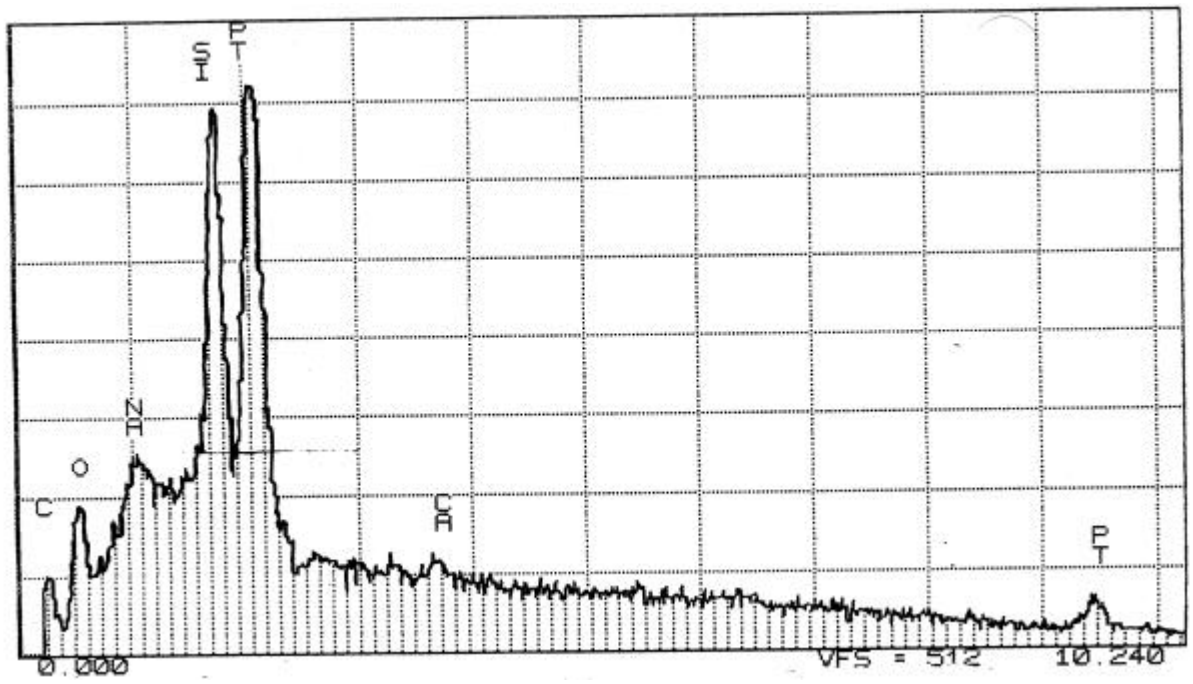


Fig. 4.14c: A typical EDX spectrum of the co-lyophilized lipase with peracetylated *b*- cyclodextrin.

Fig. 4.14: An EDX study showing the difference between the composition of the lipase only (Fig. 4.14a), peracetylated *b*-cyclodextrin only (Fig. 4.14b) and co-lyophilized lipase with peracetylated *b*-cyclodextrin.

4.2.5. Evidence for the complexation of 1-(2-furyl)ethanol in peracetylated *b*-cyclodextrin

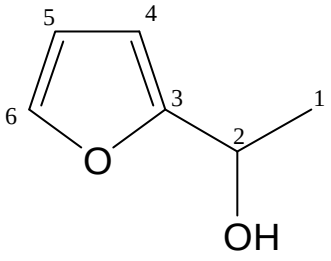


Table 4.4: ^{1}H NMR of (**1**) and complexation-induced ^{1}H chemical shift difference (ppm)

	3H-CH_3	OH	H-C_2	H-C_4	H-C_5	H-C_6
(**1**)	1.32-1.33	2.47	4.59-4.64	6.00-6.01	6.03-6.05	7.05-7.15
(**1**) **+** peracet-ylated *b*-CD	1.34-1.35	2.64	4.63-4.68	6.03-6.04	6.05-6.07	7.08-7.08
Chem. shift	0.02	0.17	0.04	0.03	0.02	0.03

The ^{1}HNMR (400 MHz) signals of the hydroxyl group of (**1**) recorded in the presence of peracetylated *b*-cyclodextrin, showed a downfield shift (0.17 ppm) which suggests the involvement of the OH group of (**1**) in complexation with CD.

The neighboring proton at C_2 showed a downfield shift of 0.04 ppm, that of CH_3 was 0.02 ppm. The aromatic protons showed a different pattern in the presence of peracetylated *b*-cyclodextrin, the ortho and para hydrogen experienced a deshielding of 0.03 ppm while the meta hydrogen showed a deshielding of 0.02 ppm. The evidence of the inclusion of (**1**) in cyclodextrin cavity is also provided by the observed ^{13}C NMR shift differences reported in table 4.5 for solution of the same concentration as used for recording the proton spectra.

Table 4.5: ^{13}C NMR of (**1**) and complexation-induced ^{13}C chemical shift difference (ppm)

	C_1	C_2	C_3	C_4	C_5	C_6
(**1**)	21.62	63.66	158.71	105.00	110.27	141.63
(**1**) **+** peracetyl-ated *b*-CD	21.66	63.64	158.79	104.99	110.28	141.63
Chem. shift	+0.04	-0.02[a]	+0.08	-0.01	+0.01	0.00

a: Upfield displacements are negative

The carbons of the aromatic ring were less sensitive to the presence of CD, only C_3 was more sensitive and showed a downfield shift of 0.08 ppm. Other carbons like C_4 showed an upfield shift of 0.01 ppm, C_5 showed a downfield shift of 0.01 ppm while C_6 showed no displacement. C_1 (methyl group) showed a downfield shift of 0.04 ppm while C_2 showed an upfield shift of 0.01 ppm.

In addition to the complexation-induced chemical shift of (**1**) in presence of peracetylated *b*-CD, the NMR of peracetylated *b*-CD and

its complexation-induced chemical shift difference can also provide information on the complexation geometry. The analysis of the shifts corresponding to the protons of peracetylated *b*-CD (host) can be related to the location of (**1**) (guest) in the CD cavity (Table 4.6).

Table 4.6: ^{1}H NMR of peracetylated *b*-CD and complexation-induced ^{1}H chemical shift difference (ppm)

	H-C_1	H-C_2	H-C_3	H-C_4	H-C_5	H1-C_6	H2-C_6	3H-CH_3
Peracetylated *b*-CD	5.17-5.24	4.99-5.03	5.59-5.63	3.72-3.76	4.30-4.33	4.79-4.82	4.40-4.44	1.87-1.95
Peracetylated *b*-CD + (**1**)	5.21-5.22	4.98-5.02	5.56-5.60	3.74-3.78	4.27-4.30	4.76-4.79	4.39-4.40	1.88-1.95
Chemical shift	+0.04	0.0	-0.03	+0.02	-0.03	-0.03	-0.01	+0.01

An upfield shift for the CD protons is expected when an aromatic guest molecule is located close to the protons in question[35] since they are located in the shielding area of the aromatic ring of the guest. It is known that the H_3 and H_5 protons are located within the cavity of CDs, H_3 is situated near the larger rim, while H_5 is more deeply embedded in the cavity. H_6 protons are located at the narrower entrances and H_2, H_4 protons are located at the wider entrance.

In presence of (**1**), the H_3, H_5 and H-1_6 protons were shifted upfield by 0.03 ppm, however, a much smaller downfield shift (0.02 ppm) was observed for H_4 while H_2 showed no displacement. H_1 showed a much higher downfield shift (0.04 ppm). These results suggest that the alcohol (**1**) is incorporated axially into the peracetylated *b*-CD cavity. Since the ^{1}H NMR spectroscopic data showed only small differences between the chemical shifts of the free and the complexed alcohol, it was decided to use more pronounced shifts to

observe the interaction between furyl ethanols protons and that of CD. This is based on ROESY spectra. The alcohol (**1**) in ROESY spectra showed an intermolecular cross-peaks between the free hydroxyl group and both protons located at C_6 in the host as well as the methyl group of the acetyl moiety existing in the host. An interaction has been observed between the H-methyl of (**1**) and those of acetyl group existing in the host.

Only the aromatic protons located on C= 110 and C=105 ppm (ortho and meta) in (**1**) showed a strong interaction with the H-3 and a weaker on with H-1 of the host, while the proton at C= 141 (para) did not show any interaction with the host. The proton of C-H in (**1**) showed an interaction with H-1, H-2, H-5 and H-6 of the host. No interaction has been observed between the aromatic protons of (**1**) and those of the 6th position in the host. This might give a postulation about how (**1**) is included in the cavity of peracetylated *b*-CD (Fig. 4.15)

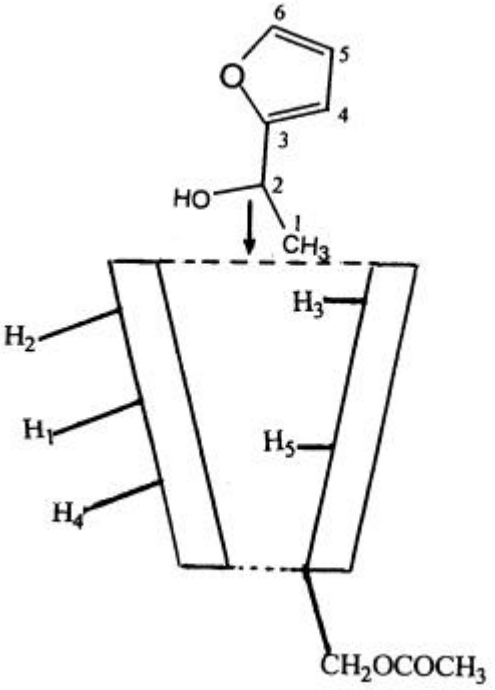
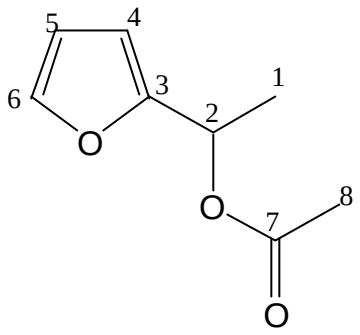


Fig. 4.15: A schematic representation of the inclusion of (**1**) in the cavity of peracetylated *b*-CD.

4.2.6. Evidence for the complexation of (3) in peracetylated *b*-cyclodextrin



The ester (**3**) can also be complexed in the CD to prevent product inhibition, the evidence for its complexation was quite similar to that for (**1**).

The complexation-induced ^{1}H chemical shift differences of (**1**) in presence of peracetylated *b*-cyclodextrin were too small to assign for an inclusion. The interpretation of ^{13}C NMR shift changes were difficult due to marked sensitivity of carbon shielding towards even minor conformational distortions. The complexation-induced ^{1}H chemical shift difference recorded for peracetylated *b*-cyclodextrin in presence of (**3**) showed a similar effect as found in its spectra in presence of (**1**).

Table 4.7: The complexation-induced ^{1}H chemical shift differences (ppm) resulting from inclusion of (**3**) in peracetylated *b*-cyclodextrin

	H-C_1	H-C_2	H-C_3	H-C_4	H-C_5	H1-C_6	H2-C_6	3H-CH_3
Chem. shift	+0.05	0.01	-0.02	+0.01	-0.02	-0.03	-0.01	+0.01

The ROESY spectra recorded for (**3**) in presence of peracetylated *b*-cyclodextrin showed intermolecular cross-peaks between methyl protons of (**3**) and those of the acetyl moiety in CD. The aromatic protons located on C= 110 and C=105 ppm (ortho and meta) in (**1**) showed an interaction with the H-5 as well as H-3, H-6 and a weaker one with H-1 of the host, while the proton at C= 141 (para) did not show any interaction with the host. The proton of C-H in (**3**) did not show interaction with the host's protons.
It was concluded that the complexation of (**3**) with CD is nearly the same as it was with (**1**).

4.2.7. Postulated mechanisms for the enhancement effects caused by the peracetylated *b*-cyclodextrin

As mentioned before, the improvement of the reaction conditions of the co-lyophilized PSL with cyclodextrin-catalyzed transesterification of (**1**) in organic solvent was based on a combined effect of CDs and enzyme, both of them contributing in the improvement of the reaction parameters. SEM and EDX have proved the effect of CDs on the enzyme morphology. This change in morphology of the enzyme might be responsible for the enhancement observed in PSL-catalyzed transesterification of (**1**). Other postulations are shown in the mechanism outlined below. It has been already proved that the one aspect of the use of peracetylated *b*-cyclodextrin to alter the kinetics of an enzyme-catalyzed reaction involves the CD host selectivity complexing a component of the reaction mixture [the alcohol (**1**) or the product (**3**)] hence reducing the amount free in solution. The complexation of (**1**) or (**3**) with peracetylated *b*-cyclodextrin has been indicated as described before by NMR techniques. Here a postulated mechanisms based on the encapsulation of (**1**) or (**3**) inside the cavity of peracetylated *b*-cyclodextrin have been reported. The first mechanism is based on the encapsulation of (**1**) inside the cavity of peracetylated *b*-cyclodextrin while the second mechanism is based on the encapsulation of (**3**) inside the cavity of peracetylated *b*-cyclodextrin.

When (**1**) is encapsulated in the cavity of CD, an addition on the C=O occurs leading to the formation of the ester (**3**) and the free CD. The ester (**3**) itself acylate again the free CD returning again to the

peracetylated *b*-cyclodextrin and regenerating the alcohol (**1**), it is this cycle which remove the amounts of alcohol free in solution leading to the observed enhancement. (Fig. 4.16)

When (**3**) is the encapsulated component in the cavity of peracetylated *b*-cyclodextrin, Claisen condensation occurs as outlined in figure 4.17. The acyl moiety of the ester (**3**) is reacted with that of the peracetylated *b*-cyclodextrin affording the diketone and the free alcohol (**1**). The free alcohol itself reacts again with the diketone by addition on C=O affording again the ester and the peracetylated *b*-cyclodextrin.

Both mechanisms are outlined below.

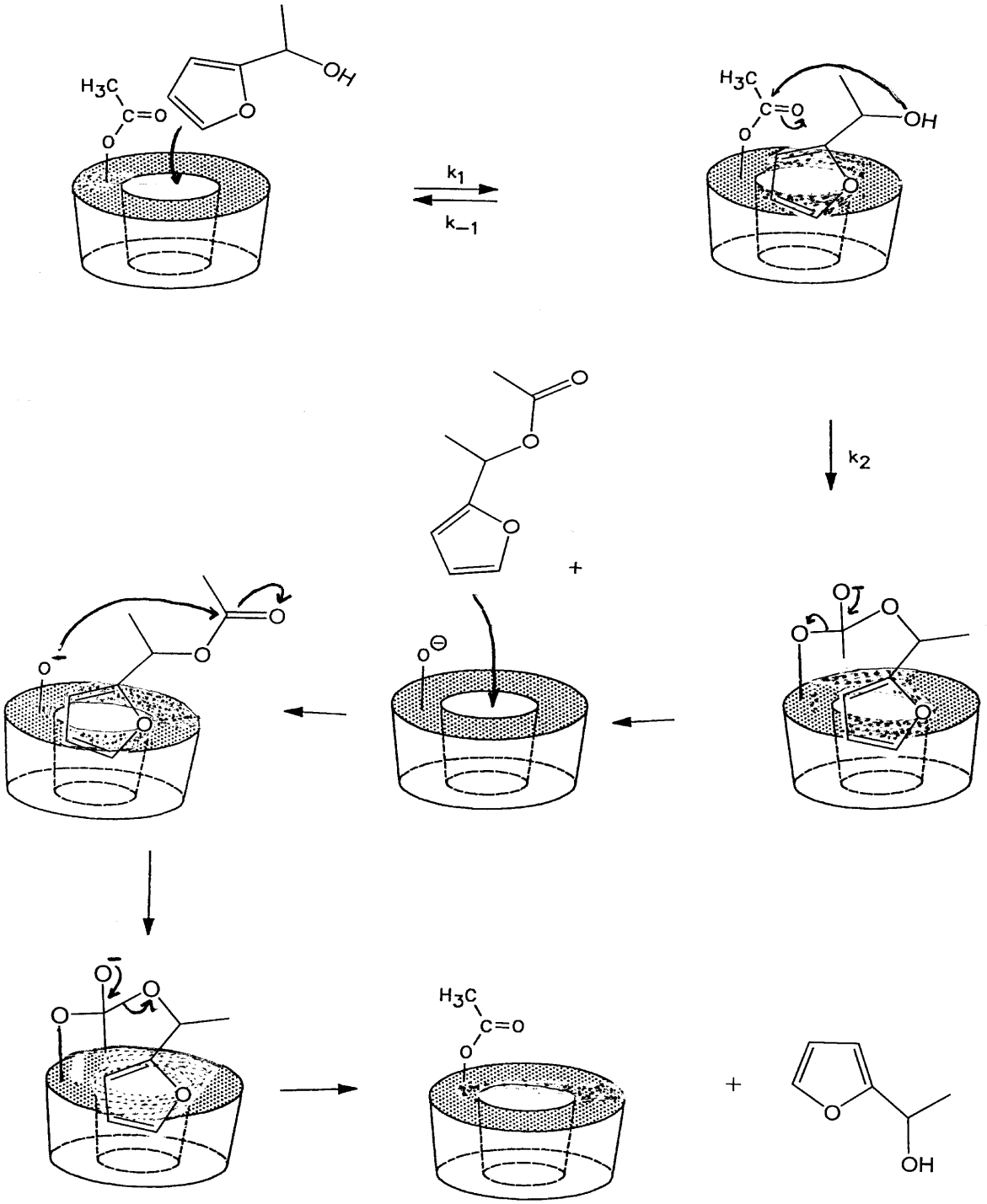


Fig. 4.16: The first postulated mechanism due to the encapsulation of (**1**) in peracetylated *b*-cyclodextrin.

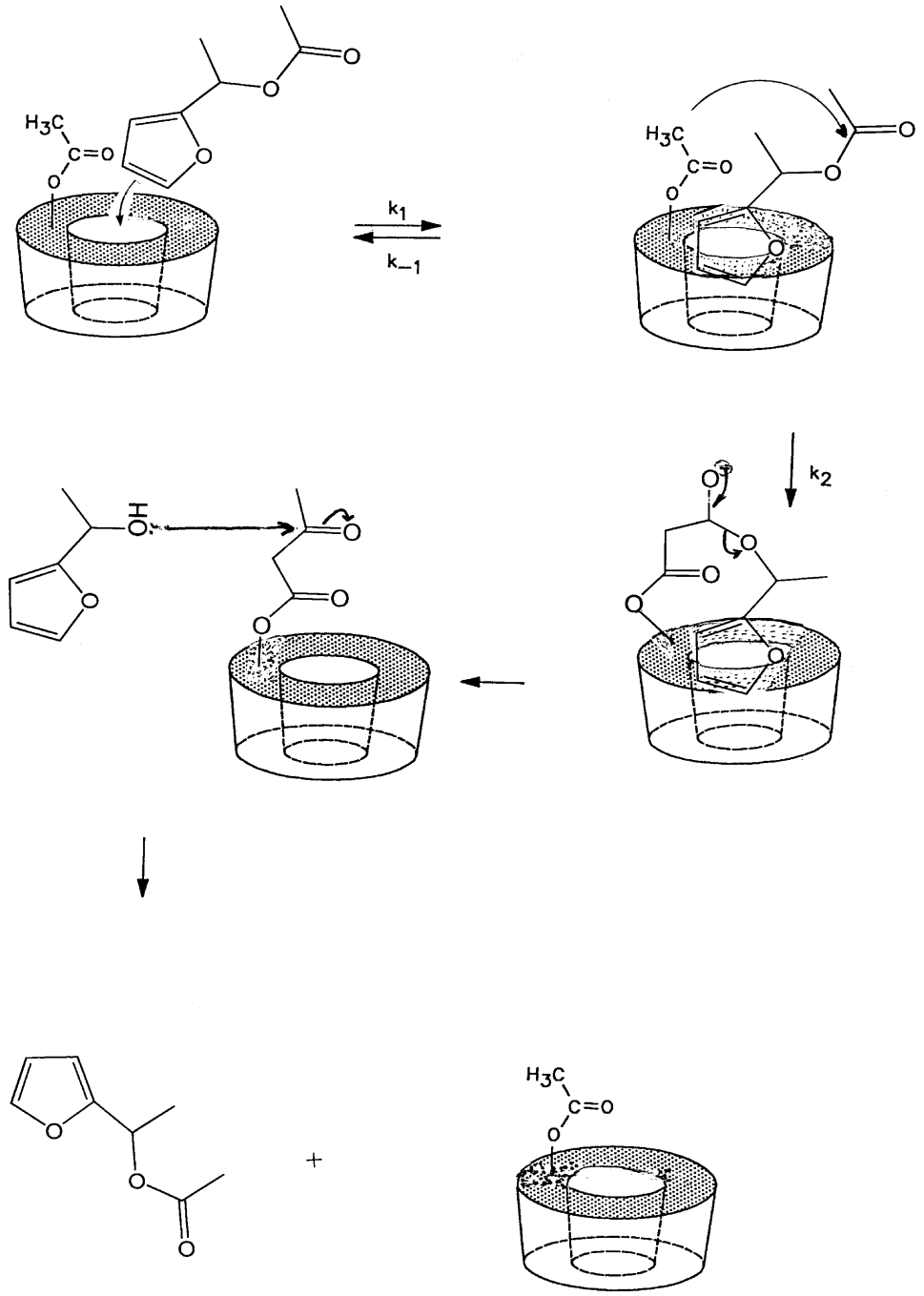


Fig. 4.17: The second postulated mechanism due to the encapsulation of (**3**) in peracetylated *b*-cyclodextrin.

Trying to prove the second postulation, the lipase PSL was added to a mixture of 100 mg peracetylated *b*-cyclodextrin and 77 µl ester (R,S)-**3** dissolved in 3 ml toluene. The reaction was thermostated at 40°C. Surprisingly, the alcohol (R)-**1** is formed after 4 days having the following data: ee_s [ester (R,S)-**3**] 41%; ee_p [alcohol (R)-**1**] 99.9%; Conv. =29.1% and E>1000. After 5 days, ee_s [ester (R,S)-**3**] 44%; ee_p [alcohol (R)-**1**] 99.9%; Conv. = 30.8% and E>1000. The formation of the alcohol (R)-**1** can be due to the Claisen condensation shown in the second postulation (Fig. 4.17) or the lipase PSL contains some water which can itself hydrolyze the ester (R,S)-**3**, however, the second assumption is not favored.

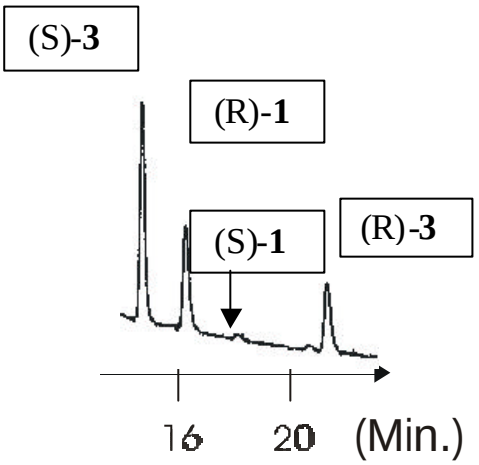


Fig. 4.18: Gas-chromatographic separation of the enantiomers of both substrate (R,S)-**3** and product (R)-**1** resulting from the reaction of peracetylated CD and ester (R,S)-**3** in presence of PSL in toluene, t= 5 days, ee_s (ester (R,S)-**3**) 44%; ee_p (alcohol (R)-**1**) 99.9%; Conv. = 30.8% and E>1000.

4.2.8. Activation of serine protease subtilisin Carlsberg and *a*-chymotrypsin in organic solvents by co-lyophilization with peracetylated cyclodextrin

In order to generalize the procedure reported before (co-lyophilized lipase (PSL) with cyclodextrin-catalyzed transesterification of 1-(2-furyl)ethanol (**1**) in toluene to be used for other less active enzymes in organic solvent, the effect of peracetylated *b*-cyclodextrin additives on the subtilisin Carlesberg and *a*-chymotrypsin-catalyzed transesterification of (**1**) using three different vinyl esters as acyl donors in toluene as solvent has been studied (Fig. 4.19).

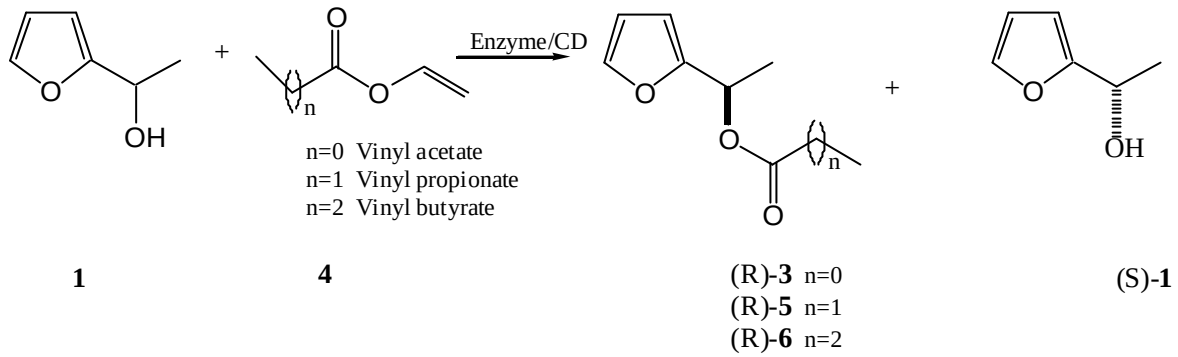


Fig.4.19: Co-lyophilized lipase with peracetylated *b*-cyclodextrin-catalyzed enantioselective transesterification of racemic (**1**) using vinyl acetate (n=0), vinyl propionate (n=1) and vinyl butyrate (n=2) as acyl donors in toluene as organic solvent.

At first, the racemic esters (R,S)-**5** and (R,S)-**6** were synthesized on an analytical scale to optimize a baseline separation of the enantiomers by gas chromatography (Fig. 4.20).

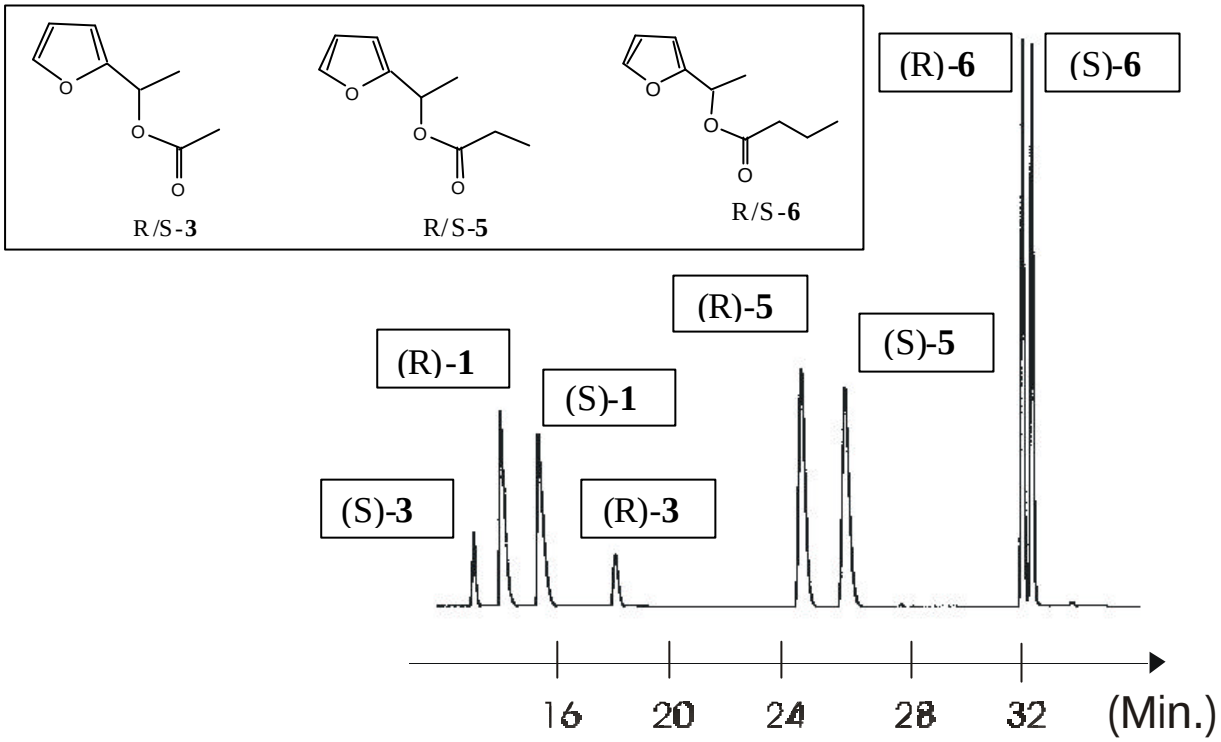


Fig. 4.20: Gas-chromatographic separation of the enantiomers of both substrate (R,S)-**1** and products (R,S)-**3**, (R,S)-**5** and(R,S)-**6.** The retention time of (S)-**3**, (R)-**1**, (S)-**1**, (R)-**3**, (R)-**5**, (S)-**5**, (R)-**6** and (S)-**6** were 13.7, 14.8, 16.0, 18.4, 24.4, 25.9, 32.0 and 32.3 min. respectively.

In the reference reaction, subtilisin Carlsberg and *a*-chymotrypsin have been used in the transesterification of (**1**) using vinyl acetate, propionate and butyrate, respectively as acyl donors in toluene as solvent. From this screening, it was noticed that the reactions were very slow (in 24 hrs, 5.8% conv., 5.1% ee_s, 83.2% $ee_{p.}$, E=11.5 in case of using subtilisin Carlsberg and 0.35% conv., 0.4% ee_s, 99.9% $ee_{p.}$, E >1000 in case of using *a*-chymotrypsin)**,** only vinyl butyrate showed the best performance in comparison with other vinyl esters used as acyl donors in the transesterification of (**1**).
The co-lyophilizing procedure has been performed using subtilisin Carlsberg and *a*-chymotrypsin with peracetylated *b*-cyclodextrin additives-catalyzing the transesterification of (**1**) in toluene. The same weight ratio (enzyme/cyclodextrin) has been used as reported before. Only an enhancement effect in the rate of conversion was noticeable when subtilisin Carlsberg or *a*-chymotrypsin were co-lyophilized with CD (1:6 weight ratio enzyme to cyclodextrin). The enhancement was 20 fold higher in magnitude than that obtained without the use of CD.

In conclusion, the utility of peracetylated *b*-cyclodextrin as additive in lipase-catalyzed transesterification of 1-(2-furyl)ethanol in organic solvent has been demonstrated. The results suggest that co-lyophilization with CD can be a useful method for the activation of enzymes in organic solvents.

5. Lipase-catalyzed enantioselective access to enantiomerically pure (R)- and (S)-*trans*-4-phenyl-3-butene-2-ol

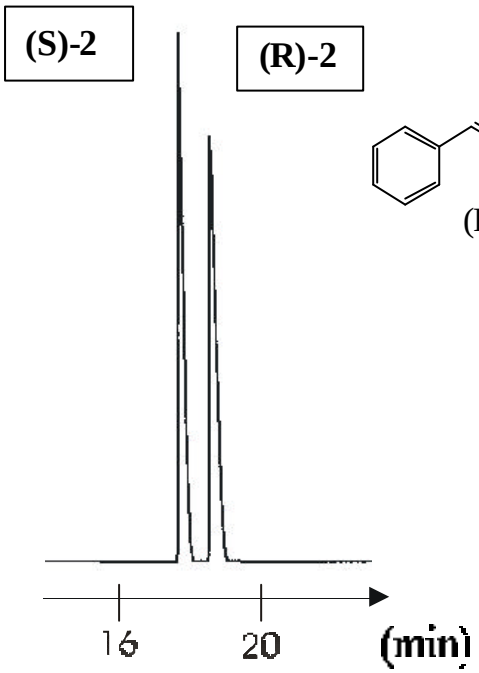
5.1. Introduction

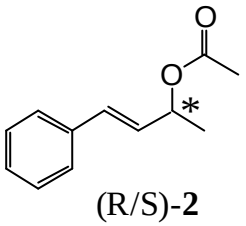
In general, enantiomerically pure allylic alcohols are useful chiral auxiliaries in organic synthesis, as many stereoselective transformations can be performed by taking advantage of the 1,3-allylic motif of such systems.[79] They are preferably obtained by kinetic resolution of the racemic alcohols using Sharpless' epoxidation[80] or via enzyme mediated transformations.[81] The latter biochemical process has become a standard reaction protocol in organic synthesis.[24]
The enantiomeric allylic alcohols (S)-(-)- and (R)-(+)-*trans*-4-phenyl-3-butene-2-ol (**1**) are frequently used as chiral building blocks for numerous biologically active compounds and they are employed for studies of reaction mechanisms.[82] Therefore, these chiral starting materials should be available as both enantiomers in high enantiomeric excess. Different accesses to chiral *trans*-4-phenyl-3-butene-2-ol (**1**) have been reported using ruthenium catalysts[83] or enzyme-catalyzed enantioselective reduction of the corresponding ketone.[84] The lipase-catalyzed transesterification of (R,S)-*trans*-4-phenyl-3-butene-2-ol using vinyl acetate[85] or dimethyl malonate[82] as acyl donor has been reported, however, the use of vinyl acetate as acyl donor in enzymatic reactions is restricted to enzymes which are not sensitive to the acetaldehyde released from the tautomerization of vinyl alcohol (section 3.1). In case of dimethyl malonate used as acyl donor, the enantiomeric excess did not exceed 93 % ee (S)-**1** and in addition, the conditions applied in the kinetic resolution, e.g. reduced pressure (8 Torr) and the use of $KHCO_3$ limit their application at a large scale. An efficient method for the gram-scale preparation of enantiomerically pure (R)- and (S)-*trans*-4-phenyl-3-butene-2-ol is reported using two different approaches based on the lipase-catalyzed transesterification of the racemic alcohol using isopropenyl acetate as acyl donor in toluene as solvent affording the (S)-alcohol and (R)-ester and the lipase-catalyzed enantioselective hydrolysis of the corresponding acetate affording the (R)-alcohol and (S)-ester, respectively. A baseline separation of both the derivatized alcohol and the ester enantiomers was achieved using heptakis-(2,3-di-*O*-methyl-6-*O*-tert-butyldimethylsilyl)-*b*-cyclodextrin as chiral stationary phase in GC.[47]

5.2. Results and discussion

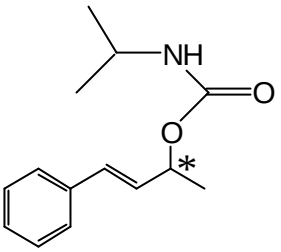
5.2.1. Synthesis and biochemical transformation reactions

The racemic alcohol (R,S)-**1** and the corresponding acetate (R,S)-**2** were synthesized on an analytical scale to optimize a baseline separation of the enantiomers by gas chromatography. The enantiomers of the allylic alcohol were not separated on heptakis-(2,3-di-*O*-methyl-6-*O*-tert-butyldimethylsilyl)-*b*-cyclodextrin used as chiral stationary phase in GC, however, upon derivatization with acetic anhydride [the acetate (**2**)] or isopropylisocyanate [alcohol (**1**) as carbamate], a base-line separation has been achieved (Fig. 5.1).





