LIPASES IN BIOTRANSFORMATIONS: RESOLUTION OF SOME NOVEL ALCOHOL AND DIOL SUBSTRATES *VIA* SELECTIVE HYDROLYSIS OF THEIR ESTERS AND RELATED MECHANISTIC STUDIES

THESIS

submitted to the

UNIVERSITY OF PUNE

for the degree of

DOCTOR OF PHILOSOPHY

in

BIOTECHNOLOGY

By

SHRIVALLABH B. DESAI

Division of Organic Chemistry: Synthesis

National Chemical Laboratory

Pune-411 008

Dedicated to my parents

ACKNOWLEDGEMENTS

It gives me great pleasure to express my deep sense of gratitude to Dr. K. N. Ganesh for introducing me to the fascinating area of bioorganic chemistry and his astute guidance. His receptive attitude, untiring enthusiasm and positive approach will always remain a source of inspiration.

I am greatly thankful to Dr. N. P. Argade for his scientific guidance, stimulating suggestions and encouragement related to this work. I am indebted for his kind care and concern in the lab.

My sincere thanks to Dr. V. Shankar and Dr. (Mrs.) V. A. Kumar for valuable scientific discussions, during the course of this work. I would like to thank Dr. A. A. Natu, Mrs. A. D. Gunjal, Dr. A. P. Likhite, Dr. S. P. Mayabhate, Dr. S. R. Deshpande for their kind help and encouragement.

My special thanks to Drs. Gopal, Rajeev, D. A. Barawkar, Vasant, Vip ul and Mr. Swami for helping me in various ways during the course of this work.

I am thankful to Prof. Dr. D. Basavaiah University of Hyderabad for the generous gift of PLAP and BLAP, I am grateful to Amano Pharmaceuticals (Japan) for the generous gifts of AmanoPS and AY30. I also thank Dr. Vairamani, I. I. C. T., Hyderabad for providing the FAB mass spectra.

My thanks are also due to Mr. A. G. Samuel, Dr. G. J. Sanjayan, Mrs. U. D. Phalgune and Mr. V. T. Sathe for their help in recording the NMR spectra and the library staff for excellent facilities.

I am grateful to Drs. R. R. Joshi, B. P. Gangamani, Dimpan Bhatia and. Leena for their encouragement.

I take this opportunity to thank my colleagues, Pradeep, Ramesh, Moonesha, Meena, Anil, Anand, Nagamani, Santosh, Revaiah, Raman and Pallavi for helping me in various capacities throughout my work.

I am grateful to Vinay, Milind, C. Ramesh, Mangaeshl, Aparna, Nagesh, Dr. Trushar, Sachin, Uday, Mahesh, Sheetal, Ravi and Suresh with whom I shared many delightful moments and who made my stay at NCL pleasant.

I thank Mr. Pawar, Mr. Khandekar and Mr. Govind for their help and cooperation.

I am indebted to my parents and my family members who have constantly encouraged me and I am grateful to them for all their sacrifices.

Finally, I acknowledge CSIR for a research fellowship and thank the Director, NCL, for providing the necessary research facilities.

SHRIVALLABH B. DESAI

CERTIFICATE

Certified that the work incorporated in the thesis entitled **"LIPASES IN BIOTRANSFORMATIONS: RESOLUTION OF SOME NOVEL ALCOHOL AND DIOL SUBSTRATES *VIA* SELECTIVE HYDROLYSIS OF THEIR ESTERS AND RELATED MECHANISTIC STUDIES"** submitted by **Mr. Shrivallabh B. Desai** was carried out by the candidate at the National Chemical Laboratory, Pune, under my supervision. Such materials as obtained from other sources have been duly acknowledged in the thesis.

(**K. N. Ganesh**) **May 2000**

Research guide

Head, Division of Organic Chemistry (Synthesis)

National Chemical Laboratory

Pune 411 008

CANDIDATE'S DECLARATION

I hereby declare that the thesis entitled "**LIPASES IN BIOTRANSFORMATIONS: RESOLUTION OF SOME NOVEL ALCOHOL AND DIOL SUBSTRATES *VIA* SELECTIVE HYDROLYSIS OF THEIR ESTERS AND RELATED MECHANISTIC STUDIES**" submitted for the degree of Doctor of Philosophy in Biotechnology to the University of Pune has not been submitted by me for a degree to any other university or institution. This work was carried out at the National Chemical Laboratory, Pune, India.

(**Shrivallabh B. Desai**)
National Chemical Laboratory
Pune 411 008

May 2000

General Remarks

All the solvents used were purified according to the literature procedures. (±)-4-Methoxymandelic acid, *p*-anisaldehyde, CD_3COOD, DCC, tetradecylaldehyde, *R*-(+)-MTPA and chiral shift reagent, tris[3-(heptafluropropylhydroxymethylene)-(+)-camphorato]europium (III) were obtained