(R/S)-**2**



(R/S)-**1**

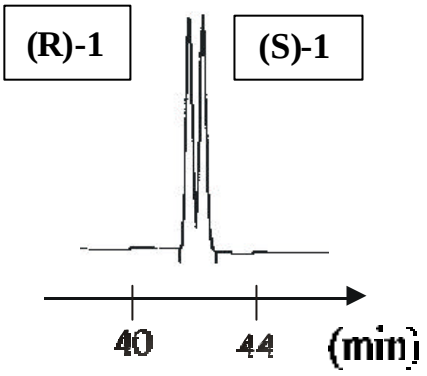
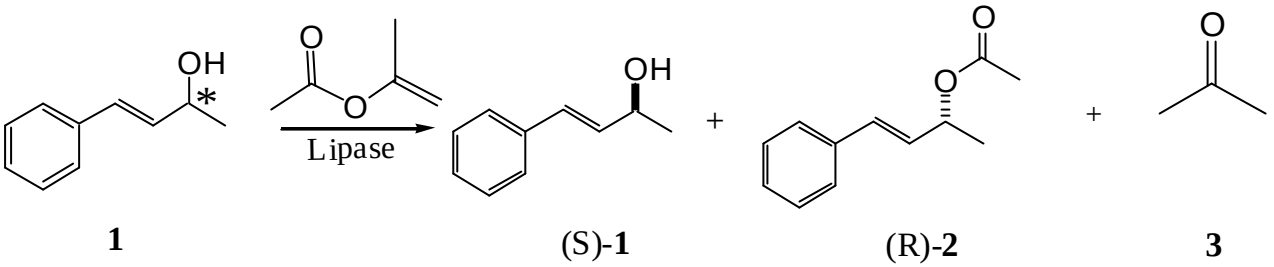


Fig. 5.1a **Fig. 5.1b**

Fig. 5.1: Gas-chromatographic separation of racemic (**1**) as isopropylcarbamate **(Fig. 5.1b)** and acetate (**2**) **(Fig. 5.1a)** on heptakis-(2,3-di-*O*-methyl-6-*O*-tert-butyldimethylsilyl)-*b*-cyclodextrin with a separation factor α= 1.05 and R_s = 2.83 for the ester (**2**) and α=1.01 and R_s = 1.07 for the alcohol (**1**). The oven temperature was 130°C (isothermal) for 20 min, the n 30°C/min until 160°C for 25 min.

5.2.2. Lipase-catalyzed kinetic resolution of (R,S)-*trans*-4-phenyl-3-butene-2-ol (R,S)-1

Different lipases were screened in resolving racemic *trans*-4-phenyl-3-butene-2-ol (**1**) either in a hydrolytic reaction using phosphate buffer (pH = 6) in the enantioselective hydrolysis of the corresponding acetate (R,S)-**2** or by transesterification of (R,S)-**1** using isopropenyl acetate as an innocuous acyl donor in toluene as solvent.



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

Fig. 5.2: Lipase-catalyzed kinetic resolution of racemic (**1**) using isopropenylacetate as acyl donor in toluene as organic solvent.

Among the lipases tested were Lipase from *Pseudomonas cepacia* (PSL), *Pseudomonas fluorescens* (PFL), *Pseudomonas cepacia* immobilized on ceramic particles (PSL-C), *Pseudomonas cepacia* immobilized on diatomaceous earth (PSL-D), *Aspergillus niger* (ANL), *Candida rugosa* typeVII (CRL), *Candida cylindracea* (CCL), Novozyme 435 (CAL-B), Novozyme 525 L and Lipozyme RM immobilized (RML). The transesterification of (**1**) was carried out at 40^{o}C in toluene at a molar ratio of isopropenyl acetate to racemic (**1**) of 2:1 to ensure the irreversibility of the reaction. A simultaneous base line separation of both substrate and product enantiomers was achieved using heptakis-(2,3-di-*O*-methyl-6-*O*-tert-butyldimethylsilyl)-*b*-cyclodextrin as a stationary phase by GC[47] with a separation factor α= 1.05 and R_s = 2.83 for the ester (**2**) and α=1.01 and R_s = 1.07 for the alcohol (**1**). The results of the lipase-catalyzed transesterification of (**1**) are summarized in table 5.1.

Table 5.1: Lipase-catalyzed transesterification of (R,S)-**1** using isopropenyl acetate in toluene

Lipase	Time (hr)	[%]ee_s (S)-**1**	[%]ee_p (R)-**2**	% conversion	E
PSL	9	97.2	86.9	52.8	61
PSL-C	4	99.9	91.8	52.1	228
PSL-D	4	99.9	96.7	51.0	680
PFL	9	99.9	92.2	52.0	285
ANL	48	52.0	41.5	55.5	4
CCL	24	50.6	57.6	47	6
CRL	48	44.8	46.4	49.1	4
CAL-B	6	99.9	80.3	55.4	93
Novozyme 525 L	9	93.2	97.2	49.0	323
RML	48	46.4	22.5	67.2	2

ee_s: enantiomeric excess of substrate (alcohol), ee_p: enantiomeric excess of product (ester), E : enantiomeric ratio.

In all cases of the transesterification of (**1**), (R)-**1** was the faster reacting enantiomer yielding (R)-**3** in high ee and leaving (S)-**1** as enantiomerically pure unreacted enantiomer. Both lipases from *Pseudomonas cepacia* immobilized on ceramic particles (PSL-C) and immobilized on diatomaceous earth (PSL-D) displayed high enantioselectivity towards (**1**) (Table 5.1, Fig. 5.6). In regard to the ee of the remaining substrate (S)-**1** and that of the product (R)-**3** as well

as the rate of conversion (51% in 4 hrs) and enantiomeric ratio E>300, PSL-D was the best lipase employed in the transesterification of (**1**). Immobilized lipase from Novozyme (CAL-B) showed also high selectivity towards (**1**), however, the enantiomeric ratio E was relatively low in comparison with the PSL-D catalyzed transesterification of (**1**). Other enzymes showed from low to moderate enantioselectivity and reaction rate in the transesterification of (**1**). (Table 5.1)

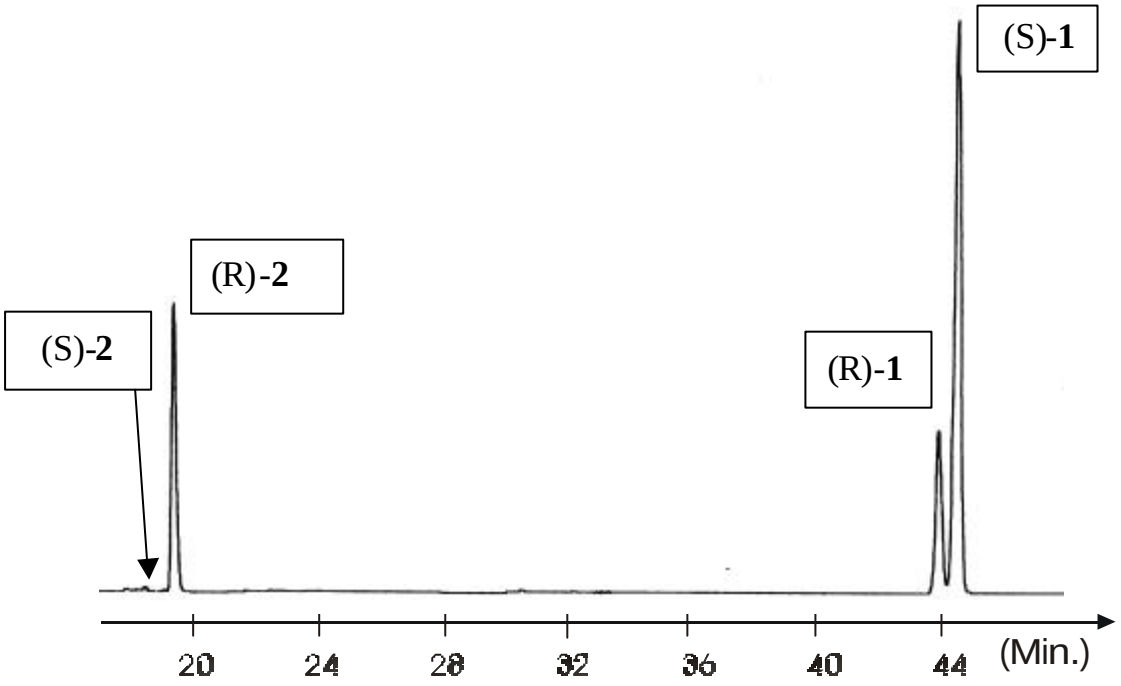


Fig. 5.3: Gas-chromatographic separation of the enantiomer of both substrate (**1**) (as carbamate) and product (**2**) on heptakis-(2,3-di-*O*-methyl-6-*O*-tert-butyldimethylsilyl)-*b*-cyclodextrin of the *Pseudomonas fluorescens* lipase (PFL)-catalyzed transesterification of (**1**) in toluene at t=1 hr, ee_s =56.7%, ee_p=96.4%, conv. =37.0%, E =98.9. The retention time of (S)-**2**, (R)-**2**, (R)-**1**, (S)-**1**, were 18.5, 19.4, 43.7, 44.4 min, respectively.

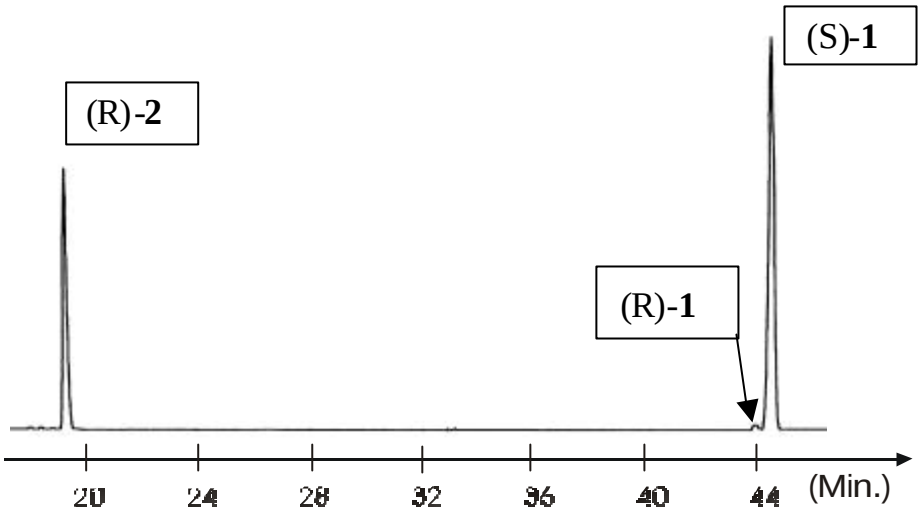


Fig. 5.4: Gas-chromatographic separation of the enantiomer of both substrate (**1**) (as carbamate) and product (**2**) on heptakis-(2,3-di-*O*-methyl-6-*O*-tert-butyldimethylsilyl)-*b*-cyclodextrin of the *Pseudomonas fluorescens* lipase (PFL) catalyzed transesterification of (**1**) in toluene at t=4 hrs, ee_s =97.0%, ee_p=96.6%, conv. =50.1%, E =247.5.

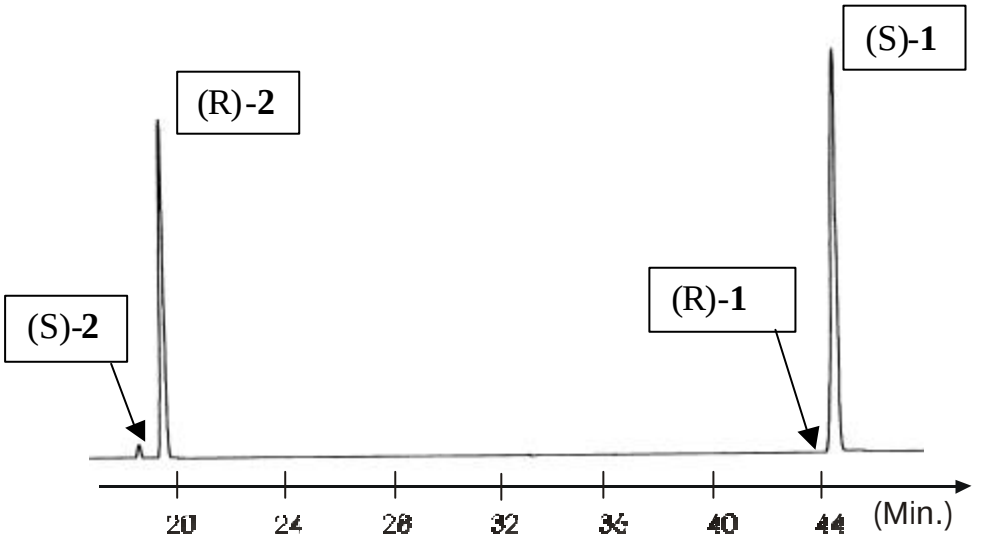


Fig. 5.5: Gas-chromatographic separation of the enantiomer of both substrate (**1**) (as carbamate) and product (**2**) on heptakis-(2,3-di-*O*-methyl-6-*O*-tert-butyldimethylsilyl)-*b*-cyclodextrin of the *Pseudomonas fluorescens* lipase (PFL) catalyzed transesterification of (**1**) in toluene at t=9 hrs, ee_s =99.9%, ee_p=92.2%, conv. =52%, E =284.8.

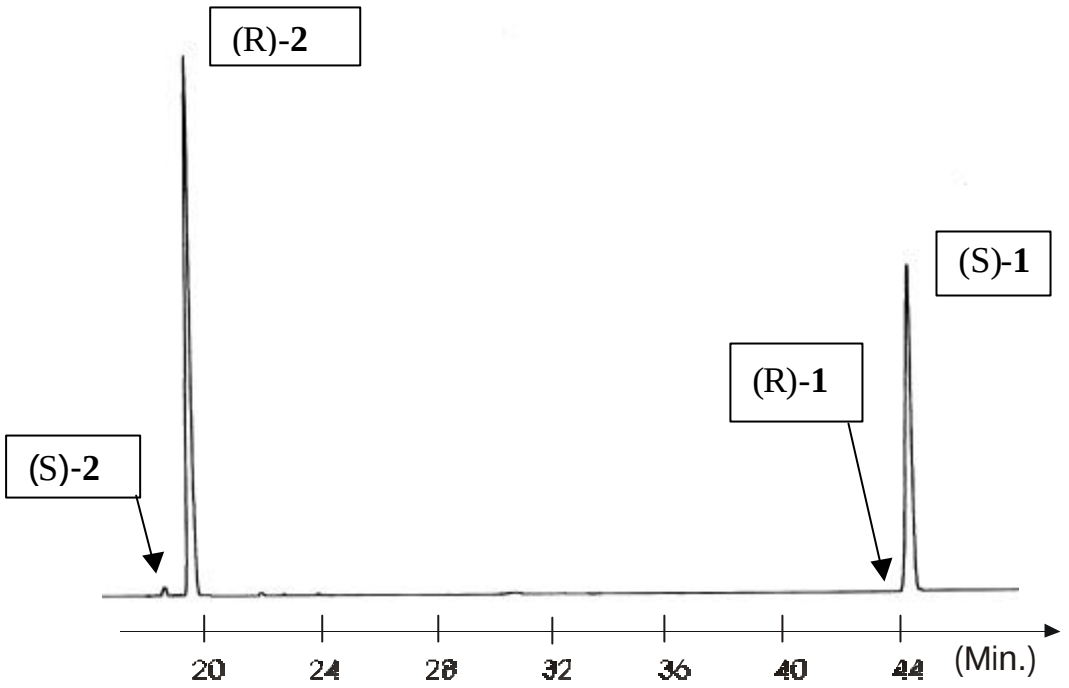
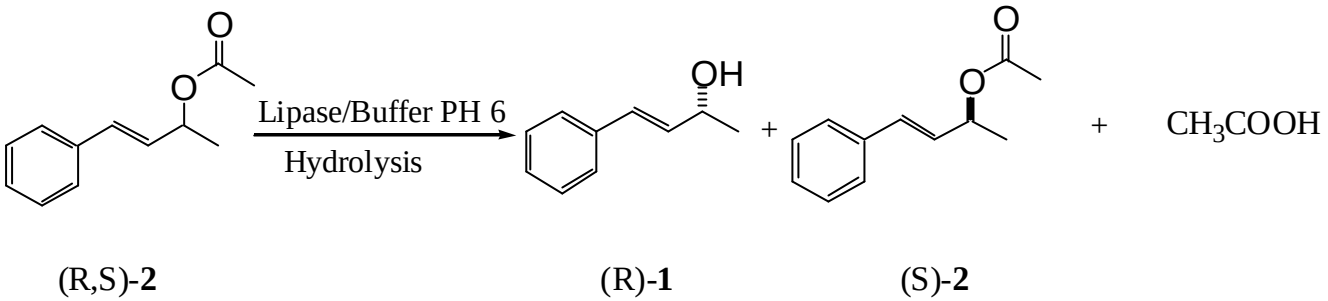


Fig. 5.6: Gas-chromatographic separation of the enantiomer of both substrate (**1**) (as carbamate) and product (**2**) on heptakis-(2,3-di-*O*-methyl-6-*O*-tert-butyldimethylsilyl)-*b*-cyclodextrin of the *Pseudomonas cepacia* lipase immobilized on diatomaceous earth (PSL-D)-catalyzed transesterification of (**1**) in toluene at t=4 hrs, ee_s =99.9%, ee_p=96.7%, conv. =50.8%, E =680.2.The retention time of (S)-**2**, (R)-**2**, (R)-**1**,(S)-**1**, were 18.5, 19.4, 43.7, 44.4 min, respectively.

Pseudomonas cepacia lipase immobilized on diatomaceous earth (PSL-D) was the enzyme of choice used in the transesterification of (**1**) using isopropenylacetate in toluene on a preparative scale. An E value of more than 300 was observed and the reaction was terminated after 4 hrs yielding (S)-**1** with more than 99% ee and the ester (R)-**3** was recovered with 86% ee determined by capillary GC after 50 % conversion. Molecular sieves 4 Å were added in order to scavenge the liberated acetone resulting as a by-product from the lipase-catalyzed reaction using isopropenyl acetate as acyl donor. The beneficial effect of molecular sieves was reported previously in

lipase-catalyzed transesterification of 1-(2-furyl)ethanol using isopropenyl acetate in organic solvents [86,87] (chapter 3).



(R,S)-**2** (R)-**1** (S)-**2**

Fig. 5.7: Lipase-catalyzed enantioselective hydrolysis of racemic (**2**) using phosphate buffer (pH =6) and toluene as organic solvent.

Compared to the transesterification experiments described above, the enzymatic hydrolysis of the acetate (R,S)-**2** (Fig. 5.7) proceeded slowly with only moderate conversion (45%), but a high ee (up to 99 % for the alcohol (R)-**1** and 80.8 % for the remaining unreacted ester (S)-**2**) (Table 5.2) was achieved after 24 hours using Novozyme IM (CAL-B). In all cases, the hydrolysis reaction was relatively fast until reaching 9 hrs, afterwards, the reaction proceeded very slowly. This is probably due to product inhibition resulting from the accumulation of products when the conversion is increased in the enzymatic hydrolysis of (R,S)-**2**, thus competing with the active site of the enzyme. Adding to that, the acid released from the enzymatic hydrolysis (Fig. 5.7) might be involved in the acylation of the resulting unreacted enantiomerically pure alcohol (R)-**1**, thus, increasing the amount of the racemic ester (**2**) leading to a decrease in the conversion and enantiomeric excess.

Table 5.2: Lipase-catalyzed enantioselective hydrolysis of (R,S)-**2** using phosphate buffer (pH =6)

Lipase	Time (days)	[%]ee_s (S)-**2**	[%]ee_p (R)-**1**	% conversion	E
PSL	2	57.5	99.9	36.5	5265
PSL-C	4	30.2	99.9	23.2	3957
PSL-D	2	27.5	99.8	21.6	2503
PFL	4	56.9	99.9	36.2	21012
ANL	2	41.4	99.7	29.3	1323
CCL	4	40.9	54.15	43.03	5
CRL	4	33.6	99.9	25.1	3753
CAL-B	1	80.8	99.9	45.0	74474
Novozyme 525 L	4	59.06	99.9	37.14	9717
RML	4	39.9	99.8	28.57	1569

ee_s: enantiomeric excess of substrate (ester), ee_p: enantiomeric excess of product (alcohol), E : enantiomeric ratio.

CAL-B (Novozyme) not only showed the best performance in the analytical runs, but also gave an excellent result for the hydrolysis of (R,S)-**2** on a multi-gram scale (in 24 hours, ee_s 74.5%, ee_p >99%, conv. =43% and E>300)

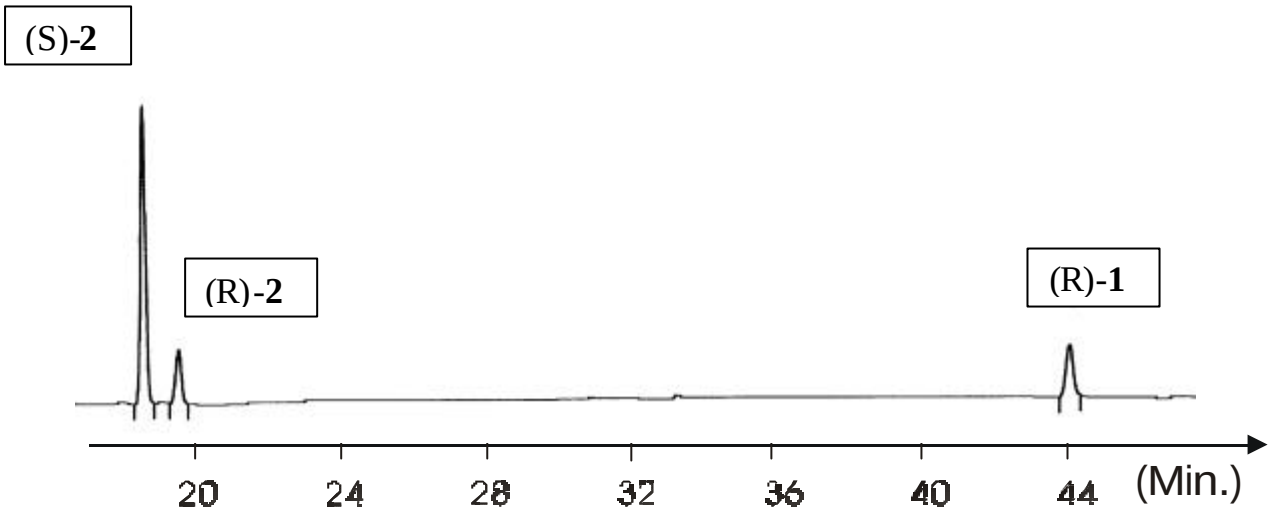


Fig. 5.8: Gas-chromatographic separation of both substrate (**2**) and product (**1**) (as carbamate) on heptakis-(2,3-di-*O*-methyl-6-*O*-tert-butyldimethylsilyl)-*b*-cyclodextrin of the lipase (CAL-B)-catalyzed enantioselective hydrolysis of (**2**) using phosphate buffer pH = 6. At t= 9 hrs, ee_s= 62.8%, ee_p= 99.8%, conv. = 38.6%, E>300.

5.2.3. Comparison with ruthenium complex-catalyzed enantioselective access to enantiomerically enriched (1)

Since the elution order of enantiomers of both substrate (R,S)-**1** (as carbamate) and product (R,S)-**2** were unknown prior to this investigation, it is interesting to compare the lipase-catalyzed transesterification of (**1**) as well as the hydrolysis of (**2**) with the $RuCl_2(\eta^1\text{-}Ph_2PCH_2CH_2OCH_3)_2$(R,R) or (S,S)-1,2-diphenyl-ethane-1,2-diamine) (**5**)-catalyzed enantioselective reduction of the prochiral ketone *trans*-4-phenyl-3-butene-2-one (**4**) (benzalacetone),all leading to enantiomerically enriched (**1**)

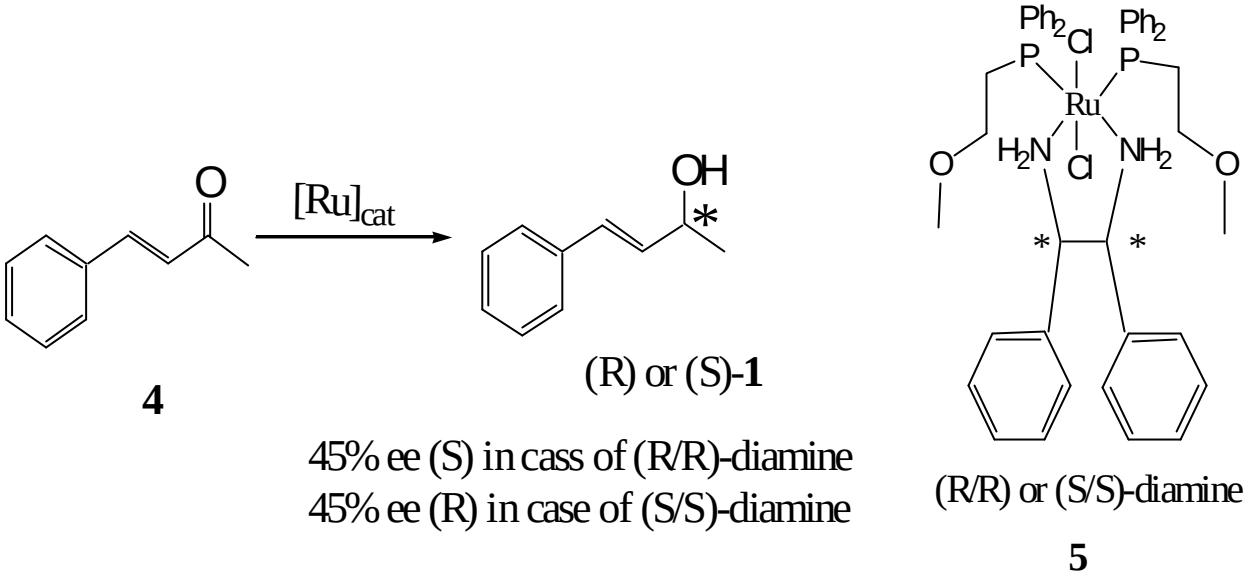


Fig. 5.9: The diamine (ether phosphine) ruthenium(II)-catalyzed enantioselective reduction of benzalacetone (**4**).

It was found that complexes with (R,R)-diamine afforded (S)-alcohol (S)-**1** with 45%ee while complexes with (S,S)-diamine afforded only the (R)-alcohol (R)-**1** with 45% ee (Fig. 5.10). The catalytic enantioselective reduction of (**4**) has been performed according to literature procedure.[22]

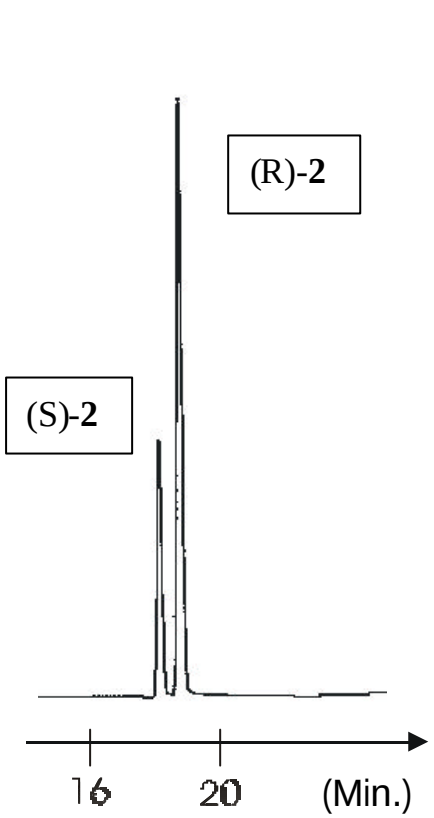


Fig. 5.10a

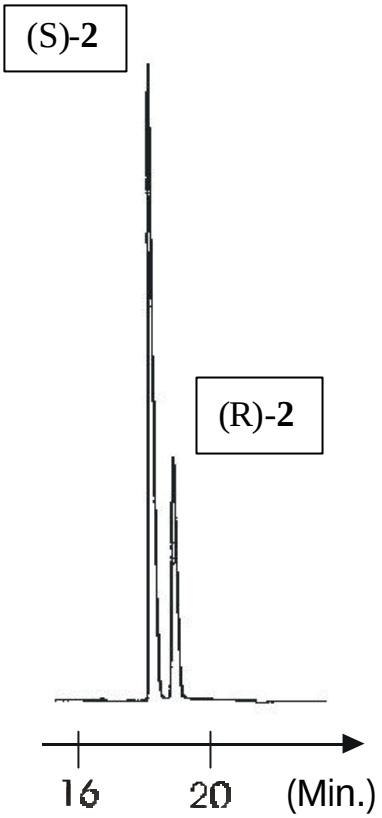


Fig. 5.10b

Fig. 5.10: Gas-chromatographic separation of enantiomerically enriched secondary alcohol (*trans*-4-phenyl-3-butene-2-ol) (**1**) (45% ee), with (R) configuration resulting from the ruthenium (S,S)-diamine-catalyzed enantioselective reduction of benzalacetone (**4**) (**Fig. 5.10a)**. Enantiomerically enriched secondary alcohol (*trans*-4-phenyl-3-butene-2-ol) (**1**) (45% ee) with (S) configuration resulting from the ruthenium (R,R)-diamine-catalyzed enantioselective reduction of (**4**) **(Fig. 5.10b)**. The enantiomers of (**1**) have been separated as acetate (**2**) on heptakis-(2,3-di-*O*-methyl-6-*O*-tert-butyldimethylsilyl)-*b*-cyclodextrin as stationary phase in GC. Oven temperature was 130^{o}C (isothermal) for 20 min.

These results were the stimulus to use other ruthenium complexes trying to reach higher enantiomeric excess of (**1**) (chapter 6). In order to obtain 100% enantiomerically pure (R) or (S)-**1**, it was decided to develop a procedure in which the lipase is used in a consecutive approach after the ruthenium-catalyzed enantioselective reduction of benzalacetone producing (**1**) already in an enantiomerically enriched form.

6. Combined enantioselective reduction of *trans*-4-phenyl-3-butene-2-one mediated by diamine(ether-phosphine) ruthenium(II) complex and lipase-catalyzed transesterification: consecutive approaches

6.1. Introduction

Despite the impressive new progress in enantioselective synthesis, enantioselective ruthenium catalyzed reactions are still considered as one of the most fascinating topics in organic synthesis.[89] Enantiomerically pure and enriched secondary alcohols are useful chiral auxiliaries in organic synthesis due to their utility as versatile synthetic intermediates for various functionalities as well as for many natural products.[90] Accordingly, there is a considerable interest in highly efficient routes to chiral alcohols. Two of the most practical methods currently available to synthesize secondary alcohols in enantiomerically pure form are:

1) The kinetic resolution of racemic alcohols by enantioselective enzymatic acylation (discussed in the previous chapter).
2) The enantioselective reduction of prochiral ketones performed either biologically, using biocatalytic systems,[84] or chemically via stereoselective reduction using either a catalytic system[91] or stoichiometric amount of the chiral reducing agent.[92]

Transition metal catalyzed transfer hydrogenation with 2-propanol is an excellent example of the latter method used to reduce ketones to secondary alcohols.[93] Recently, satisfactory results in the enantioselective reduction of prochiral ketones have been accomplished with some transition metal complexes,[94] however, this process has to be improved for practical use in organic synthesis due to their lower catalytic activity and low substrate/catalyst molar ratio. Excellent catalytic performance in enantioselective transfer hydrogenation of ketones with 2-propanol has been reported using ruthenium(II) complexes attached to chiral N-monosulfonylated C_2 symmetrical diamines as ligands.[95] However, the conditions applied in their synthesis and their application in enantioselective hydrogenation of ketones, e.g. reaction time of around 15 hours or

more and a hydrogen pressure of 8-10 atmospheres, limit their use in enantioselective synthesis.

Other ruthenium(II) complexes with ether phosphine and diamine ligands were already successfully employed in the catalytic hydrogenation of unsaturated ketones.[88] This has been performed using 2-propanol as solvent and KOH as cocatalyst at 35-40°C, low hydrogen pressure (1-3 bar) and substrate/catalyst molar ratio (S/C) 1000-4000 giving unsaturated secondary alcohol up to 99% yield.
In addition to the capability of metal complexes to catalyze the transformation of ketones to secondary alcohols, many examples of enantioselective microbial reduction of ketones have been described in literature.[84]
Here the preparation and application of novel diamine-bis(ether–phosphine)ruthenium(II) complexes in the selective hydrogenation of prochiral ketones are reported. Interest is commanded to complexes with chiral diamines, which catalyze the enantioselective transformation of ketones affording the enantiomerically enriched secondary alcohols.

Complexes of this type proved to be efficient catalysts in the hydrogenation of *trans*-4-phenyl-3-butene-2-one and *trans*-4-(4-chloro-phenyl)-3-butene-2-one. As the resulting alcohols from the ruthenium-catalyzed reaction were only enantiomerically enriched (45% or 37% ee, respectively), the access to (**1**) is completed to obtain an enantiomerically pure alcohol (> 99% ee) using lipase-catalyzed transesterification of the enantiomerically enriched secondary alcohol or lipase-catalyzed hydrolysis of the corresponding acetate in a consecutive approaches. It was expected that the enzymatic transformations will be accelerated when the substrate is already enantiomerically enriched rather than racemic.
The study of the enantiomeric excess of the resulting enantiomerically enriched and pure secondary alcohols produced by ruthenium complexes and consecutive lipase-catalyzed reactions, respectively, was performed using gas chromatography on the chiral stationary phase heptakis(2,3-di-*O*-methyl-6-*O*-tert-butyldimethylsilyl) -*b*-cyclodextrin.

6.2. Results and discussion

6.2.1. Catalytic activity of the ruthenium(II) complexes in the enantioselective reduction of prochiral ketones to enantiomerically enriched secondary alcohols*

The $RuCl_2(\eta^1\text{-}Ph_2PCH_2CH_2OCH_3)_2$(diamine) complexes have been prepared according to literature procedure[88] with high yield from the reaction of equimolar amounts of $RuCl_2(\eta^2\text{-}Ph_2PCH_2CH_2OCH_3)_2$ with various kinds of chelating diamines (1,2 diamine) to form five-membered rings with ruthenium (Fig. 6.1). These novel ruthenium(II) complexes have been used as catalytic system in the enantioselective hydrogenation of the prochiral ketones *trans*-4-phenyl-3-butene-2-one (benzaleacetone) and *trans*-4-(4-chloro-phenyl)-3-butene-2-one, using 2-propanol and different types of cocatalysts.

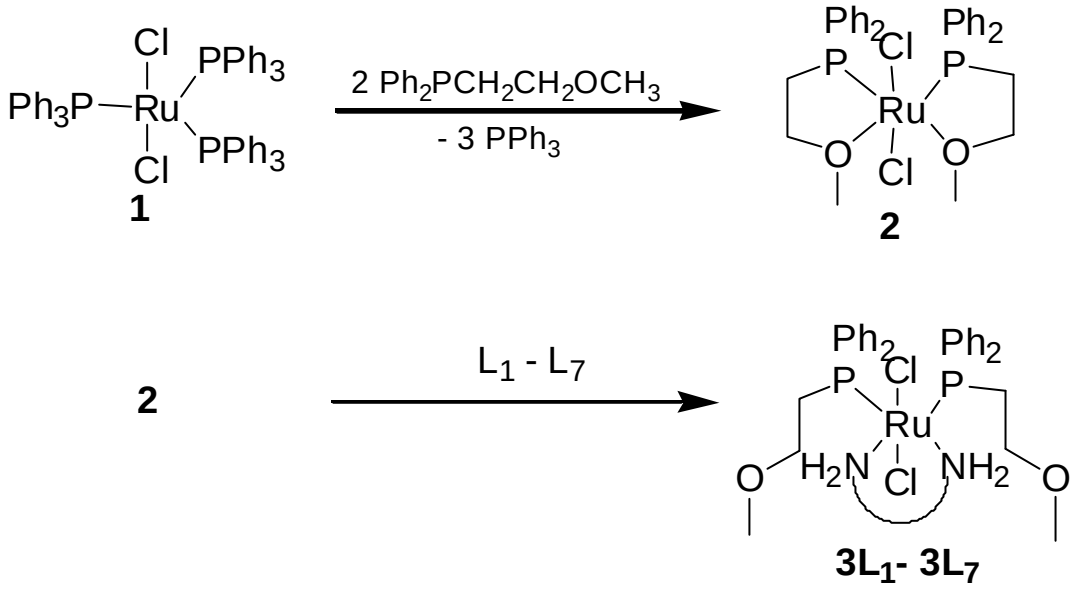
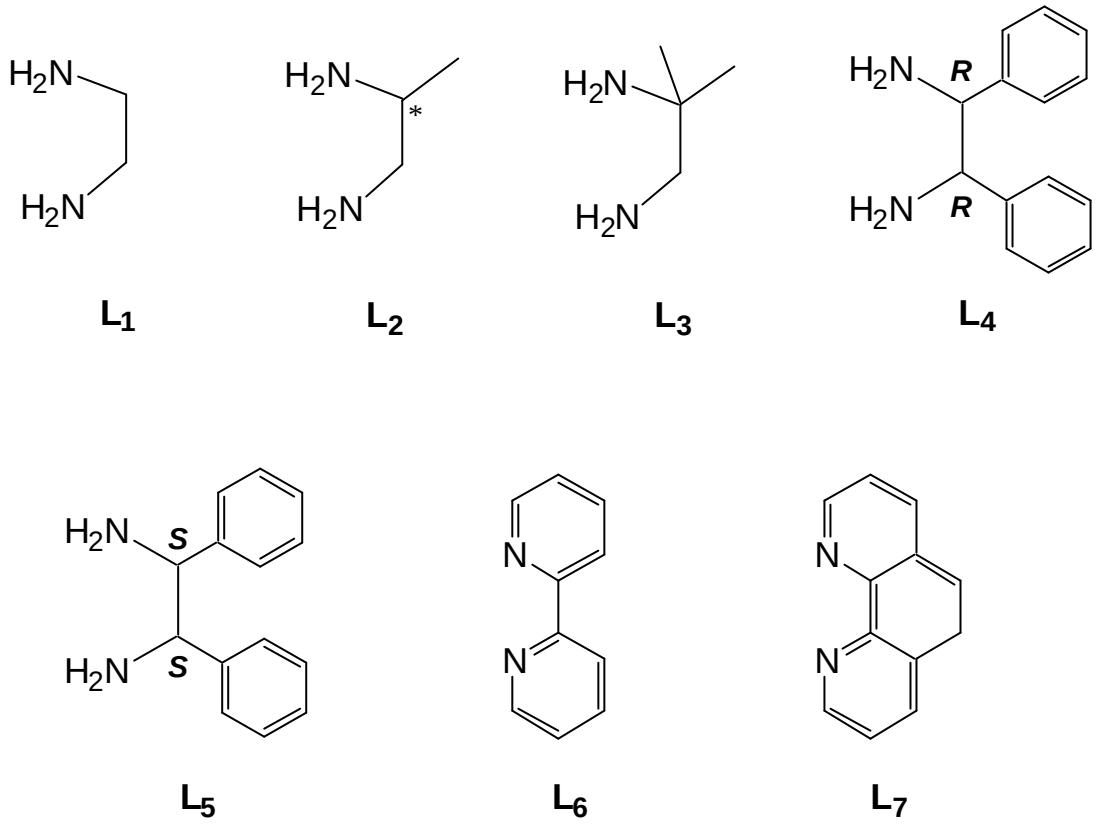


Fig. 6.1: Synthesis of $RuCl_2(\eta^1\text{-}Ph_2PCH_2CH_2OCH_3)_2$(diamine) complexes.

* This part is a cooperation with Prof. E. Lindner group



L_1 **L_2** **L_3** **L_4**

L_5 **L_6** **L_7**

N~N

Fig. 6.2: A set of diamines used in the synthesis of ruthenium complexes.

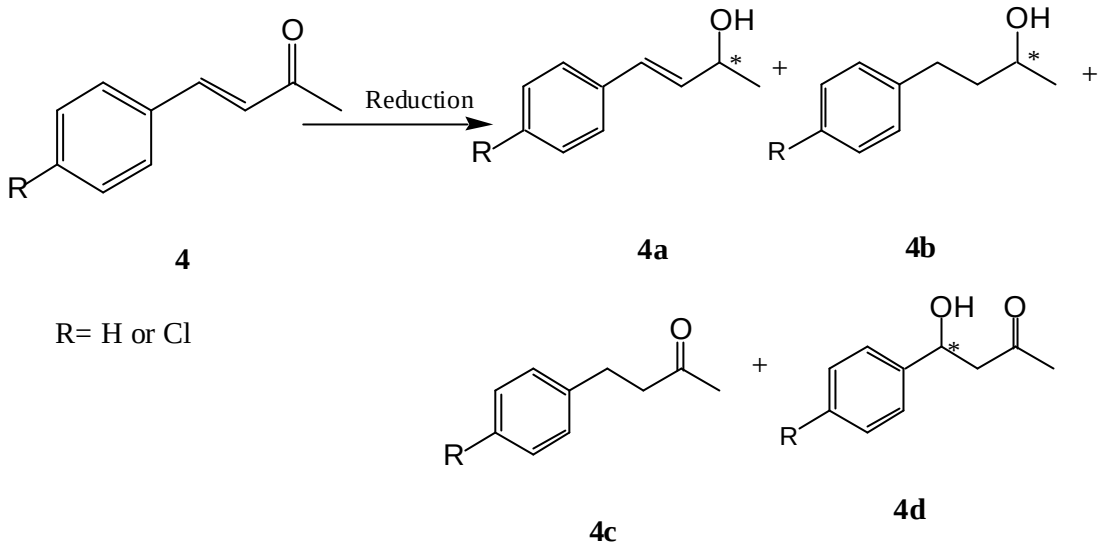


Fig. 6.3: Different products of the reduction of 4-phenyl-3-butene-2-one [benzalacetone (**4**)].

Trans-4-phenyl-3-butene-2-one and *trans*-4-(4-chloro-phenyl)-3-butene-2-one have been selected as a model examples since three different products of reduction are to be expected to form compounds (**4a**), (**4b**) or (**4c**). In addition to these three possibilities, it has been reported that the white-rot fungus [84] *M. tremellosus* ono991 was able to reduce (**4)** to form (**4a**) (5 mol%), (**4b**) (5 mol%), (**4c**) (55 mol%) and (**4d**) (45 mol%) in case of R= H.

The racemic alcohol (**4a**) has been synthesized from the corresponding ketone (**4**) using $NaBH_4$. As the free racemic alcohol (**4a**) was not separated on heptakis(2,3-di-*O*-methyl-6-*O*-tert-butyldimethylsilyl)-*b*-cyclodextrin, it was derivatized using acetic anhydride (Fig. 6.4) and isopropylisocyanate (Fig. 6.6) to ensure a simultaneous base line separation by GC. Afterwards, the resulting alcohol (**4a**) from the ruthenium-catalyzed reduction of (**4**) has been investigated by GC after derivatization.

Using ruthenium catalyst **3L$_4$**, with diamine (R,R) afforded the (S)-alcohol with 45% ee (S), $[\alpha]_D^{20}$ - 6.0, while using ruthenium catalyst **3L$_5$**, with diamine (S,S) afforded the (R)-alcohol with 45% ee $[\alpha]_D^{20}$ + 6.0. It is to be noted that compound (**4d**) was not observed at all in this investigation. Double bond hydrogenation only proceeds if the reaction time is prolonged (more than 30 min). Only in this case, the

saturated alcohol starts to appear with the decreasing of (**4a**). This has been observed in the UV spectrum as well as by GC.

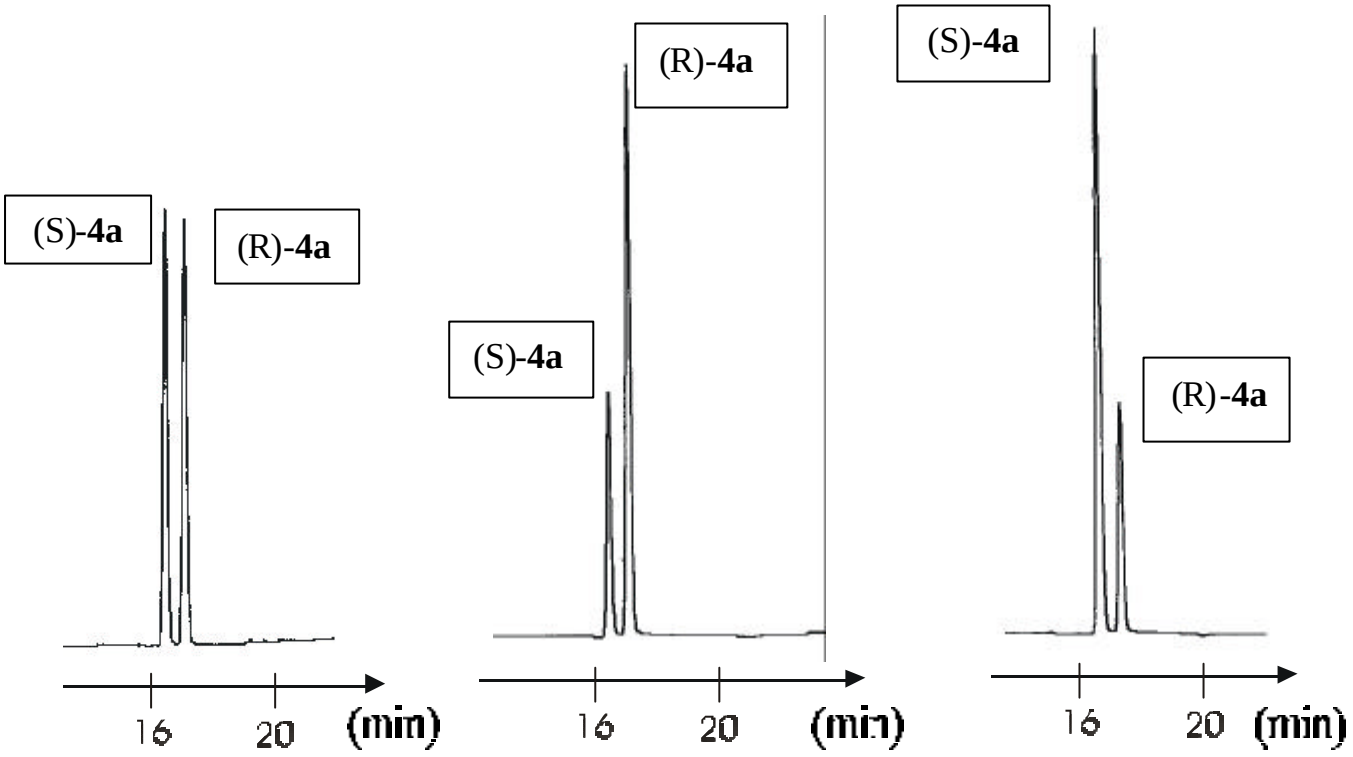


Fig. 6.4a **Fig. 6.4b** **Fig. 6.4c**

Fig. 6.4: Gas-chromatographic separation of racemic mixture of (**4a**) **(Fig. 6.4a)**, enantiomerically enriched secondary alcohol (*trans*-4-phenyl-3-butene-2-ol) (**4a**) (45% ee) with (R) configuration resulting from the ruthenium-catalyzed reaction using (S,S)-diamine **(Fig. 6.4b)**, enantiomerically enriched secondary alcohol (*trans*-4-phenyl-3-butene-2-ol) (**4a**) (45% ee) with (S) configuration resulting from the ruthenium-catalyzed reaction using (R,R)-diamine **(Fig. 6.4c)**. The enantiomers of (**4a**) have been separated as acetate on heptakis-(2,3-di-*O*-methyl-6-*O*-tert-butyldimethylsilyl)-*b*-cyclodextrin as stationary phase in GC. Oven temperature was 130°C (isothermal) for 20 min.

All hydrogenations were carried out under mild conditions between 2-3 bar hydrogen pressure and 35°C temperature using **L_1-L_7** complexes with several cocatalysts (KOH, *t*BuOK, AgOTF). It was noted that the complexes **L_1-L_5**, using 1,2-diamines were active complexes in hydrogenation of unsaturated ketones at mild conditions. Results are listed in table 6.1.

Table 6.1: Diamine(ether-phosphine)ruthenium(II) complex-catalyzed reduction of *trans*-4-phenyl-3-butene-2-one[a]

Run	Catalyst	Conversion (%)[b]	H_2 (bar)	TOF[c]	Selectivity (%)[b]		
					A	**B**	**C**
1	**3L_1**	90	3	1120	100	0	
2	**3L_2**	80	3	960	100	0	0
3	**3L_3**	67	3	673	100	0	0
4	**3L_4**	100	2	1010	100	0	0
5	**3L_5**	100	2	1688	100	0	0
6	**3L_4**[d]	100	2	1028	100	0	0
7	**3L_5**[d]	100	2	1459	100	0	0
8	**3L_4**[e]	100	2.5	680	100	0	0
9	**3L_5**[e]	100	2.5	770	100	0	0
10	**3L_4**[f]	100	2.5	1878	87	13	0
11	**3L_5**[f]	100	2.5	1993	89	11	0
12	**3L_4**[g]	100	2.5	1090	100	0	0
13	**3L_5**[g]	100	2.5	1500	100	0	0
14	**3L_4**[h]	100	2.5	760	100	0	0
15	**3L_5**[h]	100	2.5	815	100	0	0
16	**3L_6**	0	3	0	0	0	0
17	**3L_7**	0	3	0	0	0	0

[a] The reaction was conducted at 35°C using 3-10 g of substrate (S/C=1000) in 50 ml of 2-propanol [Ru : KOH : Substrate][1:10:1000]. [b] Yields and selectivities were determined by GC and GCMS. [c] TOF : turnover frequency ($mol_{sub}\ mol^{-1}_{cat}\ h^{-1}$).
[d][Ru : *t*BuOK : Substrate (**4**)] [1:10:1000]. [e][Ru : KOH : AgOTf : Substrate (**4**)] [1:10:5:1000]. [f][Ru : KOH : Substrate (4)] [1:40:1000]. [g][Ru : KOH : Substrate (**4**)] [1:10:4000]. [h][Ru : KOH : Substrate (Cl–**4**)][1:10:1000].

The TOF and the conversions were decreased when the methyl group was introduced at one carbon of the diamine. This is clearly due to the steric effect (Table 6.1, runs 1-3). The formation of the enantiomerically enriched alcohol (**4a**) (Fig. 6.4) is due to the presence of chiral diamines existing in $\mathbf{3L_4}$, $\mathbf{3L_5}$ complexes (Table 6.1, runs 4-13). In runs 6 and 7, another cocatalyst (*t*BuOK) was studied, approximately the conversion, TOF and enantioselectivity were the same as in case of using KOH as cocatalyst. In order to enhance the enantioselectivity of carbonyl hydrogenation AgOTf [96] was used (runs 8 and 9), no effect was observed while TOF was decreased.

The saturated alcohol (**4b**) was detected in a small amount, when the cocatalyst (KOH) was increased ≥ 40 mole time of the complexes (Table 6.1, runs 10, 11). Material conservation and small metal / organic ratio were accepted when using 1:4000, [Ru: Sub] instead of 1:1000 (Table 6.1, runs 12, 13). When the substrate was replaced by Cl-**4**, the TOF was decreased but the enantioselectivity was still as in the absence of Cl (Table 6.1, runs 14, 15). No product was observed when $\mathbf{3L_6}$, $\mathbf{3L_7}$ complexes have been used in the catalytic study even if the reaction was left for more than 24hrs under processing.[25,27]

Until now the mechanism of the ruthenium-catalyzed reactions has not become evident. The source of the transferred hydrogen atom in similar systems has been attributed to a ruthenium-centered hydride. The most widely accepted theory is that a nitrogen atom as well as ruthenium is intimately involved in the hydride transfer process. Noyori et al.[95] have suggested that the transferred hydride originates from Ru-N-H interaction. This suggests that an N-H moiety might be a prerequisite for an effective catalyst. However, it should be emphasized that ruthenium complexes containing ligands with aprotic sp^2-hybridized nitrogen were also successfully employed in the mentioned hydrogenation process.[100]

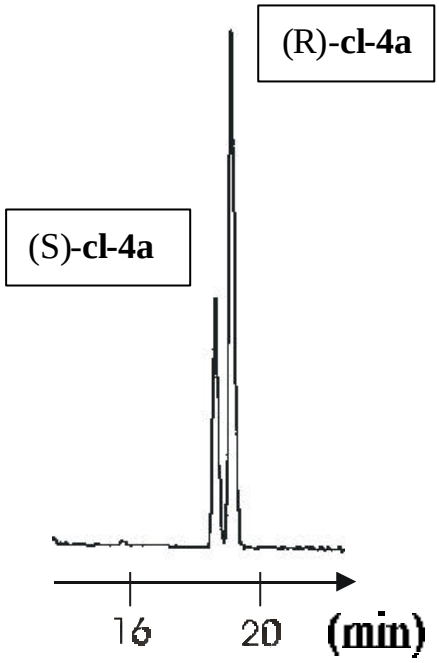


Fig. 6.5a

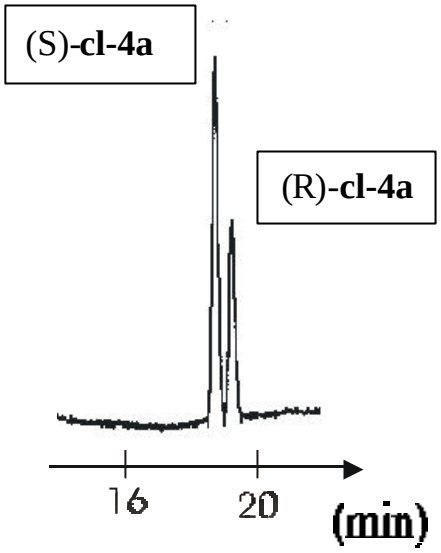


Fig. 6.5b

Fig. 6.5: Gas-chromatographic separation of the enantiomerically enriched secondary alcohol *trans*-4-(4-chloro-phenyl)-3-butene-2-ol **Cl-4a** (37% ee) with (R) configuration resulting from the ruthenium catalyzed reaction using (S,S)-diamine **(Fig. 6.5a)** and the enantiomerically enriched secondary alcohol *trans*-4-(4-chloro-phenyl)-3-butene-2-ol **Cl-4a** (45% ee) with (S) configuration resulting from the ruthenium-catalyzed reaction using (R,R)-diamine **(Fig. 6.5b)**. The enantiomers of (**4a**) have been separated as acetate on heptakis-(2,3-di-*O*-methyl-6-*O*-tert-butyldimethylsilyl)-*b*-cyclodextrin as stationary phase in GC. Oven temperature was 150^{o}C (isothermal) for 20 min.

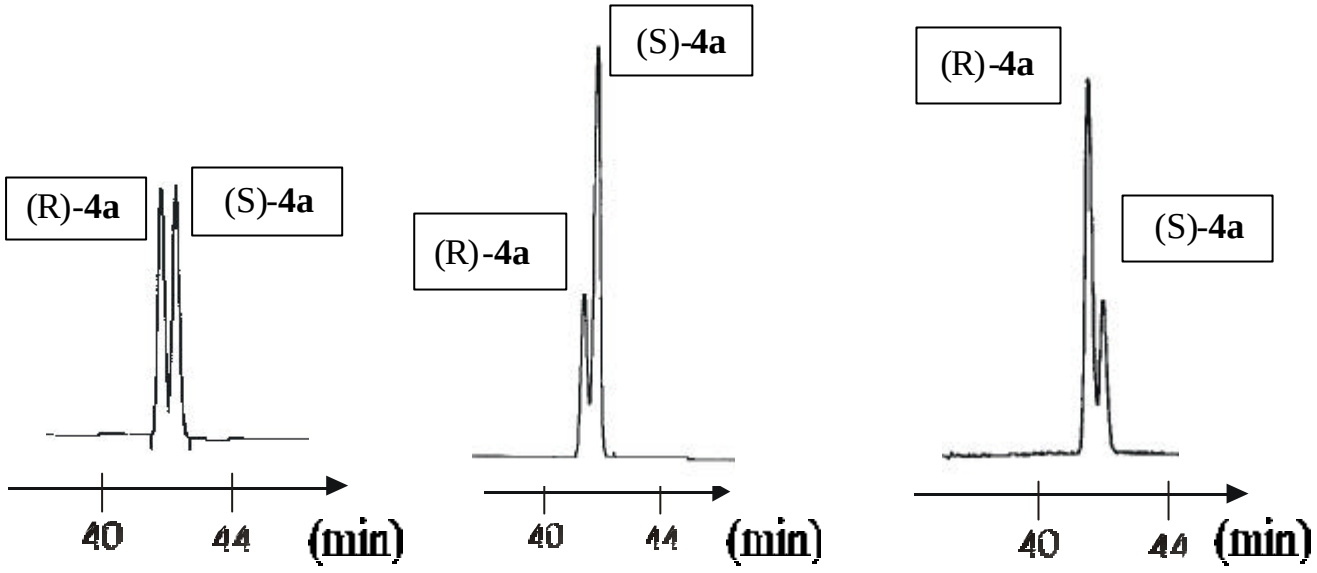


Fig. 6.6a **Fig. 6.6b** **Fig. 6.6c**

Fig. 6.6: Gas-chromatographic separation of the racemic mixture of (**4a**) **(Fig. 6.6a)**, the enantiomerically enriched secondary alcohol (*trans*-4-phenyl-3-butene-2-ol) (**4a**) (45% ee) with (S) configuration resulting from the ruthenium-catalyzed reaction using (R,R)-diamine **(Fig. 6.6b)**, the enantiomerically enriched secondary alcohol (*trans*-4-phenyl-3-butene-2-ol) (**4a**) (45% ee) with (R) configuration resulting from the ruthenium-catalyzed reaction using (S,S)-diamine **(Fig. 6.6c)**. The enantiomers of (**4a**) have been separated as isopropylcarbamate on heptakis-(2,3-di-*O*-methyl-6-*O*-tert-butyl-dimethylsilyl)-*b*-cyclodextrin as stationary phase in GC. Oven temperature was 130°C (isothermal) for 20 min, 30°C/min until 160°C for 25 min.

6.2.2. X-ray structural determination of ruthenium complexes

Table 6.2: Crystallographic data for complex (R,R)-**3**

Empirical formula	$C_{44}H_{50}Cl_2N_2O_2P_2Ru$
Formula weight	872.77
Crystal system	Monoclinic
Space group	$P2_1$
a (Å)	12.136(3)
b (Å)	29.307(3)
c (Å)	12.898(3)
α (°)	90
β (°)	114.194(17)
γ (°)	90
Volume (Å^3)	4184.7(13)
Z	4
Calculated density (g cm^{-3})	1.385
Absorption coefficient (mm^{-1})	0.617
Reflections collected / unique	20134 / 18465 [R(int) = 0.0356]
Data / restraints / parameters	18465 / 1 / 959
Goodness-of-fit on F_o^2	1.009
Final *R* indices [$I > 2\sigma(I)$][a]	$R_1 = 0.0443$, $R_w(F_o^2) = 0.0861$
R indices (all data)[a]	$R_1 = 0.0704$, $R_w(F_o^2) = 0.0958$
Absolute structure parameter	0.01(3)
Largest diff. peak and hole (*e* Å^{-3})	0.334 and -0.596

[a] $R_1 = \Sigma(||F_o|-|F_c||) / \Sigma|F_o|$; $R_w(F_o^2) = [\Sigma[w(F_o^2-F_c^2)^2] / \Sigma[w(F_o^2)^2]]^{0.5}$.

Table 6.3: Selected bond distances [Å], bond angles and torsion angles [°] for (R,R)-**3**.

Distances			
molecule 1		molecule 2	
Ru(1)-Cl(1)	2.4230(17)	Ru(2)-Cl(3)	2.4121(17)
Ru(1)-Cl(2)	2.4153(17)	Ru(2)-Cl(4)	2.4199(17)
Ru(1)-P(1)	2.277(2)	Ru(2)-P(3)	2.261(2)
Ru(1)-P(2)	2.277(2)	Ru(2)-P(4)	2.272(2)
Ru(1)-N(1)	2.175(6)	Ru(2)-N(3)	2.203(6)
Ru(1)-N(2)	2.154(6)	Ru(2)-N(4)	2.169(6)
Bond Angles			
Cl(1)-Ru(1)-Cl(2)	165.65(7)	Cl(4)-Ru(2)-Cl(3)	166.34(7)
P(1)-Ru(1)-P(2)	92.83(7)	P(3)-Ru(2)-P(4)	93.10(7)
N(1)-Ru(1)-N(2)	77.1(2)	N(3)-Ru(2)-N(4)	77.8(2)
N(1)-Ru(1)-Cl(1)	85.95(16)	N(3)-Ru(2)-Cl(3)	86.66(15)
N(2)-Ru(1)-Cl(1)	81.95(15)	N(4)-Ru(2)-Cl(3)	82.60(15)
N(1)-Ru(1)-Cl(2)	82.01(16)	N(3)-Ru(2)-Cl(4)	83.91(15)
N(2)-Ru(1)-Cl(2)	87.74(16)	N(4)-Ru(2)-Cl(4)	85.76(15)
P(1)-Ru(1)-Cl(1)	91.93(7)	P(3)-Ru(2)-Cl(3)	91.78(7)
P(2)-Ru(1)-Cl(1)	99.09(7)	P(4)-Ru(2)-Cl(3)	97.23(7)
P(1)-Ru(1)-Cl(2)	97.24(7)	P(3)-Ru(2)-Cl(4)	98.66(7)
P(2)-Ru(1)-Cl(2)	91.49(7)	P(4)-Ru(2)-Cl(4)	91.02(6)
Torsion Angles			
N(2)-Ru(1)-N(1)-C(31)	-16.9(4)	N(4)-Ru(2)-N(3)-C(75)	-22.0(4)
N(1)-Ru(1)-N(2)-C(32)	-13.3(4)	N(3)-Ru(2)-N(4)-C(76)	-10.9(3)
Ru(1)-N(1)-C(31)-C(32)	42.7(5)	Ru(2)-N(3)-C(75)-C(76)	50.5(5)
Ru(1)-N(2)-C(32)-C(31)	39.3(5)	Ru(2)-N(4)-C(76)-C(75)	40.7(5)
N(1)-C(31)-C(32)-N(2)	-52.1(5)	N(3)-C(75)-C(76)-N(4)	-59.8(5)
N(1)-Ru(1)-P(1)-C(10)	-0.9(3)	N(3)-Ru(2)-P(3)-C(54)	5.8(2)
N(2)-Ru(1)-P(2)-C(19)	-9.3(3)	N(4)-Ru(2)-P(4)-C(69)	1.1(3)

6.2.3. Crystal structure of (R,R)-3

The crystal structure of (R,R)-**3** is shown in figure 6.7, selected bond distances, bond angles and torsion angles are collected in table 6.3. Complex (R,R)-**3** crystallizes with two independent molecules in the unit cell of the chiral, non-centrosymmetric monoclinic space group $P2_1$. Both molecules are *trans*-chloro-*cis*-phosphine isomers of only approximate C_2 symmetry and differ in the orientation of the substituents at phosphorus relative to the conformation of the diamine ligand. Common to both molecules is the regular octahedral coordination geometry about ruthenium, only perturbed by the phosphine ligands pushing the chlorine ligands from their axial positions toward the diamine chelate, as expressed by the deviation of the Cl-Ru-Cl angles from linearity, 165.65(7)° and 166.34(7)°.[88,97] One of the phenyl groups at phosphorus is bisected by the equatorial P_2RuN_2 plane, embracing the diamine like a pair of tongs (e.g., the torsional angles C_{ipso}-P-Ru-N_{cis} are in the range 1-9°). As expected, the phenyl substituents at the diamine carbon chain favor equatorial positions. Because of the fixed absolute (*R,R)* conformation of the diamine, chelate ring formation with twist conformation results in the predetermined λ conformation. In the case of molecule 2, this twist conformation is actually slightly distorted toward an envelope conformation with C(75) at the tip: C(75) is displaced from the N-Ru-N plane by 0.53 Å, while C(76), C(31) and C(32) show smaller deviations in the range 0.27-0.39 Å. The methoxyethyl chains at phosphorus adopt different conformations relative to the diamine chelate system: in molecule 1, the methoxyethyl chain is oriented parallel to the axial C-H bond of the diamine that is located cis to it, while in molecule 2 the methoxyethyl chains and C-H bonds are antiparallel chelated, but the bite angles of both diamine ligands are obviously sufficiently small to leave the opposing P-Ru-P angles unaffected, 92.39° ($3L_2$) and 91.72° ($3L_8$).

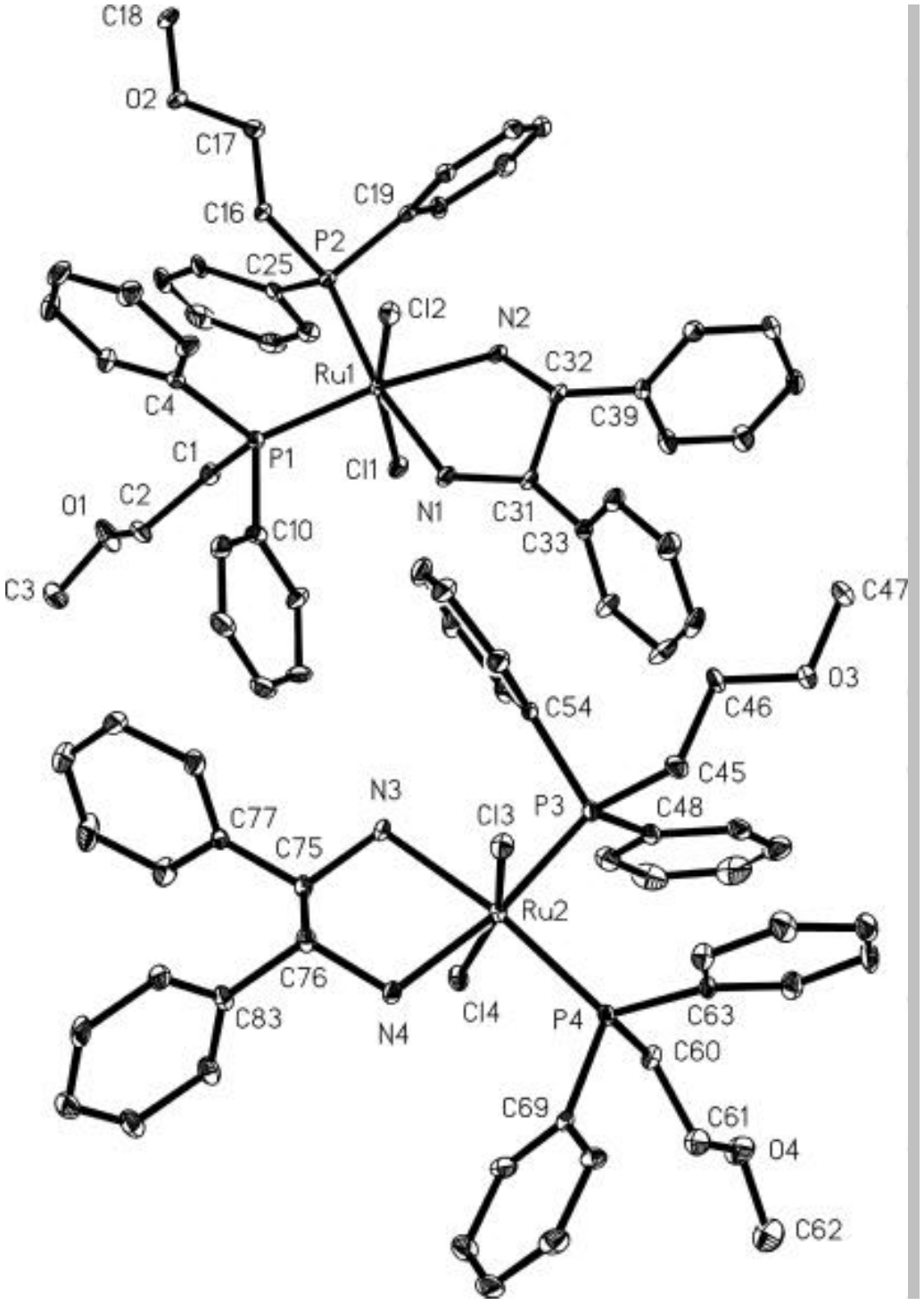


Fig. 6.7: ORTEP plot with atom-numbering scheme showing the two independent molecules in the crystal structure of (R,R)-**3**.Thermal ellipsoids are shown at the 20% probability level, hydrogen atoms have been omitted for clarity.

In **$3L_2$**, the *trans*-diaminocyclohexane ligand forms a typical five-membered chelate with twist conformation, similar to the complex with 1,2-diaminoethane.[97] This allows the cyclohexane ring to adopt a chair conformation. In **$3L_8$**, the six-membered diamine chelate also has a chair structure, but is flattened about the position of the metal; e.g., the dihedral angle between the planes N(1)-Ru(1)-N(2) and N(1)-C(33)-N(2)-C(35) is 31.09° compared to 56.98° between N(1)-C(33)-N(2)-C(35) and C(33)-C(34)-C(35). This is in contrast to six-membered cobalt(III) diamine rings that tend to form more regular chair conformations.[98] The Ru-N distances of 2.170 and 2.209 Å in **$3L_8$**, where nitrogen is *trans* to phosphorus, are shorter than those in a complex of *trans*-diaminocyclohexane where nitrogen is *trans* to hydrogen atoms, with Ru-N distances of 2.225 and 2.284 Å[99]

6.2.4. Lipase-catalyzed kinetic resolution of the enantiomerically enriched sec. alcohol 4-phenyl-but-3-ene-2-ol (4a): Consecutive approach

The kinetic resolution of (**4a**) was performed starting from an enantiomerically enriched alcohol (R) or (S)-**4a** (45% ee) resulting from the ruthenium complex-catalyzed enantioselective reduction of benzaleacetone (**4**). This has been performed in order to enhance the enantiomeric purity of the alcohol resulting from the ruthenium-catalyzed enantioselective reduction of (**4**) aiming to reach 100% ee in a consecutive approach (Fig. 6.8).

Different lipases were screened in resolving the enantiomerically enriched *trans*-4-phenyl-3-butene-2-ol (**4a**) (45% ee), either in a hydrolytic reaction using phosphate buffer (pH=6), in the enantioselective hydrolysis of the corresponding acetate [(R)-**5**, 45% ee] or by transesterification of (S)-**4a** (45% ee) using isopropenyl acetate as an innocuous acyl donor in toluene as solvent.
Among the lipases tested were lipases from *Pseudomonas cepacia* (PCL), *Pseudomonas cepacia* immobilized on diatomaceous earth (PCL-D) and Novozyme 435 (CAL-B). The transesterification of (**4a**) [45% ee (S)] was carried out at 40°C in toluene at a molar ratio of isopropenyl acetate to (**4a**) of 2:1 to ensure the irreversibility of the reaction. A simultaneous base line separation of both substrate and product enantiomers was achieved using heptakis-(2,3-di-*O*-methyl-

6-*O*-tert-butyldimethylsilyl)-*b*-cyclodextrin as a stationary phase by GC[17] with a separation factor α = 1.08 and R_s = 2.5 for the ester (**5**) and α =1.01 and R_s = 1.07 for the alcohol (**4a**). The results of the lipase-catalyzed transesterification of (**4a**) are summarized in table 6.4.

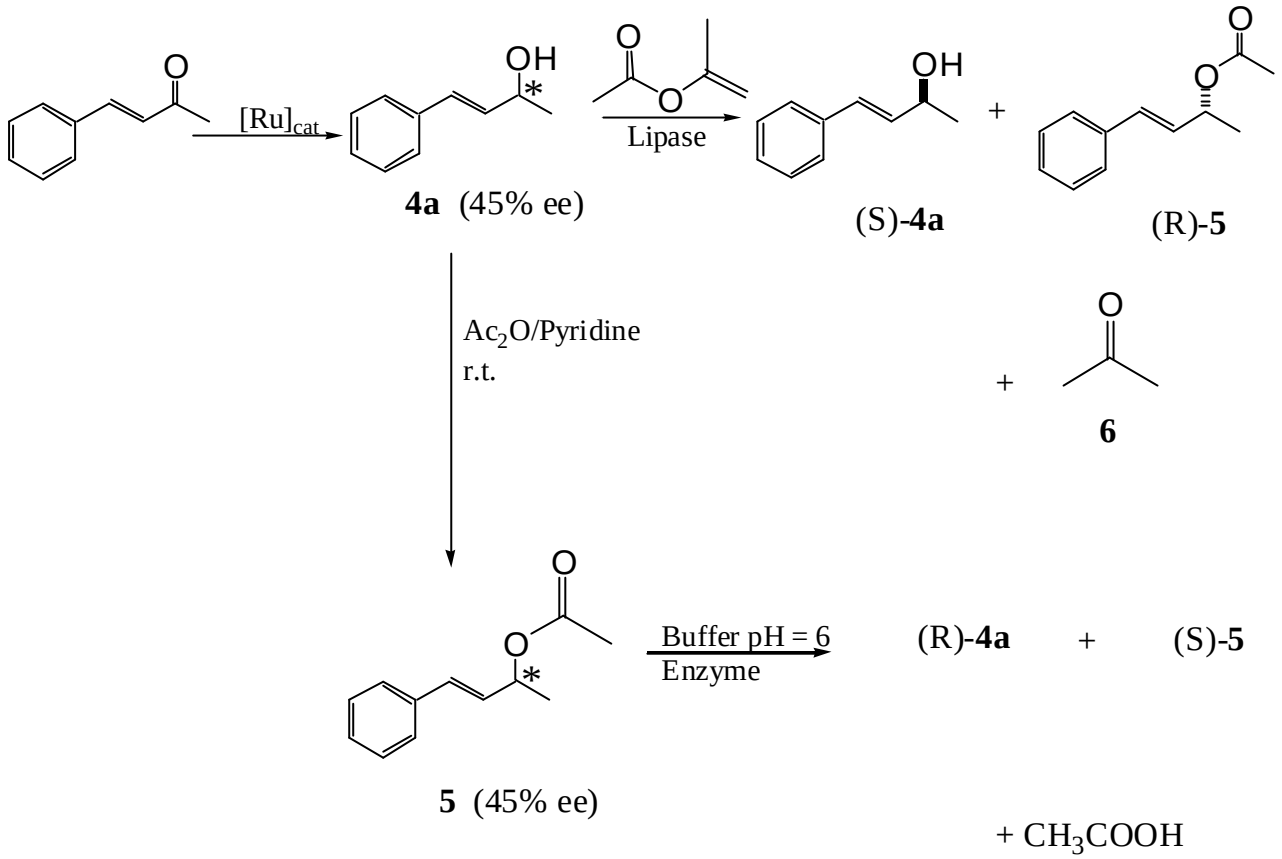


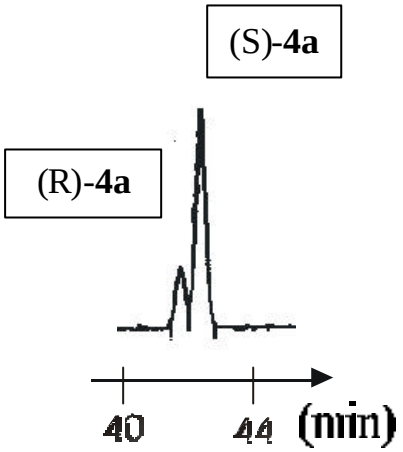
Fig. 6.8: Consecutive ruthenium/lipase-catalyzed enantioselective access of enantiomerically pure (R) and (S)-4-phenyl-but-3-ene-2-ol (**4a**).

Table 6.4: Lipase-catalyzed transesterification of enantiomerically enriched (**4a**) using isopropenyl acetate in toluene.

Lipase	Time (hr)	[%]ee_s (S)-**1**	[%]ee_p (R)-**2**	% conversion	E
PCL	2	97.2	86.9	52.8	61.1
PCL-D	2	99.9	91.8	52.1	227.8
CAL-B	4	99.9	80.3	55.4	93.2
RML	4	49.2	99.9	33.0	3782.9

ee_s: enantiomeric excess of substrate (alcohol), ee_p: enantiomeric excess of product (ester), E : enantiomeric ratio.

In all cases of the transesterification of (**4a**), (R)-**4a** was the faster reacting enantiomer yielding (R)-**5** in high ee and leaving (S)-**4a** as enantiomerically pure unreacted enantiomer. Both lipases from *Pseudomonas cepacia* immobilized on diatomaceous earth (PCL-D) and CAL-B displayed high enantioselectivity towards (**4a**) (Table 6.4). In regard to the ee of the remaining substrate (S)-**4a** and that of the product (R)-**5** as well as the rate of conversion (52.1% in 2 hrs) and enantiomeric ratio E>200, PCL-D was the best lipase employed in the transesterification of (**4a**).
Pseudomonas cepacia lipase immobilized on diatomaceous earth (PCL-D) was the enzyme of choice used in the transesterification of (**4a**) using isopropenylacetate in toluene on a preparative scale. An E value of more than 300 was observed and the reaction was terminated after 3 hrs yielding (S)-**1** with more than 99% ee and the ester (R)-**3** was recovered with 86% ee determined by capillary GC after 50 % conversion. Molecular sieves 4 Åwere added in order to scavenge the liberated acetone resulting as a by-product from the lipase-catalyzed reaction using isopropenylacetate as acyl donor.



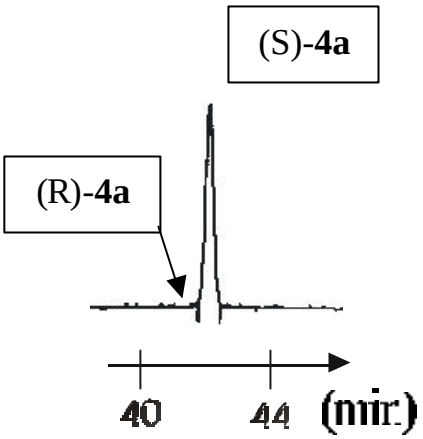


Fig. 6.9a **Fig. 6.9b**

Fig. 6.9: Gas-chromatographic separation of the enantiomerically enriched secondary alcohol (*trans*-4-phenyl-3-butene-2-ol) (**4a**) (64 % ee) with (S) configuration resulting from the ruthenium catalyzed reaction of (**4**) using (R,R)-diamine and the consecutive approach using lipase-catalyzed transesterification reaction in toluene **(Fig. 6.9a)**, the enantiomerically pure alcohol with (S) configuration at the end of the enzymatic reaction **(Fig. 6.9b)**.
The enantiomers of (**4a**) have been separated as acetate on heptakis-(2,3-di-*O*-methyl-6-*O*-tert-butyldimethylsilyl)-*b*-cyclodextrin as stationary phase in GC. Oven temperature was 130^{o}C (isothermal) for 20 min, 30^{o}C/min until 160^{o}C for 25 min.

Compared to the transesterification experiments described above, the enzymatic hydrolysis of the acetate (**5**) [45%ee (R)] proceeded slowly, in a similar way to that described before for the lipase-catalyzed hydrolysis of the racemic acetate (section 5.2.2). Only moderate conversion (45%) and high ee [up to 99 % for the alcohol (R)-**4a** and 80.8 % for the remaining unreacted ester (S)-**5**] (Table 6.5) was achieved after 24 hours using Novozyme IM (CAL-B).

CAL-B (Novozyme) not only showed the best performance in the analytical runs, but also gave an excellent result for the hydrolysis of (**5**) on a multi-gram scale (in 24 hours, ee_s 74.5%, ee_p >99, conv. 43 and E>300)

Table 6.5: Lipase-catalyzed enantioselective hydrolysis of (**5**) using phosphate buffer (pH =6)

Lipase	Time (days)	[%]ee_s (S)-**5**	[%]ee_p (R)-**1**	% conversion	E
PCL	2	57.5	99.9	36.5	5265.13
PCL-C	4	30.2	99.9	23.2	3957.45
CAL-B	1	80.8	99.9	45.0	74474.6
RML	4	39.9	99.8	28.57	1569.02

ee_s: enantiomeric excess of substrate [ester (**5**)], ee_p: enantiomeric excess of product [alcohol (**1**)], E : enantiomeric ratio.

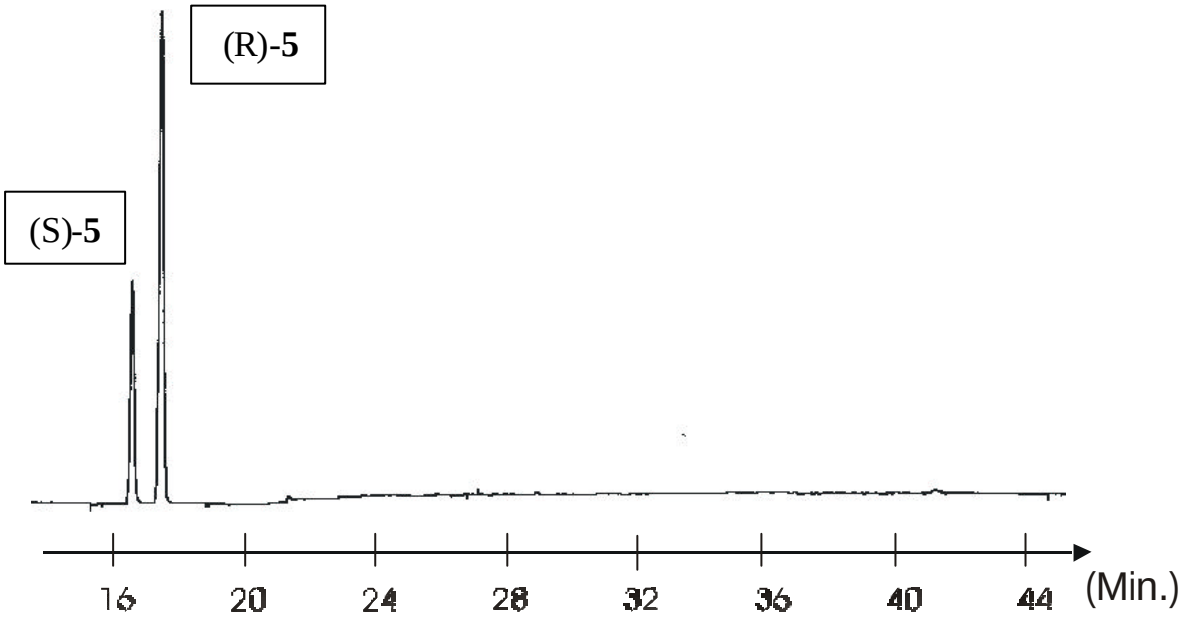


Fig. 6.10: Gas-chromatographic separation of the enantiomers of (**4a**) (derivatized with acetic anhydride) resulting from the ruthenium -(S,S)-diamine catalyzed enantioselective reduction of (**4**) [42%ee with (R) configuration (R)-**5**]. This is used as the substrate for the lipase-catalyzed enantioselective hydrolysis reaction at t=0 hrs.

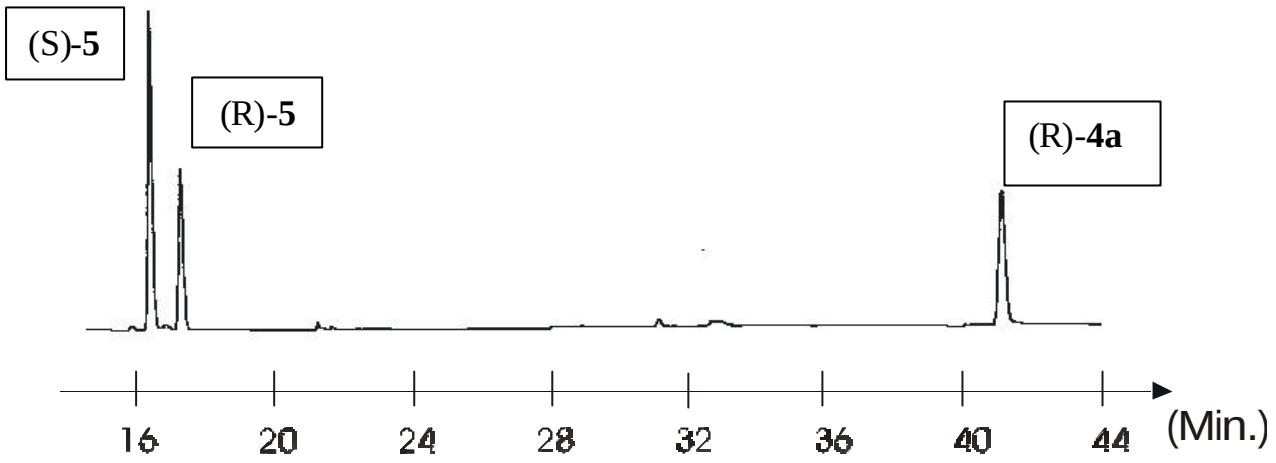


Fig. 6.11 Gas-chromatographic separation of both substrate and product of the lipase (Novozyme)-catalyzed enantioselective hydrolysis of (**5**) [enantiomerically enriched (**4a**) derivatized with acetic anhydride and resulting from the ruthenium-(S,S)-diamine catalyzed enantioselective reduction of (**4**)]. At t= 1hr**,** ee_s= 28.7%, ee_p= 99.9%, Conv. = 22.35%, E>300.

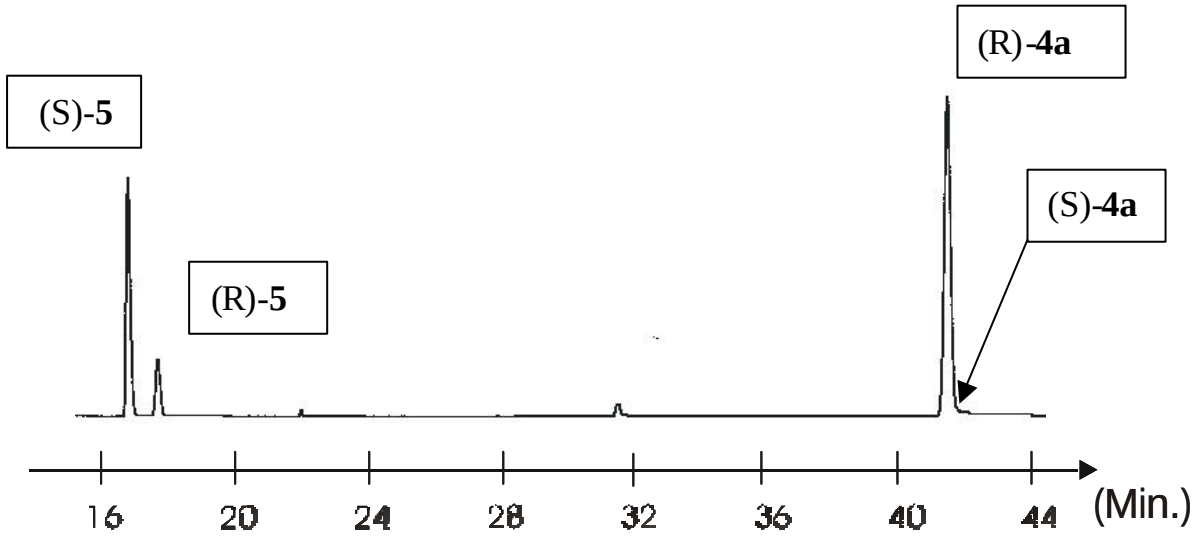


Fig. 6.12 Gas-chromatographic separation of both substrate and product of the lipase (Novozyme)-catalyzed enantioselective hydrolysis of (**5**) [enantiomerically enriched (**4a**) derivatized with acetic anhydride and resulting from the ruthenium-(S,S)-diamine catalyzed enantioselective reduction of (**4**)]. At t= 24 hrs, ee_s= 58.0%, ee_p= 99.8%, conv. = 37.0%, E>200.

It has been demonstrated that metal based catalyst together with the biocatalytic system represented by the lipase can be used in a consecutive way for the synthesis of enantiomerically pure *trans*-4-phenyl-3-butene-2-ol starting from prochiral ketones.

The use of enantiomerically enriched substrate (e.g. 45% ee) rather than a racemic one leads to the acceleration of the reaction rate. Instead of reaching >99% ee of the alcohol (**4a**) in 4 hrs when using PSL-D-catalyzed transesterification of the racemic alcohol (section 5.2.2), the time can be decreased to the half (2 hrs only) when using the same enzyme (PSL-D)-catalyzed transesterification of the enantiomerically enriched alcohol.

7. Co-lyophilized lipase with peracetylated β-cyclodextrin entrapped in sol-gel catalyzed transesterification in organic solvents

7.1. Introduction

As already discussed in chapter 3, lipases are well established as valuable catalysts in organic synthesis as they are able to perform regio- and stereoselective transformations. An additional improvement of the reaction condition can be achieved by using certain additives, which might have a beneficial influence on the microenvironment of the enzymes and hence improve their catalytic activity and enantioselectivity in organic solvents. Among these additives peracetylated *b*-cyclodextrin showed an enhancement effect in the reaction rate and enantioselectivity in lipase-catalyzed transesterification reaction. However, such processes are economically and ecologically viable only if lipases can be immobilized efficiently using cheap methods, and if such immobilizates can be separated from the reaction products and recycled without significant loss of activity. This renders the development of supports for immobilization of lipases an area of high interest. Immobilization of lipase allows for its recycling and reuse in a continuous way.

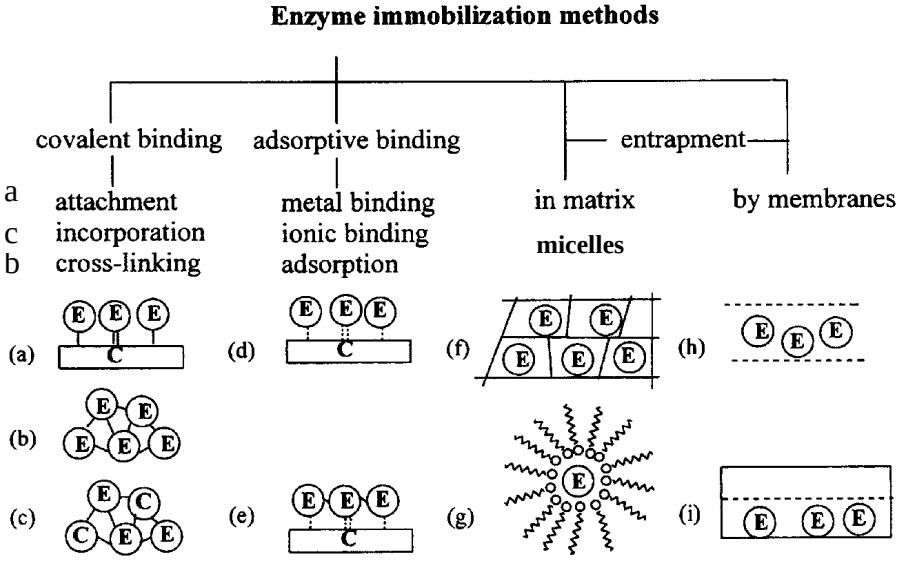


Fig. 7.1: Methods of enzyme immobilization.

Common immobilization techniques include physical adsorption onto the solid support, covalent binding to a solid support, and physical entrapment within a polymer matrix support. (Fig. 7.1)

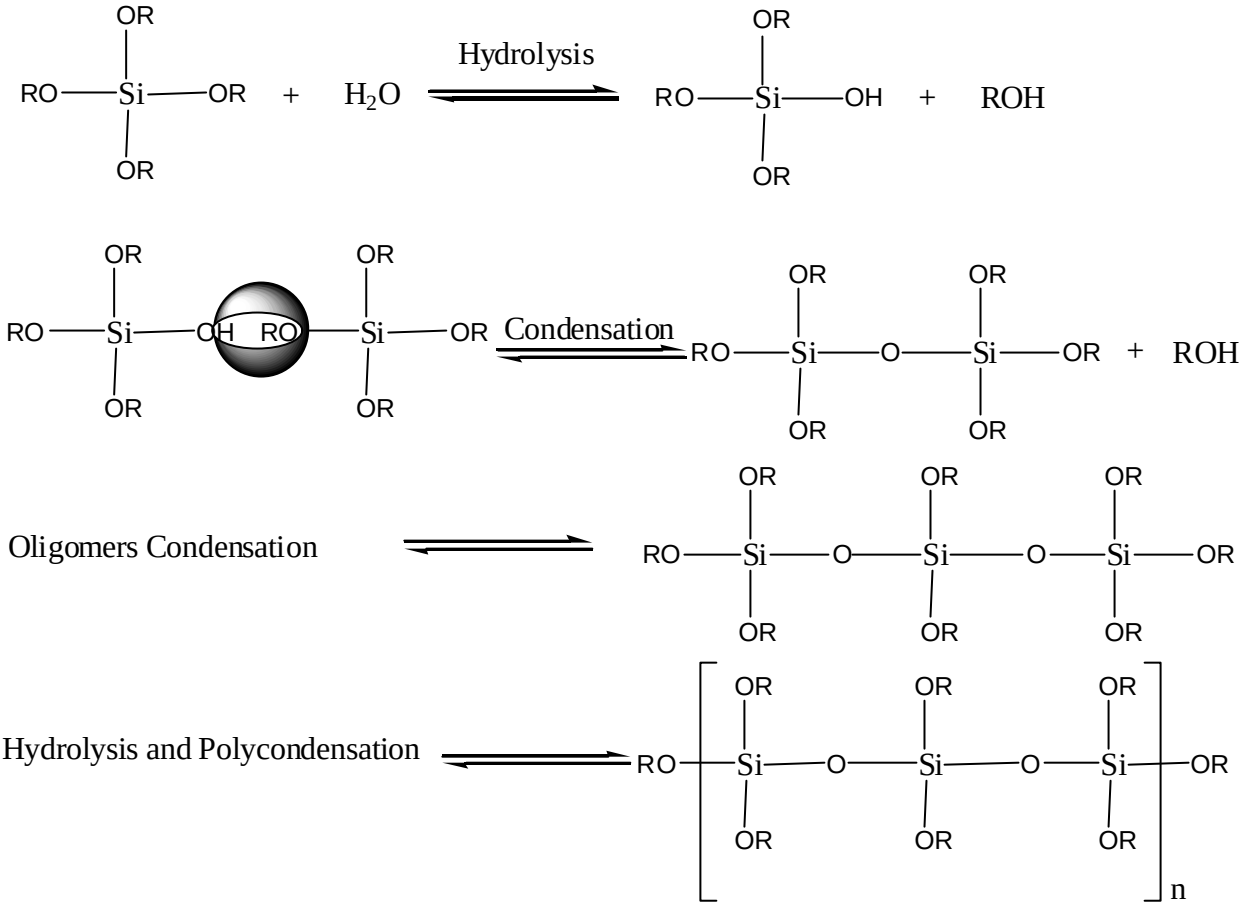


Fig. 7.2: Sol-Gel process.

Entrapment of lipase entails its capture within the matrix of cross-linkable resin. This method has several advantages over the other two methods. Unlike the covalent binding method, this method uses a relatively simple procedure that allows the immobilized lipase to maintain its activity and stability. A variety of methods have been used for entrapping lipases in a polymer matrix. In one method, lipase is entrapped in a photo-cross-linkable or solution cross-linkable resin. This technique consists of mixing a liquid photo-cross-linkable resin such as polyethylenegylcol dimethacrylate, which contains photosensitive functional groups, and appropriate initiator such as

benzoin ethyl ether, and an enzyme solution followed by irradiation with near-ultraviolet light for a few minutes or addition of a polymerization accelerator such as dimethylaniline. The immobilized lipase produced by this method has been successfully applied in the hydrolysis of triglycerides,[101,102] the esterification of fatty acids,[103] and the conversion of other water-soluble[104] and water insoluble compounds.[105]

Entrapment of enzymes in an inorganic polymer matrix has received substantial attention in recent years. This method pioneered by Avnir and coworkers is based on the sol-gel process.[106] The sol-gel procedure is well documented.[107]

A typical immobilization procedure uses an aqueous solution of enzymes, sodium fluoride (NaF) as catalyst, and alkoxysilane derivatives such as $RSi(OMe)_3$ where R= alkyl, aryl, or alkoxy as gel precursors (Fig. 7.2).

Inorganic matrices offer a number of advantages, among these advantages are: (1) they have controllable surface area, average pore size, narrow pore size distribution, and fractal dimension; (2) they are thermally stable; (3) leakage of enzyme is prevented due to rigidity of the caging; (4) they are simple to be prepared from inexpensive chemicals; (5) they retain high enzyme activity; (6) they are easily obtained in a variety of forms, e.g., monoliths, thin films, fibers, powders, etc., (7) enhance the stability of the entrapped molecule and increase the activity of enzyme.

Reetz et al. reported a significant increase in the activity of *Pseudomonas cepacia* lipase by entrapment in a sol-gel matrix using tetramethoxysilane (TMOS), methyltrimethoxysilane (MTMS), and other alkyl modified silane precursors.[108-112]

The relative stability of the entrapped lipase increased with an increase in the pendant alkyl group of the polymerization precursors. The entrapped lipase also showed good stability when it was reused.

Hsu et al. developed a procedure for the immobilization of lipase from *Pseudomonas cepacia* (PS-30) within a phyllosilicate sol-gel matrix. The procedure was based on cross-linking polysilicate clay with silicate polymer produced by the controlled hydrolysis of TMOS. The immobilized lipase showed more stability compared with free lipase in the esterification of lauric acid with octan-1-ol. [113]

Kawakami et al. studied the enhancement in thermal stability of immobilized lipases by sol-gel entrapement.[114,115] Other studies in this area have also indicated a higher activity and stability for the entrapped lipases within the inorganic matrix. [114,115]

In fact, all previous studies with these heterogeneous catalysts focused on the engagement of single species. Only, recently, Avnir et al. reported the encapsulation of two catalytic functionalities within the same matrix.[118] Since, it was demonstrated in chapter 4, the utility of peracetylated *b*-cyclodextrin in enzymatic reaction when they are used in co-lyophilized form with the lipase leading to an enhancement in the reaction rate and enantioselectivity in lipase-catalyzed transesterification, it was decided to take the benefit of CD towards the enzyme and entrap both within the sol-gel matrix. The gel-entrapped lipase/cyclodextrin was prepared by the hydrolysis of methyltrimethoxysilane (MTMS) in presence of the co-lyophilized lipase with peracetylated *b*-cyclodextrin. This type of enzyme preparation was subsequently used in the transesterification of 1-(2-furyl)ethanol in toluene as organic solvent.

7.2. Results and discussion

Three methods of enzyme preparation have been performed. The first consisted of the lyophilization of *Pseudomonas cepacia* lipase (PSL), the present model enzyme, from phosphate buffer (pH 6) without the CD additive. The second enzyme preparation was concerned with the co-lyophilization of PSL with peracetylated *b*-cyclodextrin using the same phosphate buffer. Three different mixtures have been investigated, i.e., 1:1; 1:2; 1:6 (enzyme to cyclodextrin, w/w). The third enzyme preparation is the entrapment of the co-lyophilized lipase with peracetylated *b*-cyclodextrin (1:1; 1:2; 1:6) in the sol-gel matrix. The sol-gel process (initiated by the hydrolysis of MTMS) is performed in the presence of the co-lyophilized PSL with peracetylated *b*-cyclodextrin. Hydrolysis and condensation of the Si monomers in the presence of NaF catalyst trigger cross-linking with the formation of SiO_2, a porous inorganic matrix which grows around the enzyme/CD in a three dimensional manner.

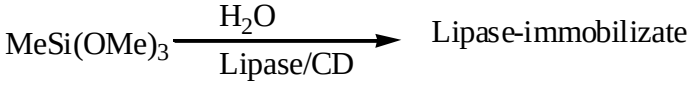


Fig. 7.3: Preparation of lipase containing gels.

The effect of the entrapment of PSL/CD in sol gel was investigated in the transesterification of 1-(2-furyl)ethanol using vinyl butyrate and isopropenyl acetate as acyl donors in toluene as solvent (Fig. 7.4).

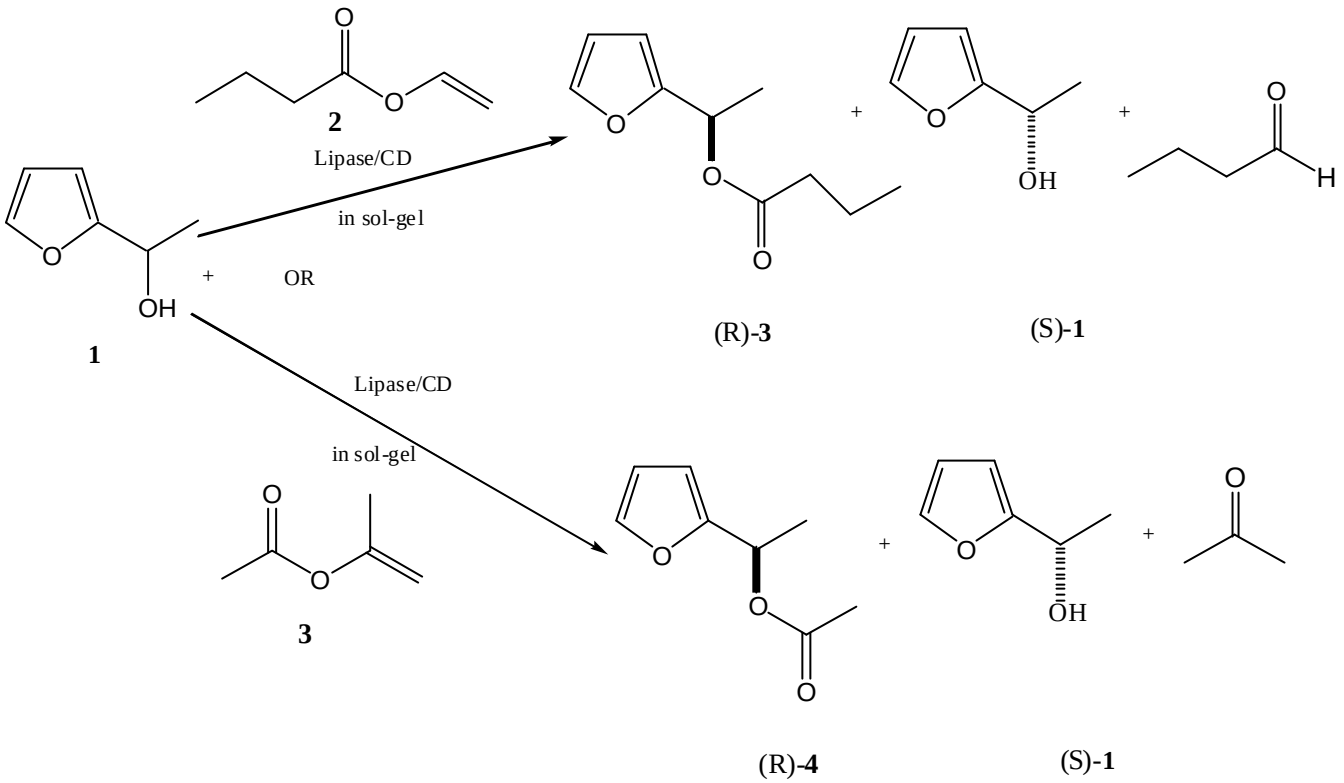


Fig. 7.4: Co-lyophilized lipase with peracetylated *b*-cyclodextrin entrapped in sol-gel catalyzed transesterification of (**1**) using either vinyl butyrate or isopropenyl acetate as acyl donors in toluene.

7.2.1. Effect of cyclodextrin additives

The activity of lipase immobilized by the sol-gel process in presence of macromolecular additives like gelatin was reported to be efficient in comparison with the immobilization without additives. Using another macrocyclic additive such as CD added during the lyophilization of lipase is expected to have another benefit. In the co-lyophilization step the CD may interact with the enzyme in a specific but yet unknown way by changing its conformation and hence influencing its catalytic behavior as already discussed in chapter 4. Since the CD is used in excess, host-guest complexation in solution with substrate

and product may not be excluded. This prevents substrate and/or product inhibition in enzymatic reactions.

Compared to the commercially available PSL (used as purchased), a slight decrease in the catalytic activity has been observed when the enzyme was lyophilized from phosphate buffer (pH 6). This finding is accounted for by partial denaturation of PSL during lyophilization. Interestingly, no such decrease in catalytic activity was observed when the enzyme was co-lyophilized with peracetylated *b*-cyclodextrin using the same phosphate buffer or both are entrapped in the sol-gel matrix. This finding may be explained by the ability of CD and sol-gel to protect the enzyme from aggregation or from denaturation effects.
Since the magnitude of the enzyme activation in organic solvents by macrocyclic compounds depends on the ratio of the enzyme and the additive[87], three different mixtures have been investigated, i.e., 1:1; 1:2; 1:6 (enzyme to cyclodextrin, w/w), and added during the gel process. When the peracetylated *b*-cyclodextrin was employed at a 1:6 weight ratio of enzyme to cyclodextrin entrapped in sol-gel, a significant activation of PSL has been detected as compared to the data of the lyophilized PSL from buffer alone.

The lyophilized lipase from buffer alone catalyzed kinetic resolution of (**1**) using vinyl butyrate as acyl donor is characterized by the following data: time = 24 hrs, ee_s = 91 %, ee_p = 93 %, conv. = 50 % and E = 92, and for the PSL/CD 1:6 (w/w) entrapped in sol-gel is characterized by the following data: time = 8 hrs, ee_s = 98 %, ee_p = 92 %, conv. = 51 % and E >100.

The lyophilized lipase from buffer alone catalyzed kinetic resolution of (**1**) using isopropenyl acetate as acyl donor is characterized by the following data: time = 24 hrs, ee_s = 99%, ee_p = 61%, conv. = 60% and E=28, and for the PSL/CD 1:6 (w/w) entrapped in sol-gel is characterized by the following data: time = 9 hrs, ee_s = 99 %, ee_p = 72 %, conv. = 57 % and E >100.

In all cases of transesterification, the enhancement of the reaction rate and the enantiomeric ratio E of the PSL/CD in sol-gel-catalyzed transesterification of (**1**) in toluene was in the order 1:6 > 1:2 > 1:1 (enzyme to peracetylated *b*-cyclodextrin, w/w).

In a control experiment it was found that no reaction took place when the gel or peracetylated *b*-cyclodextrin alone was employed without any lipase. It is concluded from this finding that neither peracetylated *b*-cyclodextrin nor the gels themselves are able to catalyze the enantioselective transesterification of (**1**) but their use regulate enzyme activity probably by changing the conformation of the enzymatic catalytic site or the CD undergoes host-guest complexation with substrate and/or product. In an additional experiment, PSL was first lyophilized from buffer alone, suspended in toluene and only afterwards peracetylated *b*-cyclodextrin entrapped in sol-gel was added to the reaction mixture at the 1:6 weight ratio as before. A slight enhancement of the reaction rate and enantiomeric ratio E has been observed. However, this enhancement was much smaller than that observed when lipase was co-lyophilized with CD in phosphate buffer using the same weight ratio.

It is to be noted that the enhancement effect observed in the co-lyophilized lipase with peracetylated *b*-cyclodextrin entrapped in sol-gel was almost the same as observed before when using co-lyophilized lipase with peracetylated *b*-cyclodextrin (without entrapment in sol-gel). Therefore it was concluded that the sol-gel is only working as an immobilizing agent for the co-lyophilized lipase with peracetylated *b*-cyclodextrin. Hence, the enhancement in reaction rate and enantiomeric ratio is believed to be based mainly on the beneficial effect of the CD additive on the enzyme. This can arise from the direct interaction with the enzyme rather than complexation with either substrate or product. Thus, in the co-lyophilization step, the CD may interact with the enzyme in a specific but yet unknown way by changing its conformation and hence influencing its catalytic behavior as already inferred above.

Since the CD is used in excess, host-guest complexation in solution with substrate and product may not be excluded. This might explain why the reaction rate was higher when using the 1:6 as compared to the 1:1 ratio (enzyme to cyclodextrin, w/w). In fact, the ability of CDs to form host-guest complexes may indeed prevent substrate as well as product inhibition in enzymatic reactions.[20] The inclusion of product in the cavity of CD may shift the equilibrium in the desired direction. This thermodynamic effect, however, would not explain the enhancement of the reaction rate. The ability of 1-(2-furyl)ethanol (**1**) as well as its ester (**3**) to undergo inclusion in the peracetylated *b*-

cyclodextrin cavity was indicated by (upfield/downfield) complexation-induced shifts in ^{1}H as well as ^{13}C NMR-spectroscopic studies (studied in details in chapter 4).

7.2.2. Stability of sol-gel entrapped PSL

The sol-gel entrapped lipase from *Pseudomonas cepacia* is highly stable and can be stored at room temperature for months without significant loss of activity. The entrapped lipase in MTMS gels has been repeatedly used several times in the transesterification of 1(2-furyl)ethanol in toluene. Adding to that, this kind of enzyme preparation was thermally stable. The lipase/CD entrapped in sol-gel catalyzed the transesterification of (**1**) using isopropenyl acetate in toluene has been repeated at several temperatures from 30 to 70^{o}C. A parallel experiment was performed using free lipase. The results suggest that as the reaction temperature is increased the activity of lipase is also increased and the enhancement in activity was much stronger for the entrapped lipase than for the free one.

7.2.3. Structural and morphological properties

a) Solid-State ^{29}Si NMR

Solid-state NMR and especially Si-NMR data yields information about the chemical structure and degree of cross linking in the Si-containing gels, but none about the actual enzymes, which are present in small amounts within the gel. In ^{29}Si NMR, it was interesting to determine the actual degree of cross linking in the prepared gel. For this purpose PSL in sol-gel only as well as PSL/CD 1:1, 1:2, 1:6 entrapped in sol-gel has been investigated. The results suggest that a high degree of cross-linking was present.

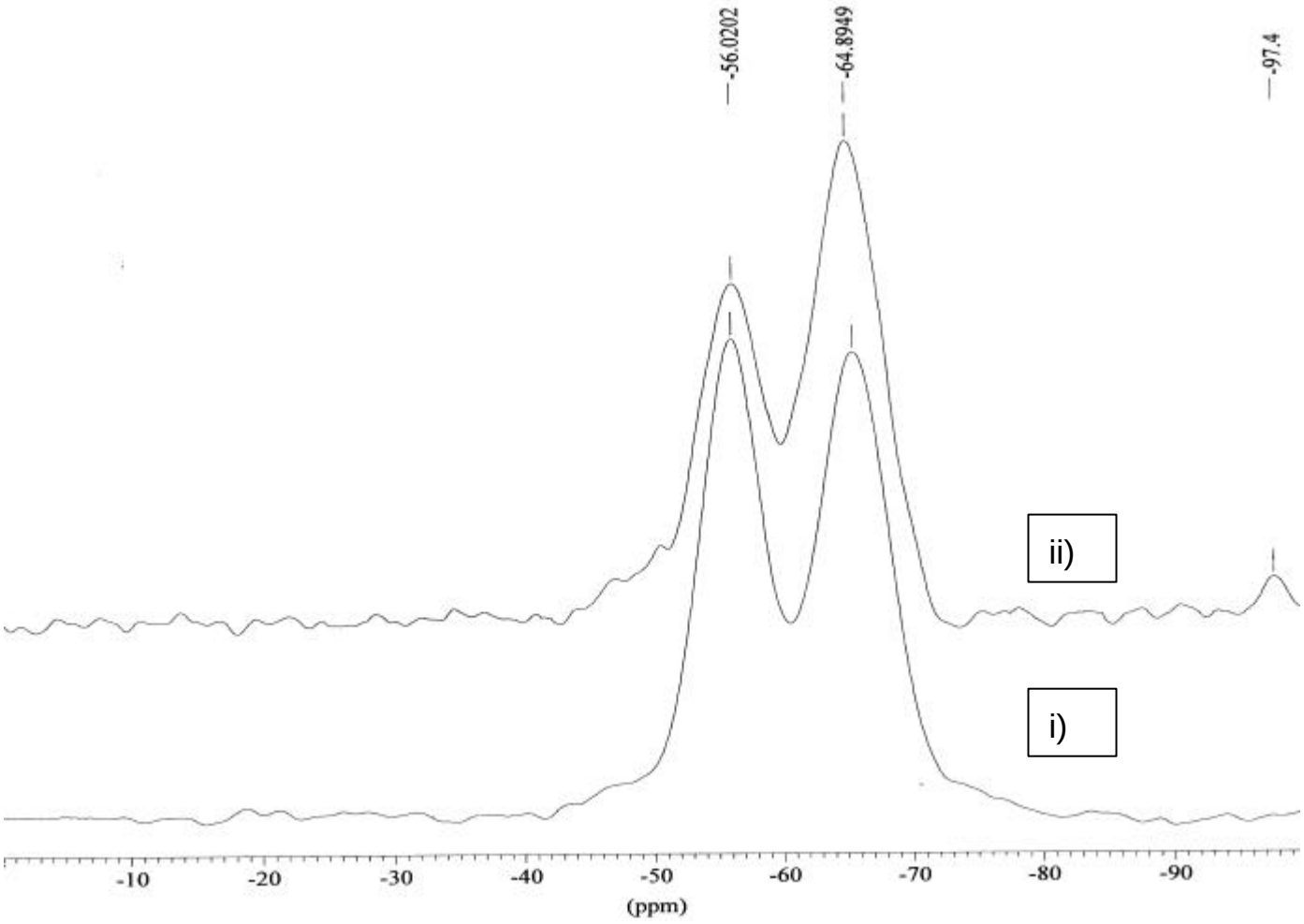


Fig. 7.5: a) ^{29}Si NMR spectrum of i) PSL-containing gel derived from MTMS ii) co-lyophilized PSL with peracetylated *b*-cyclodextrin entrapped in sol gel, derived from MTMS.

b) Scanning electron microscopy (SEM) and energy dispersive X-ray studies (EDX)

SEM studies were carried out (Fig. 7.6-7.9) to determine the morphology of lipase-containing gel derived from MTMS (control) and compare it with co-lyophilized PSL with peracetylated *b*-cyclodextrin (1:1, 1:2, 1:6 w/w)-containing gel. They revealed large amorphous regions as well as spherical particles in case of PSL (only) entrapped in sol-gel. As soon as the lipase is co-lyophilized with CDs, the morphology of the particles is changed to form agglomerates. As much as the peracetylated *b*-cyclodextrin increased (from 1:1 to 1:6 w/w lipase/CD ratio) the agglomeration is increased.

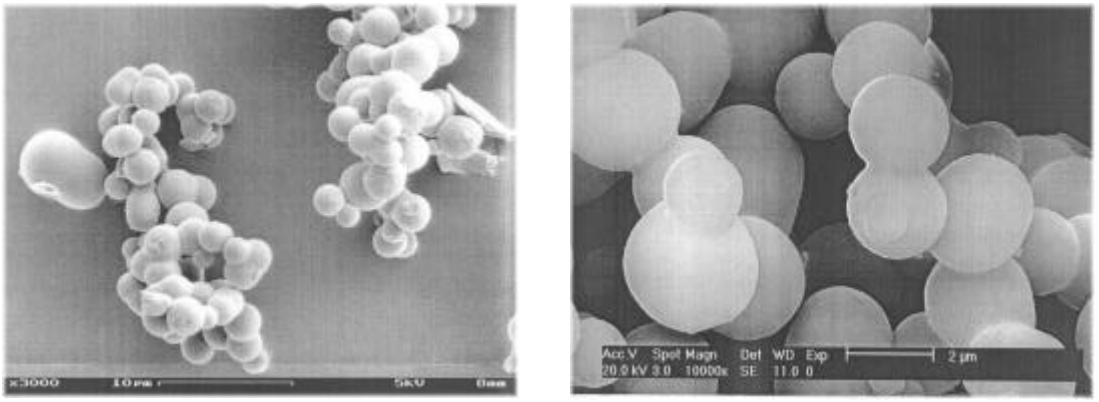


Fig. 7.6: A typical SEM-micrograph of co-lyophilized lipase (PSL) with peracetylated *b*-cyclodextrin (1:6 w/w) entrapped in sol-gel.

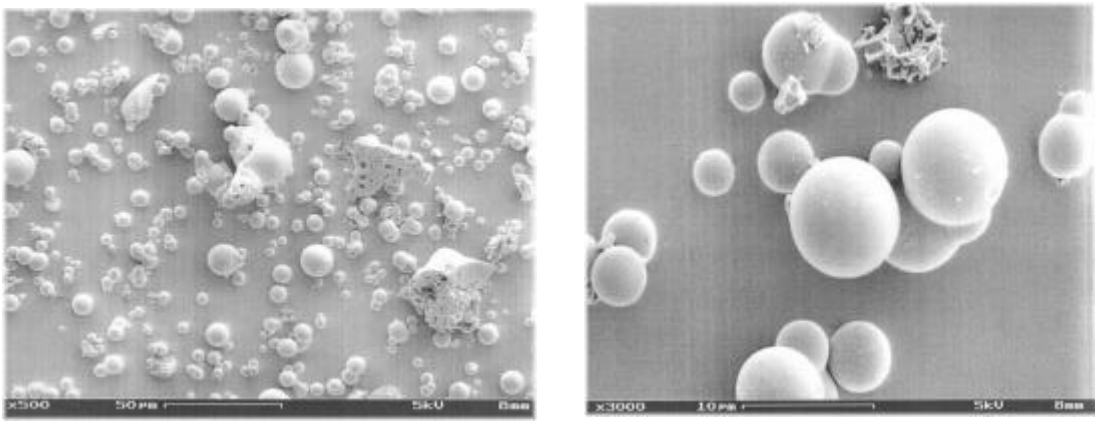


Fig. 7.7: A typical SEM-micrograph of co-lyophilized lipase (PSL) with peracetylated *b*-cyclodextrin (1:2 w/w) entrapped in sol-gel.

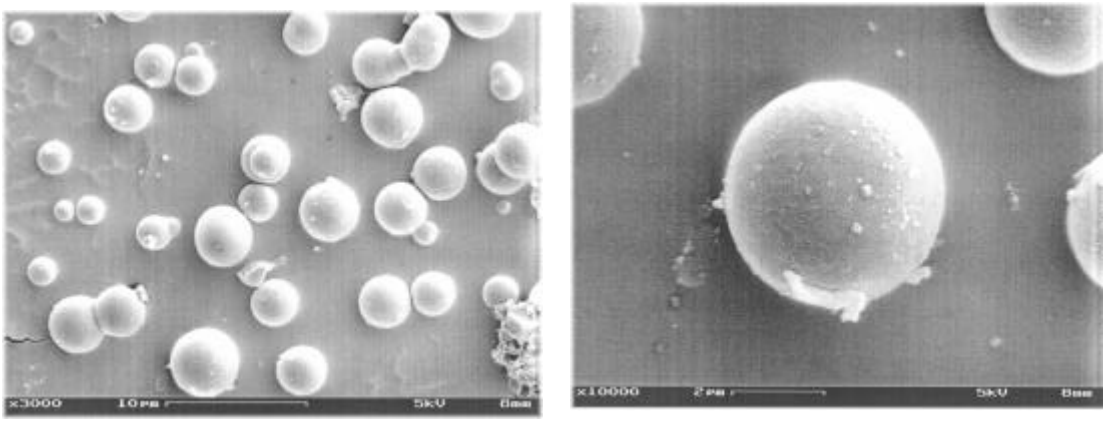


Fig. 7.8: A typical SEM-micrograph of co-lyophilized lipase (PSL) with peracetylated *b*-cyclodextrin (1:1 w/w) entrapped in sol-gel.

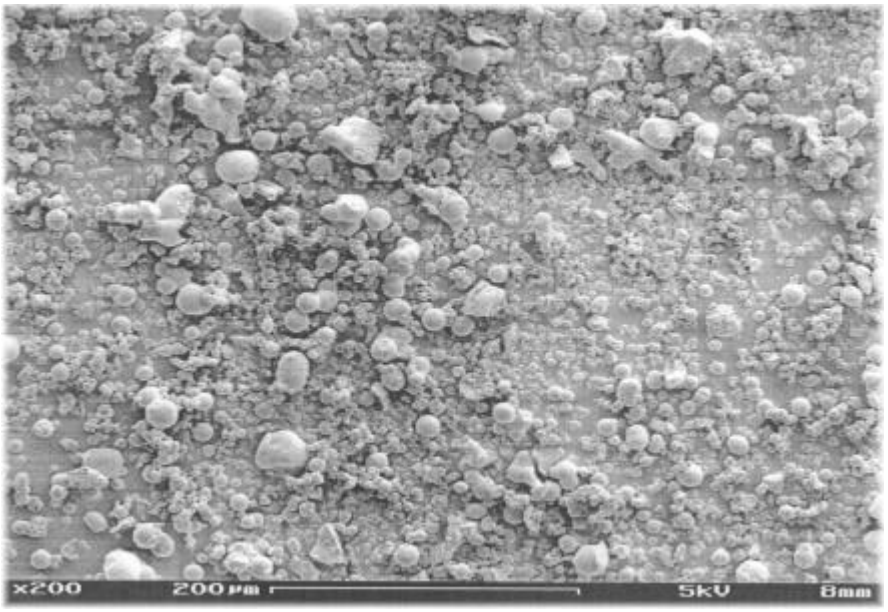


Fig. 7.9: A typical SEM-micrograph of PSL entrapped in sol-gel.

In order to define whether the lipase is simply physically entrapped or adsorbed on the surface of the particle, an EDX study (Fig. 7.10) has been performed. This study reveals the existence of lipase inside the particles as well as on the surface of the spherical particle.

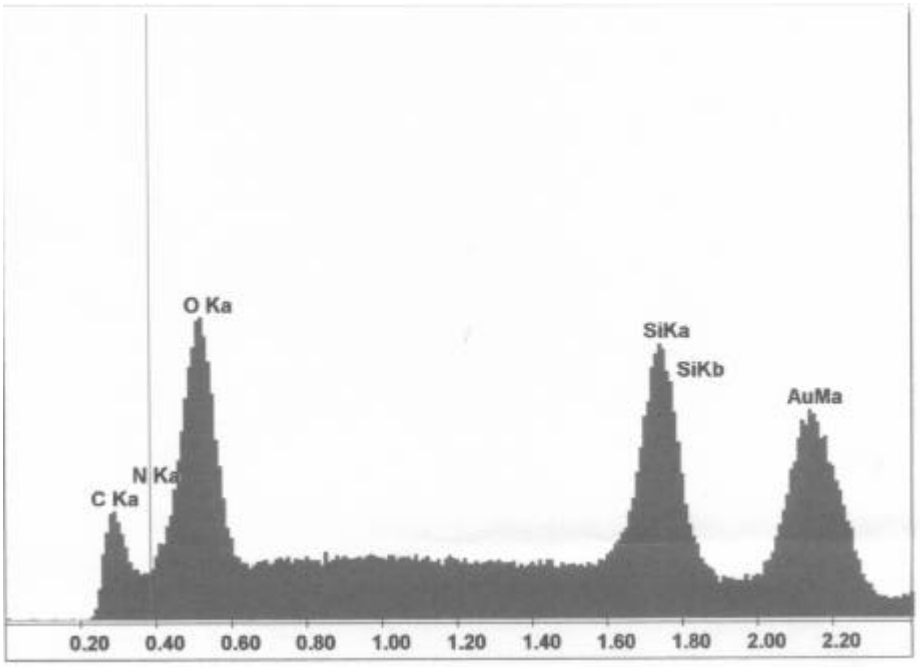


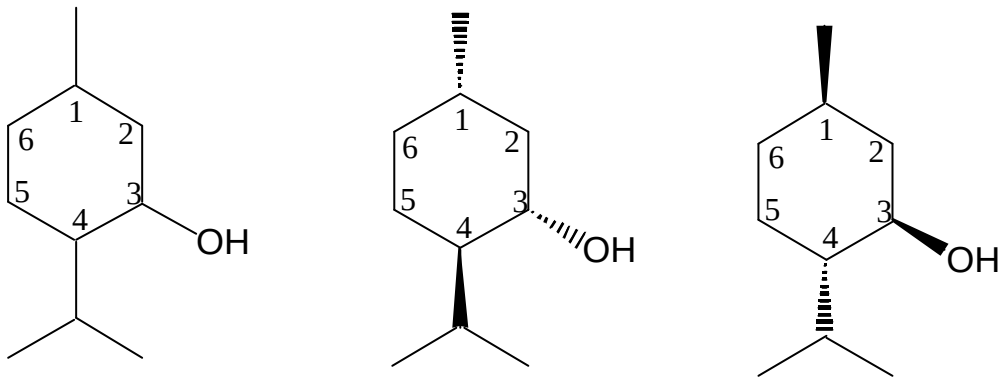
Fig. 7.10: A typical EDX spectrum of the co-lyophilized lipase (PSL) with peracetylated *b*-cyclodextrin (1:6 w/w) entrapped in sol-gel.

8. Application of Chirasil-b-Dex with 11-spacer in the lipase mediated enantioselective access to (-)-menthol

8.1. Introduction

Terpenes and steroids are two classes of important natural product research. The naturally occurring terpenes were especially investigated by Wallach, V. Bayer, Semmler and Tiemann[3]. They include not only hydrocarbons but also derivatives such as alcohols, ethers, aldehydes and ketones. They occur in plants, especially in flowers and fruits, and are obtained as essential oils by steam distillation or extraction of crushed plants. Because of their pleasant odours many terpene derivatives have been used as perfumes and fragrants.

Menthol is one of the most important saturated cyclic alcohols belonging to this series. There are three stereogenic centers in the molecule and in consequence there are eight stereoisomers which have all been obtained synthetically. The racemic form of menthol can be obtained from the catalytic hydrogenation of thymol. The only forms abundantly encountered in nature are (-)-menthol and (+)-neomenthol. By far the most important is (-)-menthol, which is the main constituent of peppermint oils (50-65 %).



menthol (+)-(1S, 3S, 4R)-menthol (-)-(1R, 3R, 4S)-menthol

Fig. 8.1: Structures showing the three stereogenic centers of menthol.

It possesses a distinct peppermint flavor and gives the impression of cooling the mouth and skin. (-)-menthol is widely used as flavoring ingredient in toothpastes, mouthwashes and cigarettes, as a rubefacient, and as cooling agent in the chest.

Commercially available (-)-menthol is isolated principally from the oil of *Mentha arvenis* grown in Japan. The process involves cooling the oils and purifying the crystals formed by subsequent crystallization. The crystals are then centrifuged and therefore the menthol crystals are separated from the supernatant, also called dementholized cornmit oil.

It can be synthesized from (+)-citronellal and by fractionated crystallization of racemic menthyl benzoate followed by hydrolysis with NaOH (Haarmann & Reimer). The latter involves the seeding of the bulk with one of the diastereomers (Fig. 8.2). This process leads to more than 90% overall yield. In Japan, it is also obtained by the Takasago process based on the Rh-BINAP (catalyst developed by Noyori et al.)-catalyzed enantioselective isomerization of diethylgeranylamine to citronellaldiethylamine. This process is used for the production of about 2000 tons per annum of *l*-menthol.[7]

The use of biological catalysts provide further opportunities for the access to (-)-menthol. The lipase-catalyzed kinetic resolution of racemic menthol by enantioselective esterification was reported using free acid[119] or acid anyhdride[120] , however, using free acid in esterification leads to the generation of water as a by-product which hydrolyze the enantiomerically pure ester and hence decreases the conversion and the enantiomeric excess. Adding to that, the low reactivity of carboxylic acid lowers the rate of the esterification reaction.

Although the use of acid anhydride as highly reactive and non-aqueous producing acyl donor, is attractive for bioreaction systems[120], the generation of free acid as a by-product still represents a drawback for this reaction since the acid released might be involved in the esterification of the remaining unreacted enanatiomerically pure menthol leading to a decrease in the enantiomeric excess and the conversion.

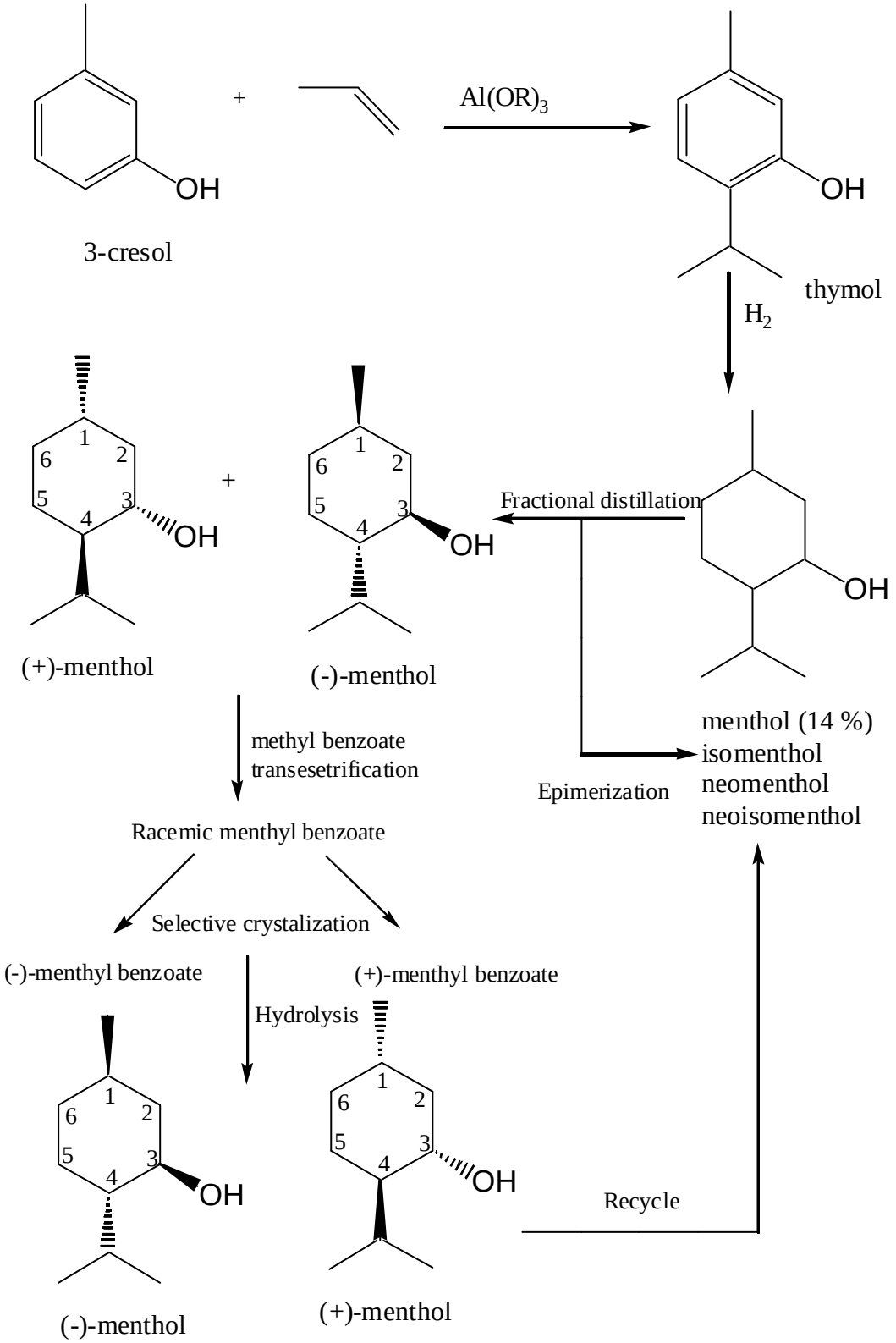


Fig. 8.2: The Haarmann and Reimer process for the synthesis of enantiomerically pure (-)-menthol.

To overcome this problem (the presence of free acid as a by-product), addition of dissolved or suspended inorganic bases could significantly improve the enantioselectivity[121], $NaHCO_3$, as a weak base was added[122] to neutralize the free acid released, however, no enhancement of the enantioselectivity (E) was observed. This is probably due to the generation of water resulting from the addition of bicarbonate to the free acid released. In addition, a base-line separation of both substrate and product was not achieved in this report.

8.2. Results and discussion

At first, the lipase (free and immobilized either on EP100 or LDPE)-catalyzed transesterification of rac. menthol has been performed using isopropenyl acetate as an innocuous acyl donor in isooctane as solvent. Unfortunately, menthol was found to be a poor substrate for the isopropenyl acetate when the latter was used as acyl donor in the transesterification reaction. Results are summarized in table 8.1.

Table 8.1: Lipase-catalyzed transesterification of rac. menthol using isopropenyl acetate as acyl donor.

Lipase	Time (d)	[%]ee_s (+)-**1**	[%]ee_p (-)-**2**	% conversion	E
CCL (free)	2	3	99.9	2.8	>100
CCL (EP100)	2	4	99.9	4	>100
CCL (LDPE)	2	1.5	99.9	1.5	>100
CRL (free)	1	20.8	99.9	17.2	>100
AYS (Amano)	2	6	99.9	6	>100
PSL (EP100)	2	12	99.9	10.3	>100
AY30 (Acros)	2	4	99.9	3.7	>100

As the transesterification was unsuccessful, other methods in lipase-catalyzed reactions have been investigated.

Different lipases were screened in resolving racemic menthol (**1**) either in the enantioselective esterification of (+)-(-)-**1** using acid anhydride (acetic, propionic and butyric anhydride) as acyl donors in isooctane as solvent in non-aqueous medium or the enantioselective hydrolysis of the corresponding menthyl acetate (+)-(-)-**2** and menthyl benzoate using phosphate buffer (pH 7) in aqueous medium.

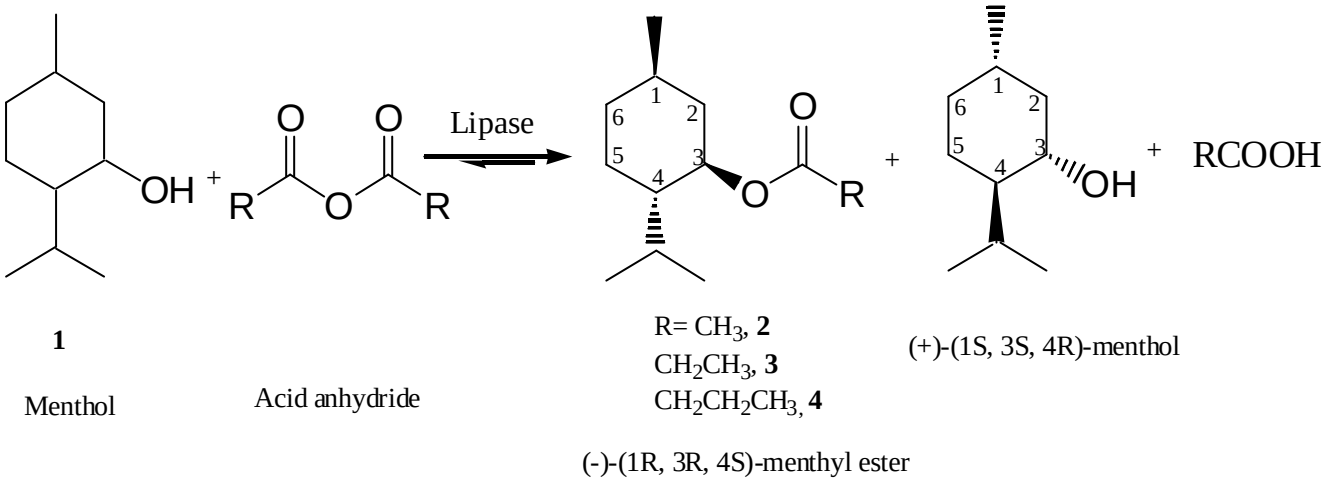


Fig. 8.3: Lipase-catalyzed enantioselective esterification of menthol using acid anhydrides as acyl donors in isooctane.

Among the lipases tested in the enantioselective esterification of menthol (**1**) were recombinant *Candida rugosa* lipase (CRL, expressed in *Pichia pastoris*), *Pseudomonas cepacia* (PSL), *Pseudomonas cepacia* immobilized on ceramic particles (PSL-C), *Pseudomonas cepacia* immobilized on diatomaceous earth (PSL-D), Lipozyme RM IM, and CRL (commercially available).
The esterification of (**1**) was carried out at 40 ^{o}C using acetic, propionic and butyric anhydride as acyl donors in isooctane as solvent. Molecular sieves were added to scavenge the liberated acids. All *Pseudomonas cepacia* lipases and the lipozyme RM IM showed low reactivity and stereoselectivity in the esterification of (**1**). The esters formed were almost racemic in cases of all acyl donors used.

Lipase from *Candida rugosa* commercially available (CRL) (free and immobilized on EP 100) showed reactivity towards rac. menthol. The best results were obtained when using propionic anhydride as acyl donor.

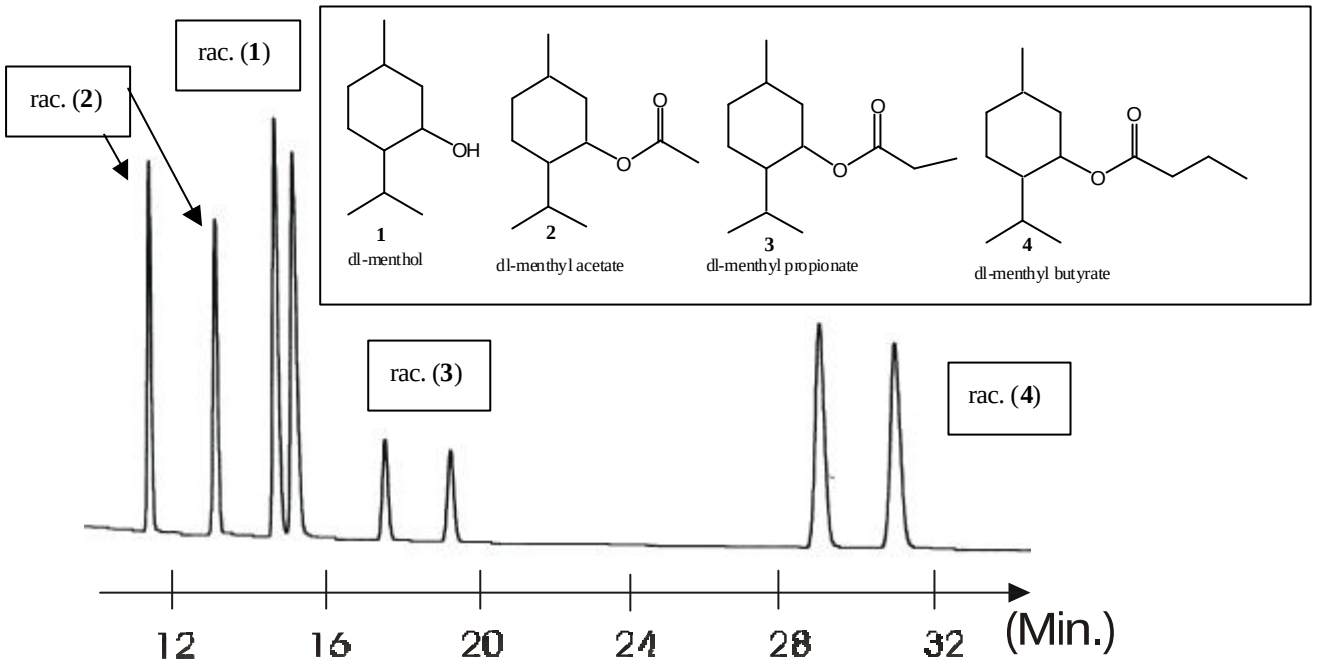


Fig. 8.4: Gas-chromatographic separation of racemic menthol (**1**), menthyl acetate (**2**), menthyl propionate (**3**) and menthyl butyrate (**4**) on Chirasil-β-Dex with a new 11-spacer as chiral stationary phase in GC. The separation factor α and R_s were: $\alpha = 1.03$ and $R_s = 1.60$ for the rac. menthol (**1**); $\alpha = 1.16$ and $R_s = 8.23$ for the ester (**2**); $\alpha = 1.10$ and $R_s = 5.57$ for the ester (**3**); $\alpha = 1.07$ and $R_s = 4.0$ for the ester (**4**). The oven temperature was 100°C (isothermal) for 30 min.

Recombinant lipase (expressed in *Pichia pastoris*) also showed some reactivity towards menthol, however, the reaction was very slow (after 26 hrs, ee_s =21%, ee_p=90%, and the conversion didn't exceed 20% with E=30), and ee_s =47.1%, ee_p= 99.2%, conv. =32.2% and E>100 in one week.

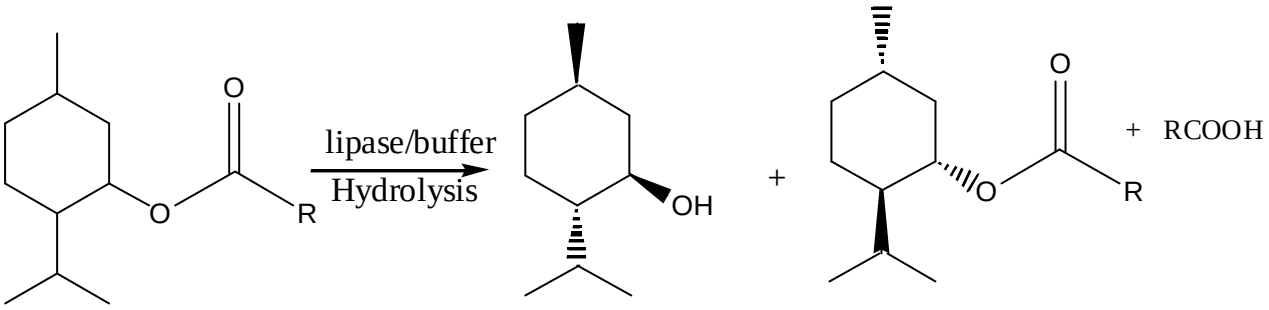
Upon immobilization of the recombinant lipase on EP100, the change was relatively small, ee_s=25%, ee_p=99.8, and Conv. =20% also in one week.

Table 8.2: Recombinant lipase-catalyzed esterification of menthol using propionic acid anhydride in isooctane

Lipase	Time (d)	[%]ee_s (+)-**1**	[%]ee_p (-)-**2**	% conversion	E
Rec. lipase (free)	1	21	90	20	30
Rec. lipase (free)	7	47.1	99.2	32.2	E>100
Rec. lipase (immobilzed)	7	25	99.8	20	E>100

Since the reaction rate was low when using acid anhydride as acyl donor, it was assumed that the enzyme is deactivated after several hrs due to some sensitivity towards the acid released, however, all enzymes used were active even after one week carrying the acylation reaction. To scavenge the released acid, $NaHCO_3$ was added to neutralize the acidic by-product. However, the conversion is decreased which could be due to unfavored hydrolysis of the resulted enantiomerically enriched ester.

Compared to the esterification experiments in non-aqueous medium described above, the enzymatic hydrolysis of the acetate (**2**) and rac. menthyl benzoate (**5**) in aqueous medium proceeded slowly (Fig. 8.4). In about 2 days, the conversion reached 56% with 69% ee (-)-menthol (-)-**1**, 83% ee (+)-menthyl acetate (+)-**2** and E= 13 in case of CRL-catalyzed enantioselective hydrolysis of rac. menthyl acetate in aqueous medium. Only 27% conversion and 70 % ee (-)-menthol (-)-**1** and 80.8 % ee for the remaining unreacted ester (+)-**5** were achieved after 24 hours using CRL (expressed in *Pichia pastoris*). It is to be noted that the conversion in case of the hydrolysis of rac. menthyl benzoate is determined using a pH stat (up on titration with 0.1 M NaOH, error 5%).



rac. menthyl ester (-)-(1R, 2S, 5R)-menthol (+)-(1S, 2R, 5S)-menthyl ester

R= CH_3 (**2**), or C_6H_6 (**5**)

Fig. 8.5: lipase-catalyzed enantioselective hydrolysis of rac. menthyl ester.

The recombinant lipase-catalyzed enantioselective hydrolysis of racemic menthyl benzoate could be an efficient method for the production of (-)-menthol on industrial scale.

9. Summary

Enantiomerically pure secondary alcohols are useful chiral auxiliaries in organic synthesis and they are preferably synthesized by enzymatic kinetic resolution of the racemates. This biochemical transformation process has become a standard reaction protocol in organic synthesis. In the presence of a suitable acyl donor, enzyme as well as solvent, and at the optimum temperature, one enantiomer of the racemic mixture is selectively transferred to the corresponding ester leaving the second unreacted enantiomer in enantiomerically pure form. The reversible enzymatic process usually requires a long reaction time and a large excess of the ester as acyl donor to achieve a reasonable degree of conversion. In order to render the process irreversible, the use of various activated esters like vinyl acetate, trifluoroethyl esters, chloroethyl esters, cyanomethyl esters, and acid anhydrides have been reported. However, these methods have some drawbacks, like the generation of toxic side products thereby deactivating the enzyme and inhibiting the formation of the required products, or the generation of water which causes the undesired hydrolysis of the enantiomerically pure ester, leading to a decrease in the conversion and in the enantiomeric excess.

The generation of acetaldehyde resulting from the tautomerization of vinylalcohol when using vinylacetate as acyl donor has the following disadvantages. Acetaldehyde leads to a deactivation of various lipases especially *Geotrichum candidum* and *Candida rugosa*, the latter being one of the most important lipases having a broad applicability to substrates in organic synthesis. Acetaldehyde undergoes aldolisation-crotonisation liberating water (unfavored in enzyme-catalyzed reactions in organic solvents) which hydrolyze the enantiomerically pure ester formed and hence decreases the conversion and the enantioselectivity. Acetaldehyde can react with the enantiomerically pure alcohol formed to form hemiacetals.

In this work, the kinetic resolution of secondary alcohols used as chiral building blocks of numerous natural products was investigated. The kinetic resolution was based on the lipase-catalyzed transesterification using isopropenly acetate as an innocuous acyl donor in organic solvents. Among the secondary alcohols

investigated were 1-(2-furyl)ethanol, *trans*-4-phenyl-3-butene-2-ol and menthol.

1-(2-furyl)ethanol

The enzyme of choice for the lipase-catalyzed transesterification of 1-(2-furyl)ethanol using isopropenyl acetate as acyl donor was the lipase from *Pseudomonas cepacia* (PSL) in isooctane as solvent in the presence of molecular sieves. The beneficial effect of molecular sieves and solvents on the kinetic resolution was studied. The optimized kinetic resolution of 1-(2-furyl)ethanol was characterized by the following data: time = 21 hrs, ee_s 96%, ee_p >99%, conv. = 48%, E >300. The procedure was applied for a gram-scale production of essentially enantiomerically pure 1-(2-furyl) ethanol.

trans-4-phenyl-3-butene-2-ol

Different lipases were screened in resolving racemic *trans*-4-phenyl-3-butene-2-ol either in the transesterification of the alcohol using isopropenyl acetate as an innocuous acyl donor in toluene as solvent (non-aqueous media) or in the enantioselective hydrolysis of the corresponding acetate using phosphate buffer (pH=6). In regard to the ee of the remaining substrate (S)-*trans*-4-phenyl-3-butene-2-ol and that of the product (R)-2-acetoxy-4-phenyl-but-3-ene as well as the rate of conversion (51% in 4 hrs) and enantiomeric ratio E>300, PSL-D (Pseudomonas cepacia lipase immobilized on diatomaceous earth) was the best lipase employed in the transesterification of *trans*-4-phenyl-3-butene-2-ol. Compared to the transesterification experiments in non-aqueous media, the enzymatic hydrolysis of the acetate (R,S)-2-acetoxy-4-phenyl-but-3-ene proceeded slowly, only moderate conversion (45%) and high ee (up to 99 % for the alcohol (R)-*trans*-4-phenyl-3-butene-2-ol and 80.8% for the remaining unreacted ester (S)-2-acetoxy-4-phenyl-but-3-ene) was achieved after 24 hours using Novozyme IM (CAL-B). A gram-scale production of the enantiomers of *trans*-4-phenyl-3-butene-2-ol has been found. Another approach has been developed for the access to enantiomerically pure *trans*-4-phenyl-3-butene-2-ol, this was based on the enantioselective reduction of *trans*-4-phenyl-3-butene-2-one using a ruthenium complex in a consecutive approach with lipase. It was demonstrated that metal based catalyst together with the biocatalytic system, represented by the lipase, can be used in a consecutive way for the synthesis of enantiomerically pure *trans*-4-

phenyl-3-butene-2-ol starting from prochiral ketones. The use of enantiomerically enriched substrate leads to the increase of the reaction rate of the enzymatic reaction.

Menthol

Different lipases were screened in resolving racemic menthol either in the enantioselective esterification of the free alcohol using acid anhydride (acetic, propionic and butyric anhydride) as acyl donors in isooctane as solvent or the enantioselective transesterification of menthol using isopropenyl acetate as acyl donor in toluene as solvent (non-aqueous medium) or the enantioselective hydrolysis of the corresponding menthyl acetate and menthyl benzoate using phosphate buffer (pH 7) in aqueous medium. The results suggest that menthol is a poor substrate for isopropenyl acetate as acyl donor. Propionic acid anhydride was the best acyl donor in lipase-catalyzed esterification of rac. menthol. CRL was the best lipase employed for both enantioselective esterification of rac. menthol as well as the enantioselective hydrolysis of the corresponding acetate, e.g. menthyl acetate and menthyl benzoate.

The utility of cyclodextrins in enzymatic reactions

The utility of peracetylated *b*-cyclodextrin as additive in lipase-catalyzed transesterification of 1-(2-furyl) ethanol in organic solvent was demonstrated. The results suggest that co-lyophilization with CD can be a useful method for the activation of enzymes in organic solvents leading to an enhancement in the reaction rate and enantiomeric ratio of the lipase-catalyzed reactions. CDs do not catalyze the enantioselective transesterification of 1-(2-furyl)ethanol by themselves, but they influence the enzyme activity, probably by changing the conformation of the enzymatic catalytic site. CDs may interact with the enzyme in a specific unknown way, changing its conformation and hence influence its behavior. This behavior is the result of the co-lyophilization of the enzyme with per-acetylated *b*-cyclodextrin. The excess of CDs that did not bind to the enzyme remains in solution and acts as a host-guest complexing agent.

Entrapment of lipase with cyclodextrin in sol-gel

Three methods of enzyme preparation have been performed. The first consisted of the lyophilization of *Pseudomonas cepacia* lipase (PSL), the present model enzyme, from phosphate buffer (pH 6) without the CD additive. The second enzyme preparation was concerned with the co-lyophilization of PSL with peracetylated *b*-cyclodextrin using the same phosphate buffer. Three different mixtures have been investigated, i.e., 1:1; 1:2; 1:6 (enzyme to cyclodextrin, w/w). The third enzyme preparation was the entrapment of the co-lyophilized lipase with peracetylated *b*-cyclodextrin (1:1; 1:2; 1:6) in sol-gel. The sol-gel process (initiated by the hydrolysis of MTMS) is performed in the presence of the co-lyophilized PSL with peracetylated *b*-cyclodextrin. The effect of the entrapment of PSL/CD in sol-gel was studied in the transesterification of 1-(2-furyl)ethanol using either vinyl butyrate or isopropenyl acetate as acyl donor in toluene. An enhancement was observed in the reaction rate and enantiomeric ratio of lipase/CD entrapped in sol-gel catalyzed transesterification in organic solvent, however, this enhancement was almost the same as reported before for the lipase/CD (without sol-gel)-catalyzed transesterification in toluene. The results suggest that the observed enhancement effect was due to the effect of CDs in the enzyme, the sol-gel merely acts as immobilizing agent for the enzyme.

The sol-gel entrapped lipase from *Pseudomonas cepacia* is highly stable and can be stored at room temperature for months without significant loss of activity. The entrapped lipase in MTMS gels has been repeatedly used in the transesterification of 1(2-furyl)ethanol in toluene. Adding to that, this kind of enzyme preparation is thermally stable.

10. Experimental

10.1. Research equipment

NMR-spectroscopy
^{1}H and ^{13}C NMR spectra were recorded either on a Bruker AC 250, WM 400 or AMX600 FT NMR spectrometer (Karlsruhe, Germany), at a sample temperature of 300K. The Rotating Frame Overhauser Experiments (ROESY) were performed using the pulse program roesyprtp with an applied mixing time of 225 ms. The ^{13}C-spectra were measured using a broad-band (BB) decoupled spin-echo experiment. 2D-^{1}H-^{1}H and ^{1}H-^{13}C-spectra were recorded on the wm400 spectrometer with COSY (90-45) pulse program. All chemical shifts (δ) of ^{1}H and ^{13}C are given in ppm relative to TMS as internal standard. All structures were drawn using CS Chemdraw Pro® by Cambridge Soft.
^{1}H-NMR, AC250, transmitter frequency 250 MHz
^{13}C-NMR, AC250, transmitter frequency 69.9 MHz
^{1}H-NMR, WM400, transmitter frequency 400 MHz
^{13}C-NMR, WM400, transmitter frequency 100 MHz
1H-NMR, AMX600, transmitter frequency 600.13 MHz

Mass spectroscopy
EI spectra were measured on a mass spectrometer Finnigan MAT TSQ 70 (source temperature 200°C). ESI spectra were recorded on an API-III-TAGA 6000 E spectrometer with triple-quadrupole focusing. Samples were dissolved in methanol (conc. 1 mg/ml, additive: sodium, potassium, or ammonium acetate). HRMS spectra were acquired using a Bruker APEX II FT-ICR mass spectrometer (sample dissolved in MeOH/H2O 95:5 additive 1% acetic acid).

Gas-chromatographic analysis
Enantioselective analysis was performed on a gas chromatograph (Hewlett Packard 580, Waldbronn, Germany) equipped with a flame ionization detector (FID). The chiral stationary phase heptakis-(2,3-di-*O*-methyl-6-*O*-tert-butyldimethylsilyl)-*b*-cyclodextrin, 20% (w/w) was dissolved in PS 86 (Gelest, ABCR GmbH & Co., Karlsruhe, Germany) and coated on a 25 m x 0.25 mm (i.d.) fused silica capillary column (0.25 µm film thickness) according to the literature procedure.[47] The analytical conditions were: Injector temperature,

200°C; FID temperature, 250°C; oven temperature as mentioned in results and discussion of each chapter for the simultaneous separation of enantiomers of both substrate and product. Hydrogen was used as the carrier gas (40 KPa column head pressure). The substrates and product were identified by using a GC/MSD-system HP 6890/5973 (Hewlett Packard, Waldbronn, Germany) equipped with an HP 7683 autosampler. The enantiomeric excess of both substrate and product as well as conversion and enantiomeric ratio were determined by the computer program available on the internet http://www-orgc.tu-graz.ac.at, developed by Faber et al.[72]
Gases:
Hydrogen (purity 99.999%) and in-house compressed air (purified with charcoal).

Polarimetry
Angles of rotation were measured on Perkin-Elmer 241 polarimeter operating at the sodium D line.

Elemental analysis
The elemental composition was determined with Carlo Erba elemental analyzer 1104.

Thin layer chromatography
Thin layer chromatography was carried out using pre-coated TLC plates SIL G-25 UV254, layer: 0.25 mm silica gel with fluorescent indicator UV254 (Macherey-Nagel GmbH & Co. KG). Chromatograms were developed using varying eluents depending on the solute. Spots on the plates were visualized by exposure to UV light or iodine or by spraying with 5% H_2SO_4 and heated at 110°C on a heating block for 5 min in case of CDs.

pH-stat
A pH-stat (718 STAT Trino, Metrohm, Switzerland) is used to determine the hydrolytic activity of the recombinant as well as other commercially available lipases. 20 ml tributyrin solution (200 ml H_2O + 4ml Tributyrin + gum arabic) were added to the pH stat and the pH is adjusted to pH 7.2. Then the lipase (500 mg) dissolved in 2 ml buffer were added to the pH stat.
This solution was automatically titrated with NaOH solution (0.1M) to the desired pH before lipase was added. The consumption of the NaOH was recorded as a function of time. The lipase activity is then

expressed in units (U). One unit corresponds to the liberation of 1µmol fatty acids per minute.
The specific activity of the recombinant lipase was 1U/mg while that of the CRL (Amano) was about 10U/mg.

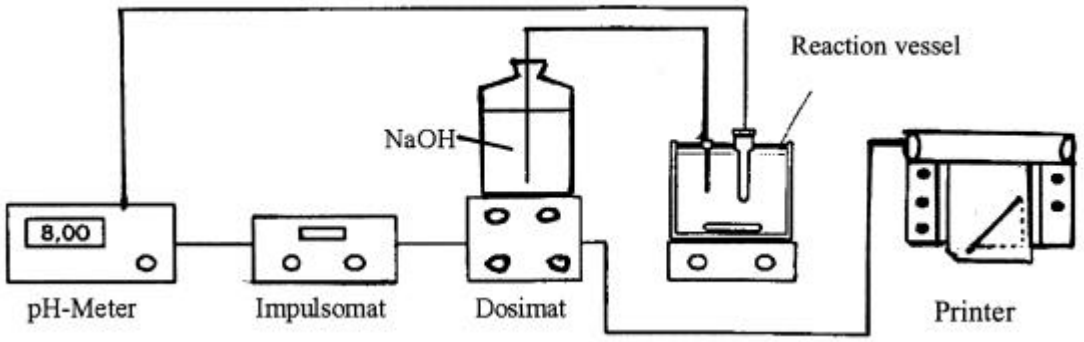


Fig. 10.1: Schematic representation showing the pH stat.

10.2. Cultivation of *Pichia pastoris* (yeast cells) harboring a lipase gene

10 g of yeast extract and 20 g Peptone were added to 800 ml distilled water in 1 L bottle, then autoclaved. 10 mg biotine was dissolved in 5 ml water (filter sterilized). 20 g glucose in 200 ml water were autoclaved separately and then added to the previous contents , i.e, a full volume 1 L.

10.2.1. Preparation of the media necessary for expression of lipase gene

5µl of the cells were added to the media prepared as follows: phosphate buffer (100 ml), biotin (2ml) and glucose solution (100ml), were mixed and then added to 800 ml yeast extract.
In three different conical flasks (3 * 50), 5µl cells and 50 µl theothin were added in each conical flask and then left for about 3 days. Afterwards a sample was taken and examined under the microscope. If no contamination is observed, 2 ml of the culture is taken and centrifuged for 10 min. Then the activity is measured in the supernatant (the upper layer) by using the pH stat.

10.2.2. Inhibition of glycosylation of recombinant lipase

To inhibit the glycosidase responsible for the glycosylation (fixing sugar to the active site of enzyme), the cell system (microorganisms, *Pichia pastoris* and enzyme) was treated with Tunicamycin.

10.2.3. Polyacrylamide gel electrophoresis (PAGE)

For polyacrylamide gel electrophoresis under denaturing conditions,[118] 12.5% gels were employed. The resolving gel is prepared as follows: 2 ml of lower Tris 4x [Tris-(hydroxymethyl)-aminomethane (36.34 g Tris), 0.8 g SDS (sodium dodecyl sulfate) in 150 ml D_2O, (pH 8.8) is mixed with 6N HCl, then filled up to 200 ml with D_2O] , 3.33 ml 30% (m/v) acrylamide in water, 2.67 ml D_2O, 40 µl APS 10% (m/v) ammoniumpersulphate in D_2O) and 4µl TEMED (N,N,N´,N´,-tetramethyl-ethylenediamine). After 30 min, the stacking gel is prepared as follow: 1 ml Upper Tris 4x (12.11 g Tris, 0.8 g SDS in 150 ml D_2O, pH 6.8 with 6N HCl then filled up to 200 ml of D_2O), 0.52 ml 30% (m/v) acrylamide in water, 2.47 ml D_2O and 40 µl APS 10% (m/v). The addition of 4 µl TEMED initiates the polymerization process and the formation of the required gel.

Staining solution:
Coomassie-Brillant Blue R-250, 0.1 %(w/v), methanol 30% (v/v), acetic acid 10% (v/v)

Destaining solution:
Methanol 30% (v/v), acetic acid 10% (v/v)

Protein standard:
The protein samples (around 10-20 µg of protein) were mixed with SDS loading buffer with a ratio (1:1), denatured for 5 min at 95°C, cooled on ice and then loaded on the gel.
The standard was a mixture of different proteins of well-known molecular weight

Phosphorylase B	97.4 KDa
Serium albumin	66.2 KDa
Ovalbumin	45.0 KDa
Cabonic anhydrase	31.0 KDa
Trypsin inhibitor	21.5 KDa
Lysozyme	14.0 KDa

The proteins were separated at 24mA of current for each gel for 1 hr. The gel was stained for 1 hr with Coomassie solution, then treated for 1-2 hrs with the destaining solution.

10.3. SEM and EDX

Scanning electron micrographs (SEM) and energy dispersive X-ray analysis (EDX) were performed on DSM 962 scanning electron microscope (SEM) (Zeiss, LEO) equipped with a DX-4 X-ray detection system by EDAX. This consists of an energy dispersive Si(Li)-detector with an active area of 10 mm^2 and the EDX software package. Micrographs were recorded detecting secondary electrons generated by a probe current of 168 pA, whereas a 623 pA probe current was applied for carrying out elemental analysis by EDX.

10.4. Chemicals and Enzymes

All chemicals were purchased from Fluka (Switzerland). The microporous polypropylene powder (EP100) [which is now called PP or MP1000] particle size was less than 200 μm and specific surface area was 91.4 m^2/g determined by B.E.T. surface area method and the microporous low density polyethylene powder (LDPE) were gifts from Membrana GmbH/ Accurel system, Obernburg, Germany, Lipase from *Pseudomonas cepacia* (PSL), *Pseudomonas fluorescens,* AK (PFL), *Pseudomonas cepacia* immobilized on ceramic particles (PSL-C), *Pseudomonas cepacia* immobilized on diatomaceous earth (PSL-D), *Aspergillus niger* (ANL) and *Candida rugosa* (AYS) were gifts from Amano (Nagoya, Japan). Lipase AY 30 according to Amano's specification was from Acros Organic (Geel, Belgium). Lipase type II, crude, from *Porcine pancreas* and typeVII from *Candida rugosa* were from Sigma (Steinheim, Germany). Lipase from *Candida cylindracea* (CCL), *Hog pancreas*, lipase immobilized in sol-gel-AK from *Aspergillus niger and* lipase immobilized in sol-gel-AK on sintered glass from *Mucor miehei* were purchased from Fluka (Buchs, Switzerland).
Novozyme 435, an immobilized non-specific lipase (*Candida antarctica B*, CAL-B) produced by submerged fermentation of a genetically modified *Aspergillus oryzae* microorganism and adsorbed on a macroporous resin, Lipozyme RM IM, RML, an immobilized 1,3-specific lipase from *Rhizomucor miehei* produced by submerged fermentation of a genetically modified *Aspergillus oryzae*

microorganism and Novozyme 525 L, the non-immobilized liquid version of Novozym 435, were gifts from Novo Nordisk, Denmark.
a-, *b*- and *g*-Cyclodextrin, permethylated *b*-cyclodextrin (average molar substitution = 1.8), methylated *a*- cyclodextrin (average molar substitution = 1.8) and (3-hydroxypropyl) *g*-cyclodextrin (average molar substitution = 0.6) were obtained from Wacker-Chemie GmbH (Burghausen/Germany). (2-Hydroxypropyl)- *a*-, *b*-, and *g*-cyclodextrin (average molar substitution = 0.6) were purchased from Fluka. The peracetylated *b*-cyclodextrin has been synthesized as described later.

10.5. Synthesis and biochemical transformations

All reactants (alcohol, ester) as well as solvents were stored over activated molecular sieves 4 Å. All racemic esters were synthesized on an analytical scale to optimize a base-line separation by gas chromatography.

10.5.1. General procedure for the synthesis of esters (analytical scale)

0.75 mmol of the alcohol was reacted with 1.05 mmol (75 µl) acetyl chloride in 750 µl pyridine. The reaction was stirred for 24 hrs at room temperature. 2 ml water were added and the extraction was performed (3 times) using 2 ml diethylether. The organic layer was washed with 2 ml 0.1 N HCl followed by 2 ml $NaHCO_3$ solution. The ether phase is washed (3 times) with 2 ml water, then dried with anhydrous sodium sulphate. The solvent was removed under reduced pressure. The residue was purified by column chromatography.

10.5.2. 1-acetoxy-1-(2-furyl)ethane

^{1}H NMR ($CDCl_3$) δ 7.3 (d, 1H), 6.1-6.3 (m, 2H), 5.6 (q, 1H), 1.9 (s, 3H), 1.5 (d, 3H).
^{13}C NMR ($CDCl_3$) δ 171.0, 157.2, 141.2, 110.1, 105.2, 76.1, 21.3, 17.2.

10.5.3. 1-propionoxy-1-(2-furyl)ethane

^{1}H NMR ($CDCl_3$) δ 7.3 (d, 1H), 6.1-6.3 (m, 2H), 5.5 (q, 1H), 1.7 (s, 3H), 2.1 (q, 2H), 1.1 (t, 3H).
^{13}C NMR ($CDCl_3$) δ 172.0, 157.2, 141.2, 110.1, 105.2, 76.1, 19.8, 26.3, 10.1.

10.5.4. 1-butyroxy-1-(2-furyl)ethane

^{1}H NMR ($CDCl_3$) δ 7.3 (d, 1H), 6.1-6.3 (m, 2H), 5.5 (q, 1H), 1.7 (s, 3H), 2.1 (t, 2H), 1.7 (m, 2H), 0.9 (t, 3H).
^{13}C NMR ($CDCl_3$) δ 172.1, 157.3, 141.2, 110.1, 105.3, 76.3, 35.1, 20.1, 18.5, 13.1.

10.5.5. menthyl acetate

^{1}H NMR ($CDCl_3$) δ 3.8 (m, 2H), 2.0 (m, 2H), 2.1 (s, 1H), 1.8 (m, 1H), 1.6 (t, 2H), 1.6 (m, 1H), 1.4 (m, 4H), 1.1 (d, 1H), 1.0 (d, 2* 3H),
^{13}C NMR ($CDCl_3$) δ 171.0, 69.2, 41.2, 36.4, 32.2, 24.5, 23.2, 20.5, 20.3, 19.2.

10.5.6. menthyl propionate

^{1}H NMR ($CDCl_3$) δ 3.8 (m, 2H), 2.3 (q, 3H), 2.0 (m, 1H), 1.8 (m, 1H), 1.7 (t, 2H), 1.6 (m, 1H), 1.4 (m, 4H), 1.1 (d, 1H), 1.1 (d, 2* 3H), 1.0 (t, 3H)
^{13}C NMR ($CDCl_3$) δ 172.0, 69.2, 41.2, 36.4, 32.2, 26.8, 24.5, 23.2, 20.5, 20.3, 19.2, 13.5.

10.5.7. menthyl butyrate

^{1}H NMR ($CDCl_3$) δ 3.8 (m, 2H), 2.3 (q, 3H), 2.0 (m, 1H), 1.8 (m, 1H), 1.7 (m, 2H), 1.6 (t, 2H), 1.5 (m, 1H), 1.4 (m, 4H), 1.1 (d, 1H), 1.1 (d, 2* 3H), 0.9 (t, 3H)
^{13}C NMR ($CDCl_3$) δ 171.0, 69.3, 41.2, 36.4, 35.8, 32.2, 25.8, 23.5, 20.5, 20.1, 19.5, 18.7, 13.4.

10.5.8. 2-acetoxy-4-phenyl-but-3-ene

^{1}H NMR ($CDCl_3$) δ 7.11-7.27 (m, 5H, aromatic), 6.4 (d, 1H), 6.1 (dd, 1H), 4.7 (m, 1H), 2.1 (s, 3H), 1.5 (d, 3H).

^{13}C NMR ($CDCl_3$) δ 170.1, 134.04, 129.64, 129.0, 128.0, 127.4, 75.2, 20.81, 17.1.

10.5.9. General procedure for the lipase-catalyzed transesterification of secondary alcohols (analytical scale)

0.5 mmol racemic alcohol and 1 mmol of acyl donor were dissolved in 3 ml organic solvent in a 5 ml reaction vial. The reaction mixture was thermostated in an oil bath at 40°C. Then, 100 µl of the reaction mixture were withdrawn for GC analysis (t=0 of sample). Afterward, 100 mg of lipase were added. 100 µl samples were taken after several time intervals. The samples were centrifuged to separate lipase. The organic layer was diluted by 100 µl solvent. The reaction progress was monitored qualitatively by thin layer chromatography. An aliquot of the supernatant was used for the GC analysis. When maximum conversion was reached, the reaction was terminated by filtration. The enzyme was washed with solvent and then with acetone. The lipase powder was then dried in air for further use.

10.5.10. General procedure for the lipase-catalyzed esterification of racemic menthol (analytical scale)

100 mg racemic menthol and 83 µl propionic anhydride were dissolved in 10 ml isooctane (2,2,4-trimethyl-pentane) in a 50 v bottle. The reaction mixture was thermostated in an oil bath to 40°C. Then, 100 µl of the reaction mixture were withdrawn for GC analysis (t=0 of sample). Afterward, 1000 units of recombinant lipase (1 g) were added. 100 µl samples were taken after several time intervals. The samples were centrifuged to separate lipase. The organic layer was diluted by 100 µl solvent. The reaction progress was monitored qualitatively by thin layer chromatography. An aliquot of the supernatant was used for the GC analysis. When maximum conversion was reached, the reaction was terminated by filtration. The enzyme was washed with solvent and then with acetone. The lipase powder was then dried in air for further use.

10.5.11. General procedure for the lipase-catalyzed enantioselective hydrolysis of racemic menthyl benzoate (analytical scale)

50 mg CRL Amano (10U/mg) or 500 Units (0.5 g in case of recombinant lipase 1U/mg) were dissolved in 900 μl phosphate buffer (pH 7.2) and added to 10 mg rac. menthyl benzoate (the ester) dissolved in 100 μl isooctane in a 2 ml reaction vial. The reaction mixture was thermostated in a thermoshaker at 40°C. Then, 10 μl of the reaction mixture (organic layer) were withdrawn at several time intervals, diluted by 10 μl solvent and analyzed at GC. The reaction progress was monitored qualitatively by thin layer chromatography. When maximum conversion was reached (calculated by pH stat, according to the amount of benzoic acid released, or from the peak of menthol by GC), the reaction was terminated by filtration. The enzyme was washed with acetone and then dried in air for further use. The unreacted substrate (the ester) and the product (menthol) were separated by flash chromatography over silica using petroleum ether/ethyl acetate 10:1.

10.5.12. General procedure for the recombinant lipase-catalyzed enantioselective hydrolysis of racemic menthyl benzoate (gram scale)

5 g (5000 Units) rec. CRL (1U/mg) were dissolved in 250 ml phosphate buffer (pH 7.2) and added to 2.1 g (8 mmol) rac. menthyl benzoate (the ester) dissolved in 10 ml isooctane in a 500 ml round bottomed flask. The reaction mixture was thermostated in a thermoshaker at 40°C. Then, 10 μl of the reaction mixture (organic layer) were withdrawn at several time intervals, diluted by 10 μl solvent and analyzed at GC. The reaction progress was monitored qualitatively by thin layer chromatography. When maximum conversion was reached (50% after 28 hrs, calculated by pH stat, according to the amount of benzoic acid released, or from the peak of menthol by GC), the reaction was terminated by filtration. The enzyme was washed with acetone and then dried in air for further use. The unreacted substrate (the ester) and the product (menthol) were separated by flash chromatography over silica using petroleum ether/ethyl acetate 10:1 affording a 25% yield of (-)-menthol $[\alpha]_D^{25}$ -53.2 (c 1, CH_2Cl_2) (98.8% ee by GC) and a 30% yield of (+)-menthyl benzoate $[\alpha]_D^{25}$ +87.2 (c 1, CH_2Cl_2).

For menthol: ^{1}H NMR ($CDCl_3$) δ 3.2 (q, 1H), 2.2 (m, 1H), 1.9 (d, 1H), 1.6 (m, 2H), 1.4 (m, 2H), 1.1 (d, 1H), 1.0 (d, 1H), 0.9 (dd, 6H), 0.8 (d, 3H).
^{13}C NMR ($CDCl_3$) δ 67, 46.8, 41.1, 34.4, 31.1, 25.1, 23.5, 22.1, 21.1, 16.4.
For menthyl benzoate: ^{1}H NMR ($CDCl_3$) δ 8.2 (t, 2H), 7.5 (m, 3H), 4.9 (d, 1H), 2.1 (m, 1H), 1.9 (m, 1H), 1.7 (m, 2H), 1.5 (m, 2H), 0.9 (dd, 6H), 0.8 (d, 3H).
^{13}C NMR ($CDCl_3$) δ 166.3, 132.8, 130.9, 129.7, 128.4, 74.9, 46.8, 41.1, 34.4, 31.1, 26.1, 23.5, 22.1, 21.1, 16.4.

10.5.13. General procedure for the lipase-catalyzed enantioselective hydrolysis of esters

100 mg lipase were dissolved in 2.8 ml phosphate buffer (pH 6.0) and added to 0.5 mmol pure substrate (the ester) in a 5 ml reaction vial. The reaction mixture was thermostated in an oil bath at 40^{o}C. Then, 100 µl of the reaction mixture (organic layer) were withdrawn at several time intervals, diluted by 100 µl solvent and analyzed at GC. The reaction progress was monitored qualitatively by thin layer chromatography. When maximum conversion was reached, the reaction was terminated by filtration. The enzyme was washed with acetone and then dried in air for further use. The unreacted substrate (the ester) and the product (the alcohol) were separated by flash chromatography over silica.

10.5.14. General procedure for the immobilization of lipase

The immobilization of lipases on EP 100 or LDPE was performed by the adsorption method. 1.5 g of EP 100 or LDPE were mixed with 5 ml ethanol. One gram of lipase powder was dissolved in 25 ml of potassium phosphate buffer (pH 6, 20mM) and added to the carrier material by stirring overnight. The immobilized lipase was collected by filtration and washed three times with the same buffer, dried and stored at 5^{o}C until use.

10.5.15. Gram-scale enzymatic transesterification of 1-(2-furyl)ethanol using *Pseudomonas cepacia* Lipase (PSL)

56.06 g of **1** (0.5 mol) and 108.82 g of **2** (1 mol) in 200 ml isooctane (2,2,4-trimethyl-pentane) were placed in a 1 L round-bottomed flask equipped with a magnetic stirring bar and thermostated in an oil bath to 40°C. 12 g of *Pseudomonas cepacia* Lipase (PSL) were added in one portion. 30 g of molecular sieves 4 Å were added. The progress of the reaction was monitored by GC. After 28 h, the enzyme was removed by Büchner-filtration and the solvent together with the excess of isopropenyl acetate were distilled off. The residue was distilled under reduced pressure affording a slightly yellowish solution. This was chromatographed on silica gel using *n*-heptane/acetone (2:1). The yield was 75% of (S)-1-(2-furyl)ethanol with > 99% ee (by GC).
^{1}H NMR ($CDCl_3$) δ 7.3 (d, 1H), 6.1-6.3 (m, 2H), 4.8 (q, 1H), 2.1 (br s, OH), 1.5 (d, 3H).
^{13}C NMR ($CDCl_3$) δ 141.90, 110.14, 105.10, 63.64, 21.30.
$[\alpha]_D^{25}$ -23.2 (c 1, EtOH 96%). Literature data $[\alpha]_D^{25}$ -24.4 (neat).[4]
Anal. Calcd for $C_6H_8O_2$: C, 64.29; H, 7.14. Found: C, 64.31; H, 7.10.

10.5.16. Acetal formation

0.217 mol 1-(2-furyl)ethanol was reacted with 0.114 mol acetone in presence of CaI_2. The reaction progress is monitored by GC.

10.5.17. Lipase-catalyzed acetal formation

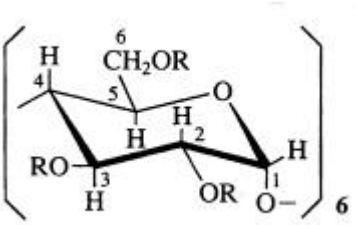
0.217 mol 1-(2-furyl)ethanol was reacted with 0.114 mol acetone in presence of lipase (PSL) dissolved in 3 ml isooctane. The reaction progress is monitored by GC.

10.5.18. General procedure for the synthesis of peracylated cyclodextrins

5 mmol of CD were reacted with 1.2 mol acetic anhydride (113.6 ml) in 227.2 ml of pyridine. The reaction was stirred for 24 hours at room temperature. Water was added and the extraction was performed using ethyl acetate.

The organic layer was washed with 1 N HCl followed by water. Drying was performed using anhydrous sodium sulfate. The solvent was removed under reduced pressure. The residue was purified by column chromatography using CH_2Cl_2/acetone 4:1 (v/v). The products were crystallized with EtOH/diethylether.

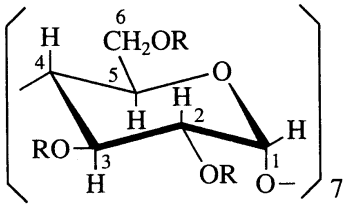
10.5.19. Peracetylated α-cyclodextrin



Peracetylated *a*-CD (R= $COCH_3$)

^{1}H NMR (Benzene d_6) δ 5.8 (dd, 1H, H-3), 5.2 (d, 1H, H-1), 4.9 (t, 1H, H-2), 4.7 (d, 2H, H-6), 4.6 (d, 1H, H-5), 3.7 (t, 1H, H-4), 1.9 (s, 3H, CH_3)
^{13}C NMR (Benzene d_6) δ 170.48 (CO), 170.34 (CO), 169.22 (CO), 96.49 (C-1), 77.21 (C-4), 71.51 (C-3), 71.10 (C-2), 69.98 (C-5), 63.60 (C-6), 20.66 (CH_3), 20.53 (CH_3), 20.47 (CH_3).

10.5.20. Peracetylated β-cyclodextrin

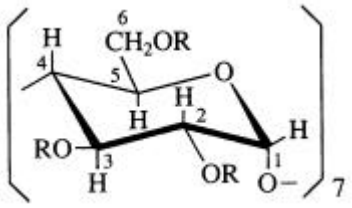


Peracetylated *b*-CD (R= $COCH_3$)

^{1}H NMR (Benzene d_6) δ 5.59 (dd, 1H, H-3), 5.17 (d, 1H, H-1), 4.98 (t, 1H, H-2), 4.79 (d, 1H, H-6a), 4.40 (dd, 1H, H-6b), 4.30 (dd, 1H, H-5), 3.72 (t, 1H, H-4), 1.93 (s, 3H, CH_3)
^{13}C NMR (Benzene d_6) δ 170.44 (CO), 170.31 (CO), 169.24 (CO), 97.48 (C-1), 77.53 (C-4), 71.23 (C-3), 70.69 (C-2), 70.33 (C-5), 63.11 (C-6), 20.62 (CH_3), 20.54 (CH_3), 20.47 (CH_3).

Yield: 75%; m.p: 201; $[\alpha]_D$ 125 ($CHCl_3$); MS (positive FAM, 0.1% in methanol + 0.1% acetic acid) m(nominal mass)/z 2017 $[M+H]^+$; *Anal*. Calcd. for $C_{84}H_{112}O_{56}$: C, 49.97; H, 5.55. Found: C, 49.80; H, 5.20.

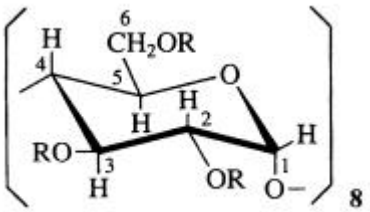
10.5.21. Perbutyrated β-cyclodextrin



Perbutyrated *b*-CD (R= $COCH_2CH_2CH_3$)

^{1}H NMR ($CDCl_3$) δ 5.24 (dd, 1H, H-3), 4.96 (d, 1H, H-1), 4.7 (dd, 1H, H-2), 4.30 (d, 1H, H-6a), 4.24 (m, 1H, H-6b), 4.06 (t, 1H, H-5), 3.65 (t, 1H, H-4), 2.10 (t, 2H, CH_2), 1.69 (m, 2H, CH_2), 1.53 (t, 3H, CH_3)
^{13}C NMR ($CDCl_3$) δ 173.19 (CO), 172.75 (CO), 171.59 (CO), 96.53 (C-1), 77.1 (C-4), 70.13 (C-3), 70.27 (C-2), 69.55 (C-5), 62.25 (C-6), 35.85 (CH_2), 35.73 (CH_2), 35.63 (CH_2), 18.30 (CH_2), 18.08 (CH_2), 18.05 (CH_2), 13.55 (CH_3), 13.48 (CH_3), 13.42 (CH_3).

10.5.22. Peracetylated γ-cyclodextrin



Peracetylated *g*-CD (R= $COCH_3$)

^{1}H NMR (DMSO d_6) δ 5.34 (dd, 1H, H-3), 5.03 (d, 1H, H-1), 4.7 (dd, 1H, H-2), 4.2 (s,1H, H-6a), 3.9 (d, 1H, H-6b), 3.89 (t, 1H, H-4), 1.99 (s, 3H, CH_3)
^{13}C NMR (DMSO d_6) δ 170.09 (CO), 169.98 (CO), 169.17 (CO), 97.28 (C-1), 77.41 (C-4), 70.44 (C-3), 69.96 (C-2), 69.35 (C-5), 62.68 (C-6), 20.63 (CH_3), 20.50 (CH_3), 20.45 (CH_3).

10.5.23. Co-lyophilization of PSL with cyclodextrin derivatives

The *Pseudomonas cepacia* lipase (PSL) was dissolved (2 mg/1µl) in 20 mM phosphate buffer (pH 6.0) and lyophilized for 48 h (control). The co-lyophilization of lipase with CD derivatives was performed by the same method, except that the CD was added prior to lyophilization in the ratio 1:1; 1:2; 1:6 weight ratio of lipase to cyclodextrin. After lyophilization, the enzyme was dried under vacuum and stored for further use.

10.5.24. Co-lyophilized lipase with peracetylated β-CD catalyzed transesterification of 1-(2-furyl)ethanol

All reactants (alcohol, ester) were stored over activated molecular sieves 4 Å.
0.5 mmol racemic 1-(2-furyl)ethanol and 1 mmol isopropenyl acetate were dissolved in 3 ml organic solvent in a 5 ml reaction vial. The reaction mixture was thermostated in an oil bath to 40°C. Then, 100 µl of the reaction mixture were withdrawn for GC analysis (t=0 of sample). Afterward, 100 mg of lyophilized lipase or co-lyophilized one were added. 100 µl samples were taken after several time intervals. The samples were centrifuged to separate lipase. The organic layer was diluted by 100 µl solvent. The reaction progress was monitored qualitatively by thin layer chromatography. An aliquot of the supernatant was used for the GC analysis. When maximum conversion was reached, the reaction was terminated by filtration. The enzyme was washed with solvent and then with acetone. The lipase powder was then dried in air for further use.

10.5.25. Synthesis of (R,S)-*trans*-4-phenyl-3-butene-2-ol (R,S)-1

2.04 g (0.014 mol) of *trans*-4-phenyl-3-butene-2-one were dissolved in 40 ml methanol and stirred in a 100 ml round bottomed flask placed in an ice bath. 0.56 g (0.015 mol) of $NaBH_4$ dissolved in 40 ml water were added dropwise through 1h, then the reaction was stirred for 3h in an ice bath and 2h at room temperature. The volume was concentrated to 30 ml. 20 ml of hot water were added and the reaction mixture was filtrated. The residue was washed several times with 20 ml of hot water and extracted twice using *n*-hexane/dichloromethane (1:4 v/v). The organic layer was separated

and the solvent was removed under reduced pressure yielding 1.9 g of a colorless oil, yield: 93%.
^{1}H NMR ($CDCl_3$) δ 7.11-7.27 (m, 5H, aromatic), 6.39 (d, 1H), 6.09 (dd, 1H), 4.31 (m, 1H), 2.39 (s, 1H, OH), 1.23 (d, 3H, CH_3).
^{13}C NMR ($CDCl_3$) δ 137.15, 134.04, 129.64, 129.0, 128.0, 127.4, 69.20, 23.81.

10.5.26. Synthesis of (R,S)-2-acetoxy-4-phenyl-but-3-ene (gram-scale)

14.8 g (0.1 mol) of (**1**) were reacted with 17 ml (0.18 mol) acetic anhydride in 65 ml pyridine. The reaction was stirred for 24 h at room temperature. Water was added and the extraction was performed using ethyl acetate. The organic layer was washed with 1N HCl followed by water. Drying was performed using anhydrous sodium sulphate. The solvent was removed under reduced pressure. The residue was purified by column chromatography using *n*-hexane/ethylacetate (9:1 v/v) affording 8.8 g of 2-acetoxy-4-phenyl-but-3-ene, yield: 60%.

10.5.27. General procedure for the lipase-catalyzed enantioselective transesterification of (R,S)-*trans*-4-phenyl-3-butene-2-ol (analytical scale)

74 mg (0.5 mmol) of (R,S)-*trans*-4-phenyl-3-butene-2-ol and 108.8 mg (1 mmol) isopropenyl acetate were dissolved in 3 ml organic solvent in a 5 ml reaction vial. The reaction mixture was thermostated in an oil bath at 40°C. Then, a 100 µl sample of the reaction mixture were withdrawn and derivatized with 10 µl isopropylisocyanate at 100°C for 30 min., diluted with toluene and analyzed at GC, (t=0 of sample). Afterwards, 100 mg of lipase were added followed by the addition of 100 mg molecular sieves 4 Å. 100 µl samples were taken after several time intervals. The samples were centrifuged to separate lipase. The organic layer was treated with isopropylisocyanate, heated to 100°C for 30 min then diluted by 100 µl toluene and analyzed by GC. The reaction progress was monitored qualitatively by thin layer chromatography using *n*-hexane/ethyl acetate (9:1 v/v) as eluent. An aliquot of the supernatant was used for the GC analysis. When maximum conversion was reached, the reaction was terminated by filtration. The enzyme was washed with acetone and then dried in air for further use. The substrate *trans*-4-

phenyl-3-butene-2-ol and product 2-acetoxy-4-phenyl-but-3-ene were separated by flash chromatography over silica (*n*-hexane/ethyl acetate 9:1).

10.5.28. General procedure for the lipase-catalyzed enantioselective hydrolysis of 2-acetoxy-4-phenyl-but-3-ene (analytical scale)

100 mg lipase were dissolved in 2.8 ml phosphate buffer (pH 6.0) and added to 0.5 mmol pure substrate (R,S)-2-acetoxy-4-phenyl-but-3-ene in a 5 ml reaction vial. The reaction mixture was thermostated in an oil bath at 40°C. Then, 100 µl of the reaction mixture (organic layer) were withdrawn at several time intervals, derivatized with isopropylisocyanate, heated to 100°C for 30 min, then diluted by 100 µl toluene and analyzed at GC. The reaction progress was monitored qualitatively by thin layer chromatography (*n*-hexane/ethyl acetate 9:1). When maximum conversion was reached, the reaction was terminated by filtration. The enzyme was washed with acetone and then dried in air for further use. The unreacted acetate (S)-2-acetoxy-4-phenyl-but-3-ene and the product (R)-*trans*-4-phenyl-3-butene-2-ol were separated by flash chromatography over silica (*n*-hexane/ethyl acetate 9:1).

10.5.29. General procedure for the gram-scale lipase-catalyzed production of enantiomerically pure (S)-*trans*-4-phenyl-3-butene-2-ol

8.8 g (0.06 mol) racemic alcohol (R,S)-*trans*-4-phenyl-3-butene-2-ol and 24 g (0.24 mol) isopropenyl acetate in 500 ml toluene were placed in a 1 L round-bottomed flask equipped with magnetic stirring bar and thermostated in an oil bath at 40°C. Then, a 100 µl sample of the reaction mixture were withdrawn and derivatized with isopropylisocyanate for GC analysis (t=0). 3.08 g lipase (PSL-D) (0.35 mass equivalent) were added in one portion followed by the addition of 5 g molecular sieves 4 Å. The progress of the reaction was monitored by thin layer chromatography (*n*-hexane/ethyl acetate 9:1) and GC. After 4 hrs the reaction reached 50 % conversion, the enzyme was separated by Büchner-filtration and the solution was concentrated under reduced pressure. The residue was chromatographed on silica using *n*-hexane/ethyl acetate 9:1 affording (S)-*trans*-4-phenyl-3-butene-2-ol (>99% ee by GC) $[\alpha]_D^{20}$ -19.9 (c 1,

CH_2Cl_2) [lit. $[\alpha]_D^{20}$ -24.5 (c 5.16, $CHCl_3$), 98% ee][18], yield 47% and (R)-2-acetoxy-4-phenyl-but-3-ene (87% ee) $[\alpha]_D^{20}$ +74.2 (c 1, CH_2Cl_2), yield 49%.

10.5.30. General procedure for the gram-scale lipase-catalyzed production of enantiomerically pure (R)-*trans*-4-phenyl-3-butene-2-ol

3.15 g lipase (Novozyme) were dissolved in 250 ml phosphate buffer (pH 6) and added to a solution of 7.8 g (40.0 mmol) of (R,S)-2-acetoxy-4-phenyl-but-3-ene dissolved in 20 ml toluene at 40°C. Samples were taken at several time intervals, the organic layer was derivatized with isopropylisocyanate as described before and analyzed by GC. When maximum conversion was reached (44% after 24 hrs), enzyme is filtered and the organic layer is separated and concentrated under vacuum. Substrate (S)-2-acetoxy-4-phenyl-but-3-ene and product (R)-*trans*-4-phenyl-3-butene-2-ol were separated by column chromatography (*n*-hexane/ethyl acetate 9:1) affording the (R)-*trans*-4-phenyl-3-butene-2-ol (>99% ee by GC) $[\alpha]_D^{20}$ + 19.9, yield 43% and (S)-2-acetoxy-4-phenyl-but-3-ene (74.5% ee) $[\alpha]_D^{20}$ -71.2, yield: 46%.

10.5.31. General procedure for the catalytic study of ruthenium complexes (cooperation with Prof. E. Lindner group)

The respective diamine-bis(ether-phosphine)ruthenium(II) complex (R,R) or (S,S) (0.026 mmol) was placed in a 150 ml Schlenk tube and solid KOH (0.26 mmol) was added as a cocatalyst. The solid mixture was stirred and warmed during the evacuation process to remove oxygen and water. Subsequently the Schlenk tube was filled with argon and 20 ml of 2-propanol were added. The mixture was vigorously stirred, degassed by two freeze-thaw cycles, and then sonicated for 20-40 min (this is important to remove any excess oxygen and to complete the dissolving of the catalyst and cocatalyst). A solution of (secondary alcohol) *trans*-4-phenyl-3-butene-2-one (26 mmol) in 60 ml of 2-propanol were subjected to a freeze-thaw cycle in a different 150 ml Schlenk tube and were added to the catalyst solution. Finally the reaction mixture was transferred to a pressure Schlenk tube which was pressurized with dihydrogen of 1-4 bar. The reaction mixture was vigorously stirred at 35°C for 1 hour. During the

reduction process, samples were taken from the reaction mixture and analyzed by GC.

10.5.32. General procedure for the lipase-catalyzed enantioselective transesterification of the enantiomerically enriched *trans*-4-phenyl-3-butene-2-ol (analytical scale)

74 mg (0.5 mmol) of the enantiomerically enriched alcohol [(S); 45% ee] (resulting from the ruthenium-(R,R)-diamine complex)-catalyzed enantioselective reduction of *trans*-4-phenyl-3-butene-2-one **4**] and 108.8 mg (1 mmol) isopropenyl acetate were dissolved in 3 ml organic solvent in a 5 ml reaction vial. The reaction mixture was thermostated in an oil bath to 40°C. Then, a 100 µl sample of the reaction mixture were withdrawn and derivatized with isopropylisocyanate for GC analysis (t=0 of sample). Afterward, 100 mg of lipase were added. 100 µl samples were taken after several time intervals. The samples were centrifuged to separate lipase. The organic layer was derivatized with isopropylisocyanate and diluted by 100 µl toluene. The reaction progress was monitored qualitatively by thin layer chromatography (*n*-hexane/ethyl acetate 9:1). An aliquot of the supernatant was used for the GC analysis. When maximum conversion was reached, the reaction was terminated by filtration. The enzyme was washed with solvent and then with acetone. Substrate (S)- and product (R)-2-acetoxy-4-phenyl-but-3-ene were separated by flash chromatography over silica gel (*n*-hexane/ethyl acetate 9:1)

10.5.33. General procedure for the lipase-catalyzed enantioselective hydrolysis of the enantiomerically enriched 2-acetoxy-4-phenyl-but-3-ene (analytical scale)

100 mg enzyme were dissolved in 2.8 ml phosphate buffer (pH 6.0) and added to 0.5 mmol of the enantiomerically enriched acetate (R)-**5** (45% ee) [resulting from the ruthenium-(S,S)-diamine complex-catalyzed enantioselective reduction of trans-4-phenyl-3-butene-2-one **4**] in a 5 ml reaction vial. The reaction mixture was thermostated in an oil bath to 40°C. Then, 100 µl of the reaction mixture (organic layer) were withdrawn at several time intervals, derivatized with isoproplyisocyanate, diluted by 100 µl solvent and analyzed at GC. The reaction progress was monitored qualitatively by thin layer

chromatography (*n*-hexane/ethyl acetate 9:1). When maximum conversion was reached, the reaction was terminated by filtration. The enzyme was washed with solvent and then with acetone. The lipase powder was then dried in air for further use and the unreacted acetate (S)-2-acetoxy-4-phenyl-but-3-ene and the alcohol (R)-*trans*-4-phenyl-3-butene-2-ol were separated by flash chromatography over silica (*n*-hexane/ethyl acetate 9:1).

10.5.34. General procedure for the gram-scale ruthenium-catalyzed production of enantiomerically enriched *trans*-4-phenyl-3-butene-2-ol. (cooperation with Prof. E. Lindner group)

The complex (R,R) or (S,S) (0.018 mmol) was placed in a 150 ml Schlenk tube and solid KOH (0.36 mmol) was added as a cocatalyst. The solid mixture was stirred and warmed during the evacuation process to remove oxygen and water. Subsequently the Schlenk tube was filled with argon and 20 ml of 2-propanol were added. The mixture was vigorously stirred, degassed by two freeze-thaw cycles, and then sonicated for 20-40 min (this is important to complete the dissolving of the catalyst and cocatalyst). A solution of *trans*-4-phenyl-3-butene-2-one (72 mmol) in 60 ml of 2-propanol [Ru: KOH: Sub][1: 20: 4000], was subjected to a freeze-thaw cycle in a different 150 ml Schlenk tube and was added to the catalyst solution. Finally the reaction mixture was transferred to a pressure Schlenk tube which was pressurized with dihydrogen of 2.5 bar. The reaction mixture was vigorously stirred at 40^{o}C for 3h. During the hydrogenation process samples were taken from the reaction mixture to control the conversion and turnover frequency. The samples were inserted by a special glass syringe into a gas chromatograph and the kind of the reaction products was compared with authentic samples.

10.5.35. General procedure for the gram-scale lipase-catalyzed production of enantiomerically pure (S)-*trans*-4-phenyl-3-butene-2-ol

8.8 g (0.06 M) of the enantiomerically enriched alcohol [(S); 45% ee] (resulting from the ruthenium-(R,R)-diamine complex)-catalyzed enantiomeric reduction of *trans*-4-phenyl-3-butene-2-one 4] and 24 g isopropenyl acetate (0.24 M) in 500 ml toluene were placed in a 1 L round-bottomed flask equipped with a magnetic stirring bar and thermostated in an oil bath to 40^{o}C. Then, a 100 μl sample of the

reaction mixture were withdrawn and derivatized with isoproplyisocyanate for GC analysis (t=0). 3.08 g lipase (PSL-D) (0.35 mass equivalent) were added in one portion. 5 g molecular sieves 4Å were added to scavenge the resulting acetone. The progress of the reaction was monitored by GC. After 2 hrs the reaction reached 50 % conversion, enzyme was filtered by Büchner-filtration and the solution was concentrated under reduced pressure. The residue was chromatographed on silica gel using *n*-hexane/ethyl acetate 9:1 affording (S)-trans-4-phenyl-3-butene-2-ol (>99% ee) $[\alpha]_\lambda^{20} = -19.9^0$ (c 1, CH_2Cl_2) [lit. $[\alpha]_\lambda^{20} = -24.5^0$ (c 5.16, $CHCl_3$), 98% ee], yield: 47% and (R)-2-acetoxy-4-phenyl-but-3-ene (87% ee) $[\alpha]_\lambda^{20} = +74.2^0$ (c 1, CH_2Cl_2), yield: 49%.

10.5.36. General procedure for the gram-scale lipase-catalyzed production of enantiomerically pure (R)-*trans*-4-phenyl-3-butene-2-ol.

3.15 g lipase (Novozyme) were dissolved in phosphate buffer (pH 6) (250 ml) and added to a solution of 7.8 g (40.0 mmol) of the enantiomerically enriched secondary alcohol (R) (45% ee) (resulting from the ruthenium-(S,S)-diamine complex)-catalyzed enantioselective reduction of *trans*-4-phenyl-3-butene-2-one dissolved in 20 ml toluene at 40°C. Samples were taken at several times interval, the organic layer was derivatized with isopropylisocyanate as described before and analyzed by GC. When maximum conversion is reached (44% after 24 hrs), enzyme is filtered and the organic layer is separated and concentrated under vacuum. Substrate (S)-2-acetoxy-4-phenyl-but-3-ene and product (R)-*trans*-4-phenyl-3-butene-2-ol were separated by column chromatography (*n*-hexane/ethyl acetate 9:1) affording the (R)-*trans*-4-phenyl-3-butene-2-ol (>99% ee) $[\alpha]_D^{20}$ + 19.9, yield: 43% and (S)-2-acetoxy-4-phenyl-but-3-ene (74.5% ee) $[\alpha]_D^{20}$ -71.2 (c 1, CH_2Cl_2), yield: 46%.

10.5.37. Co-lyophilized lipase with cyclodextrin-containing gel

The *Pseudomonas cepacia* lipase (PSL) was dissolved (2 mg/1µl) in 20 mM phosphate buffer (pH 6) and lyophilized for 48 h (control). The co-lyophilization of lipase with peracetylated *b*-cyclodextrin was performed by the same method, except that the CD was added prior to lyophilization in the ratio 1:1; 1:2; 1:6 weight ratio of lipase to cyclodextrin. The co-lyophilized lipase with peracetylated *b*-

cyclodextrin was added into a flask and stirred in 4 ml phosphate buffer (pH 6) for 5 min to generate a homogeneous lipase/CD suspension. To this mixture 400 µl of 1 M NaF solution, 800 µl gelatin (4% W/W) and 628.2 (5 mmol) MTMS were added. The two-phase mixtures were vigorously shaken by hand until the evolution of heat commences (1-3 min). The milky emulsion clears up before solidifying rather rapidly with the formation of white solid material. It is cooled down in an ice bath for 10 min and left in a closed vessel for 24 hrs at r.t. The material is dried in an oven at 40^{o}C for 3 days under atmospheric pressure. The resulting solid is grounded in a mortar and shaken with 16 ml buffer (pH 6) for 2 hrs. The product is collected on a glass frit (D4), washed with buffer 20 ml and 20 ml *n*-pentane. The gel is then dried at 40^{o}C for 24 hrs, ground and kept for further use.

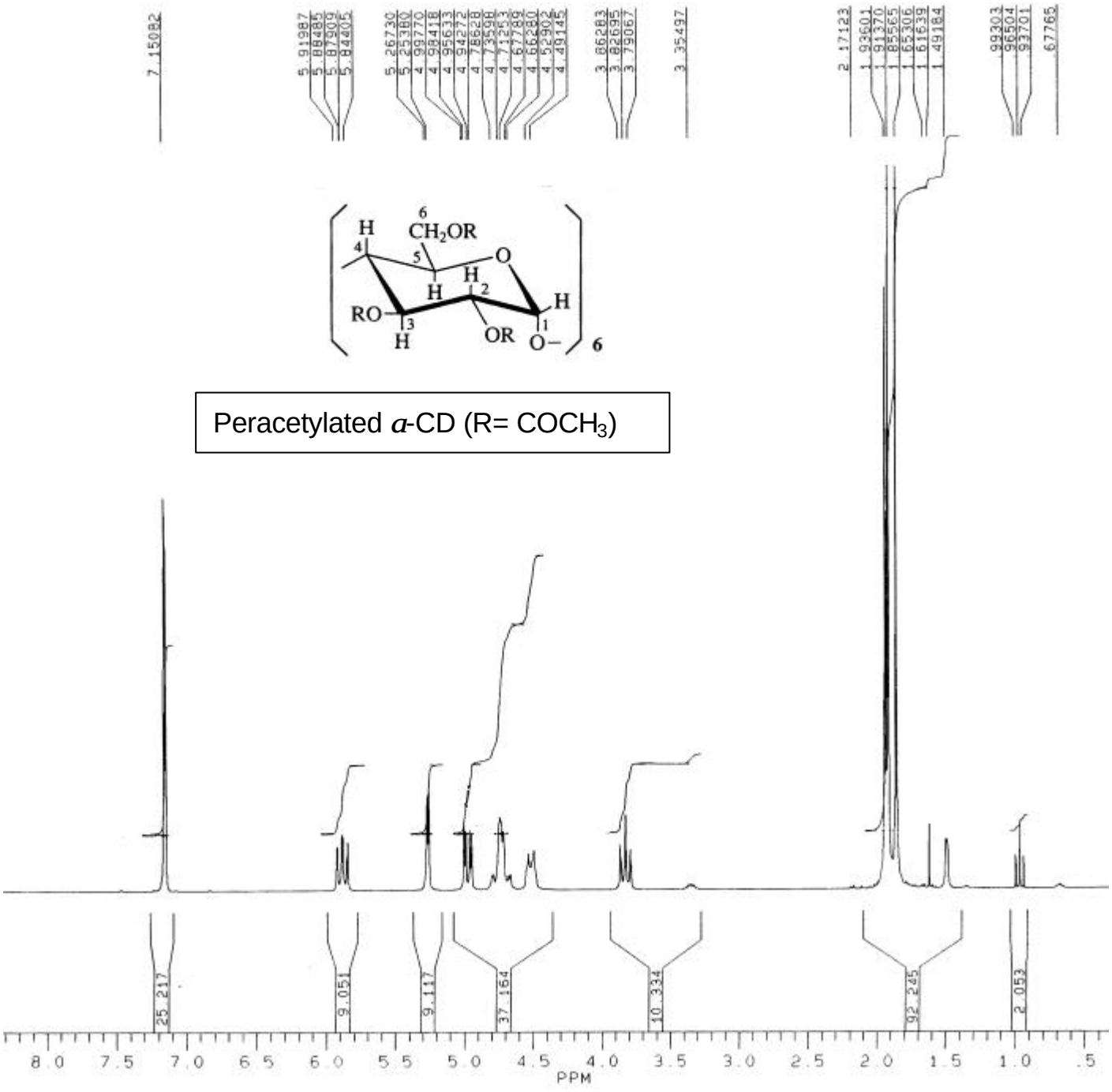


Fig. 10.2: ^{1}H-NMR-spectrum of peracetylated α-cyclodextrin.

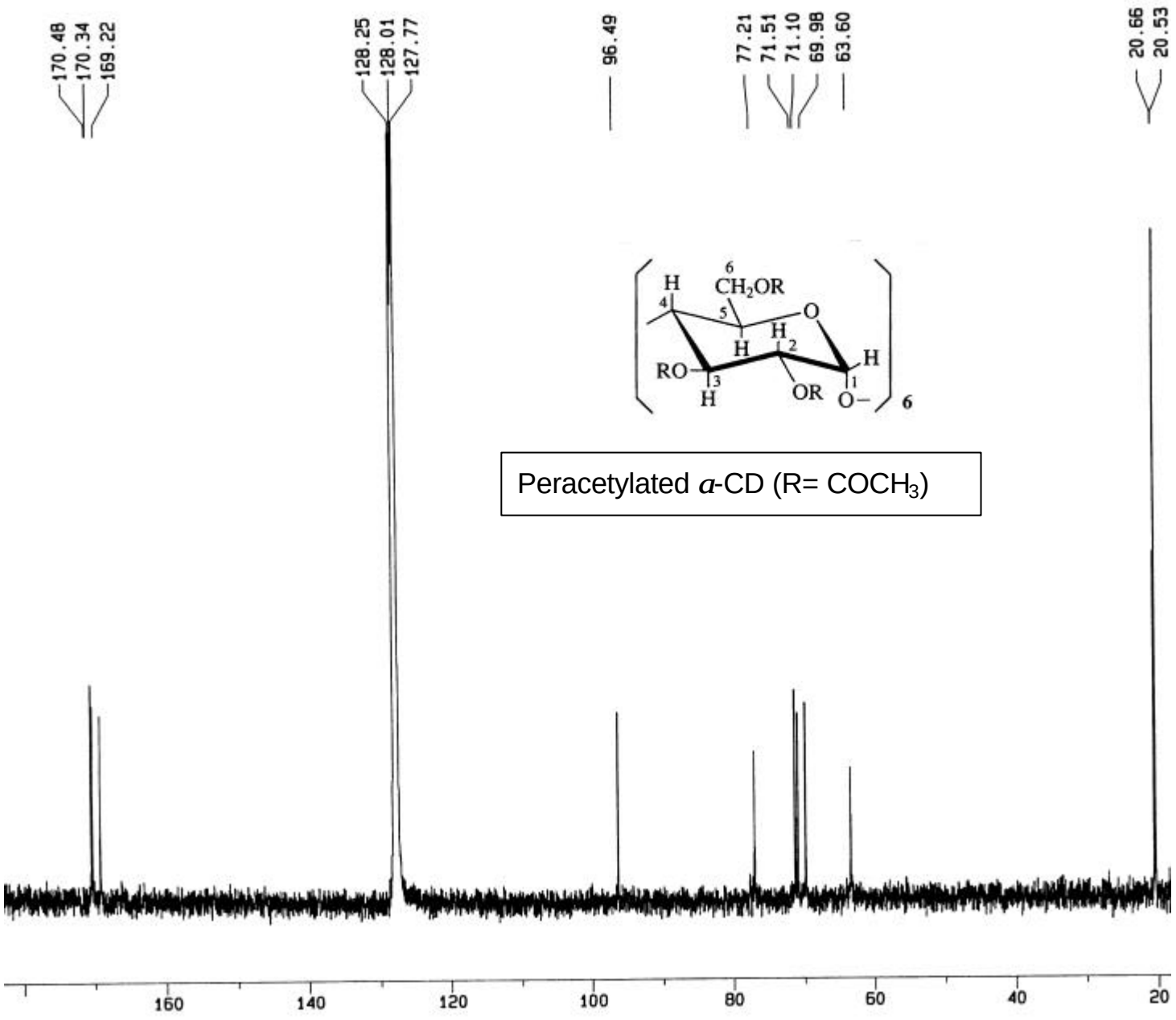


Fig. 10.3: ^{13}C-NMR-spectrum of peracetylated *α*-cyclodextrin.

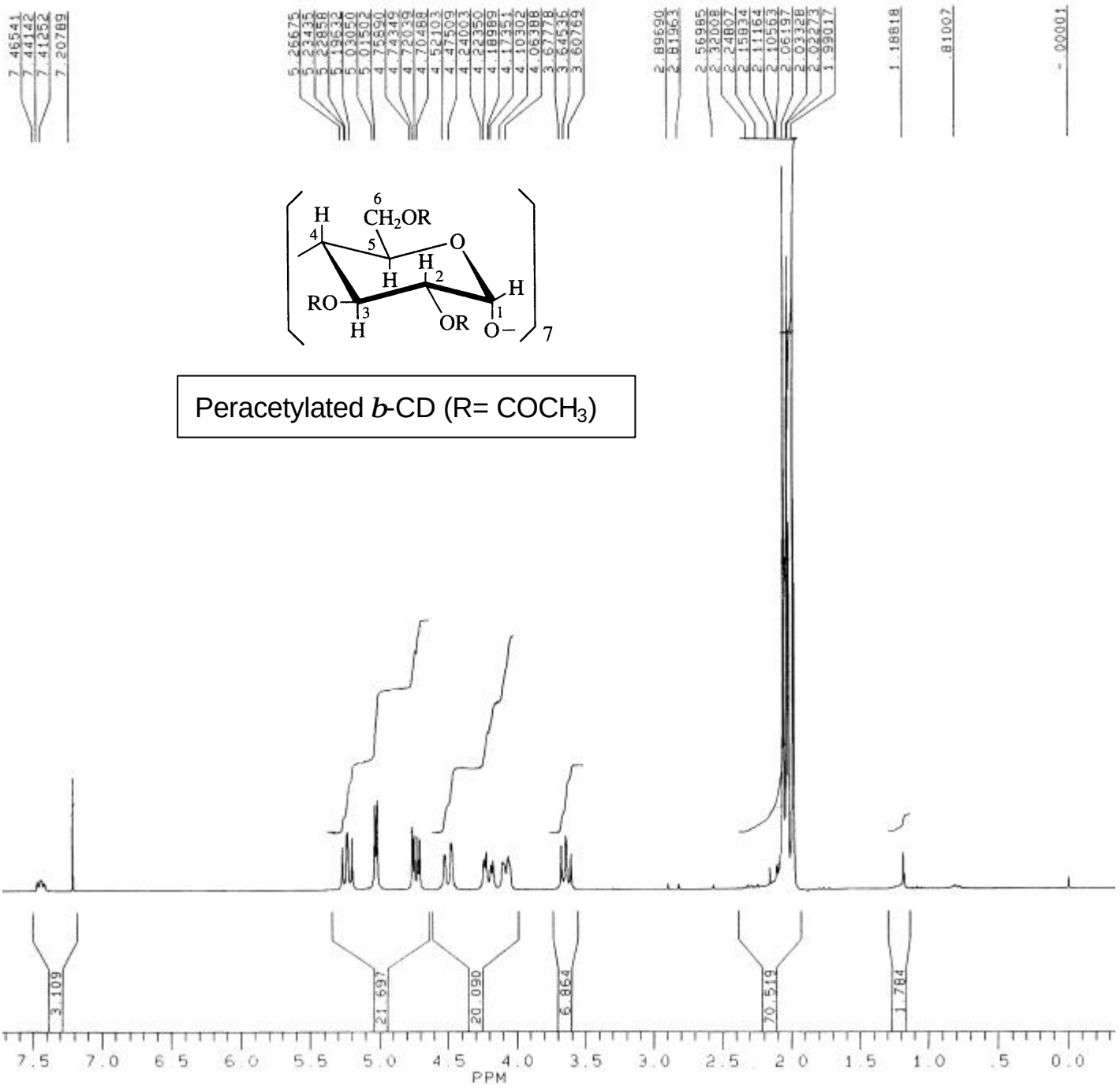


Fig. 10.4: ^{1}H-NMR-spectrum of peracetylated *b*-cyclodextrin.

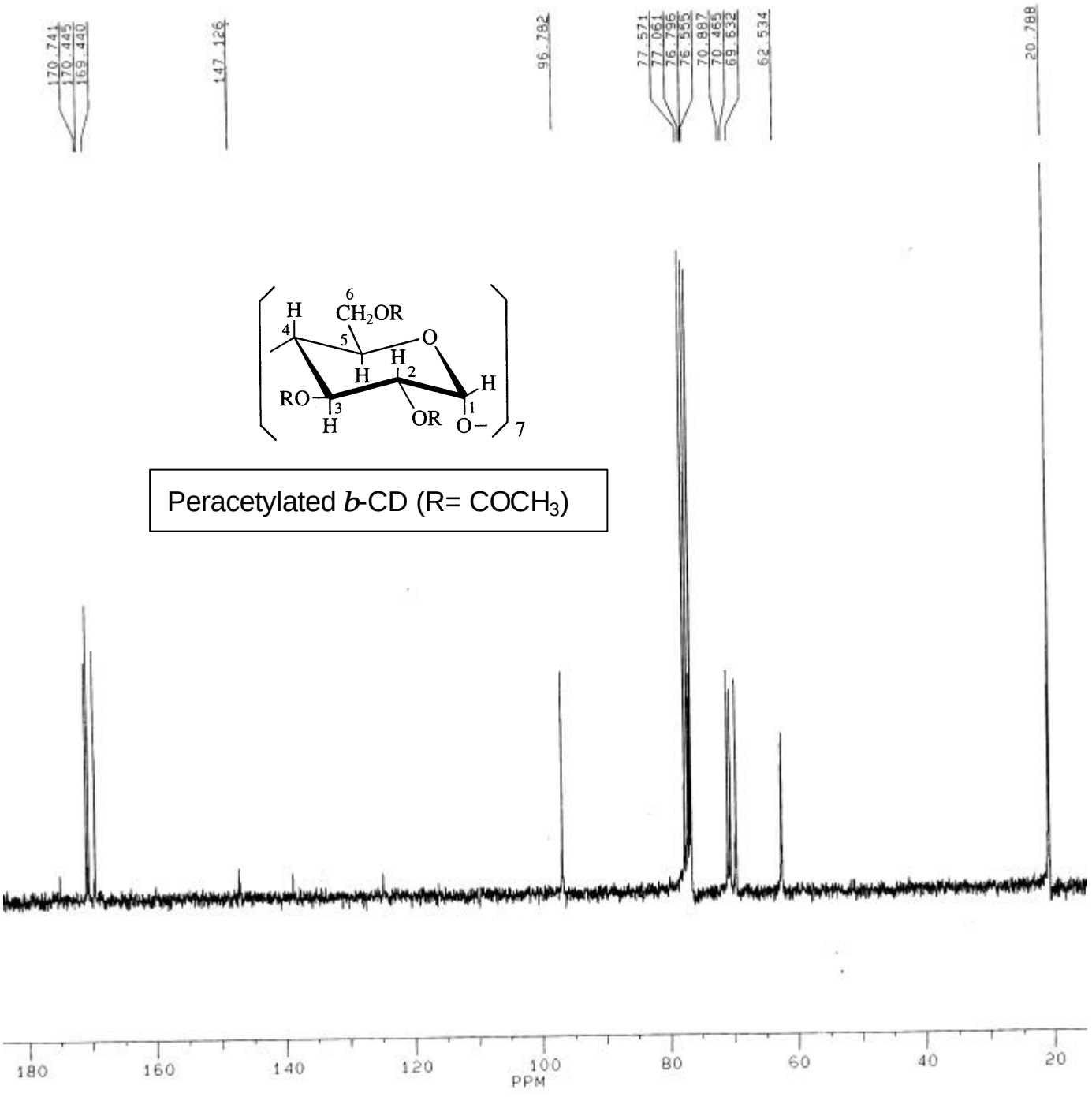


Fig. 10.5: ^{13}C-NMR-spectrum of peracetylated *b*-cyclodextrin.

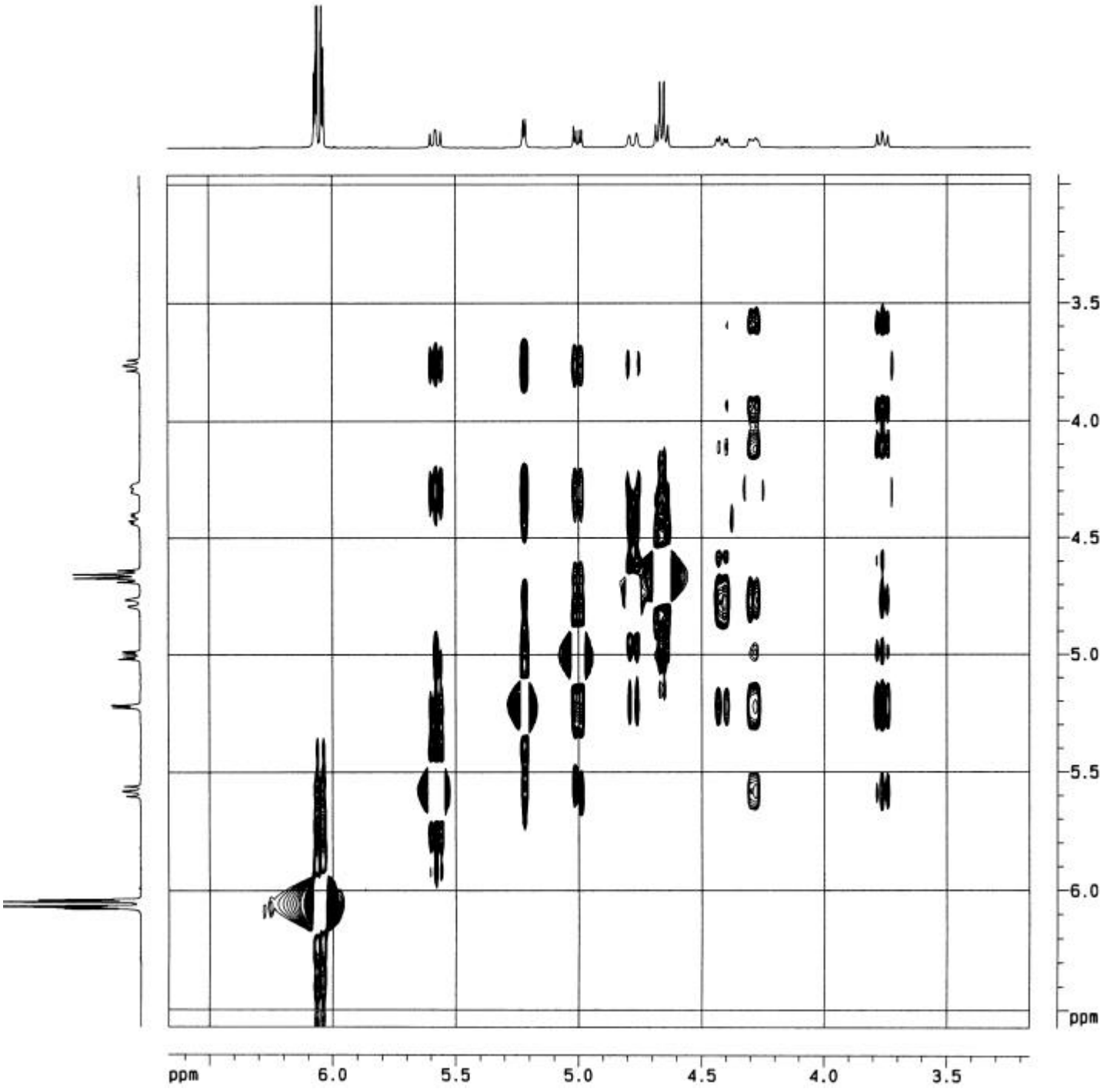


Fig. 10.6: Two-dimensional $^{1}H/^{1}H$ Roesy spectrum of peracetylated *b*-cyclodextrin containing 1(2-furyl)ethanol (1:1).

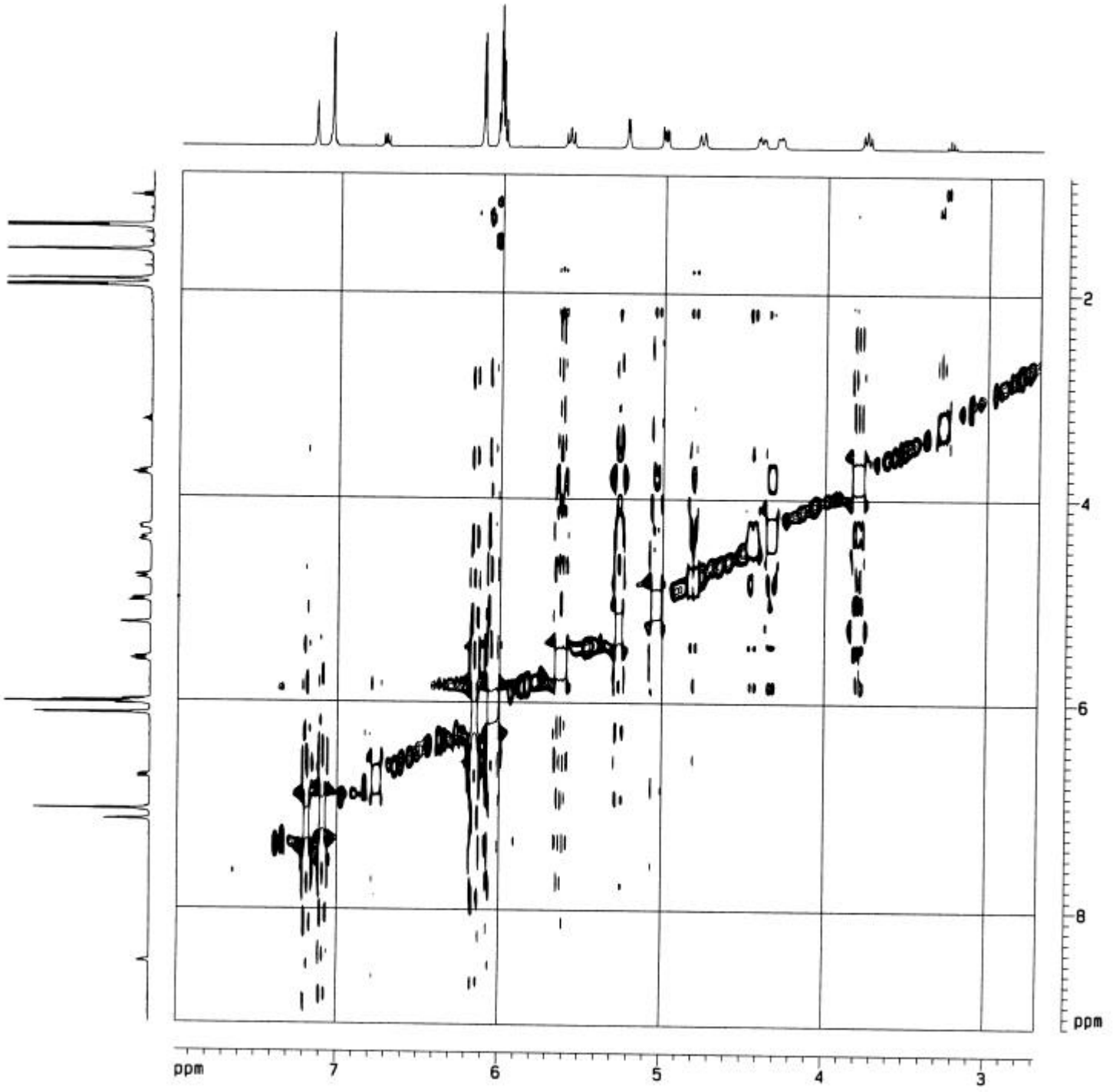


Fig. 10.7: Two-dimensional $^{1}H/^{1}H$ Roesy spectrum of peracetylated *b*-cyclodextrin containing 1-acetoxy-1-[2-furyl]ethane (1:1).

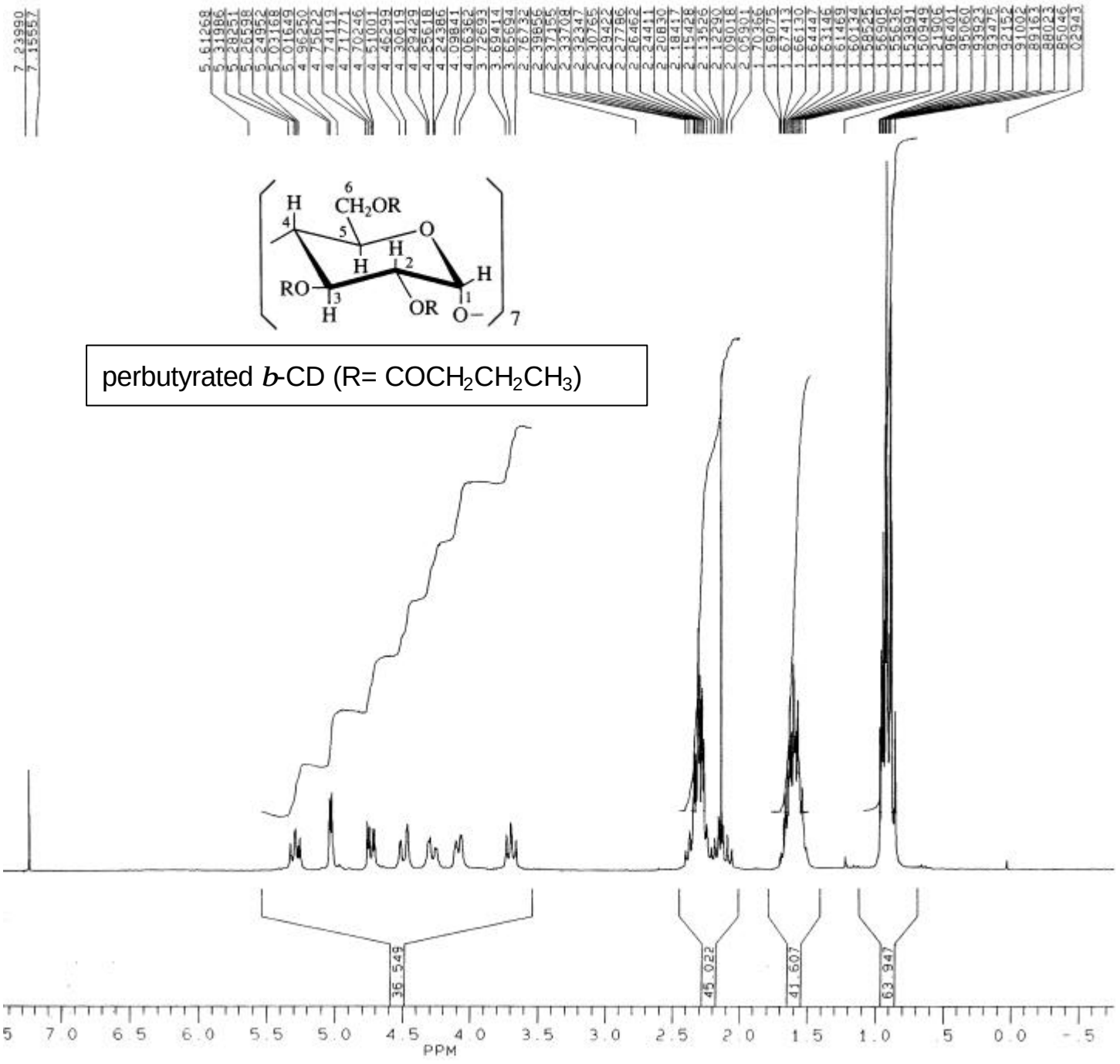


Fig. 10.8: ^{1}H-NMR-spectrum of perbutyrated *b*-cyclodextrin.

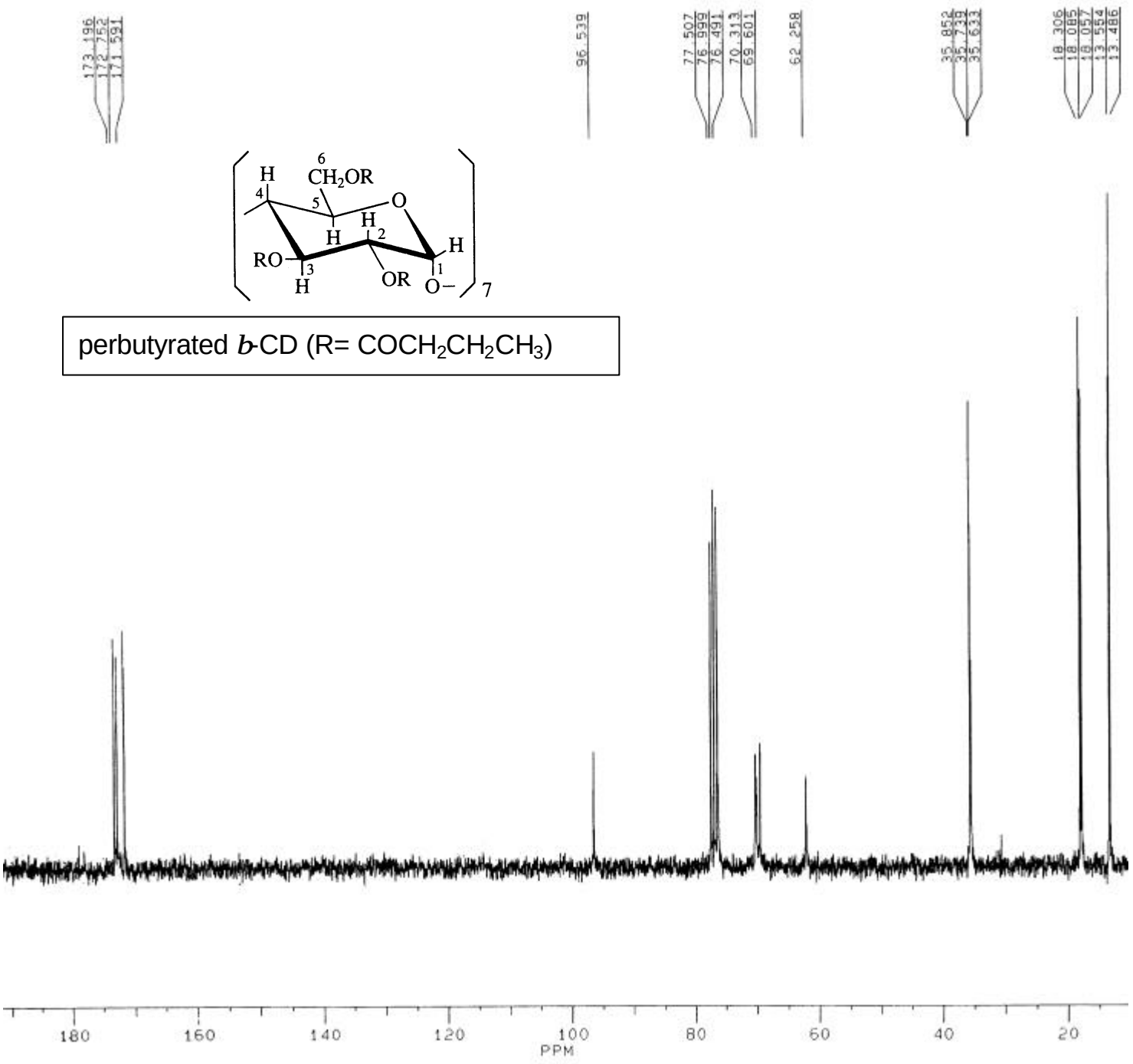


Fig. 10.9: ^{13}C-NMR-spectrum of perbutyrated *b*-cyclodextrin.

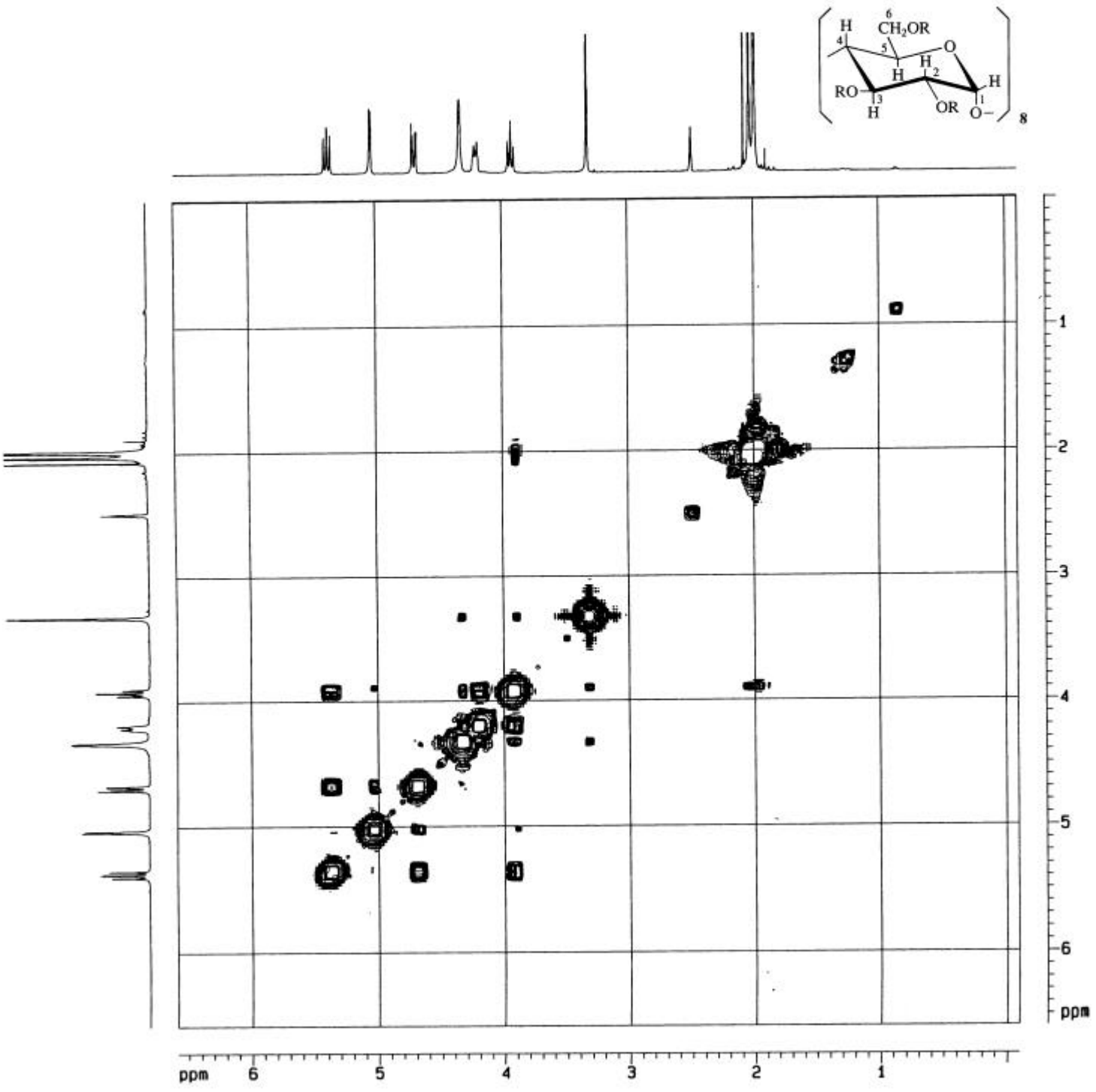


Fig. 10.10: Two-dimensional $^{1}H/^{1}H$ cosy spectrum of peracetylated *g*-cyclodextrin.

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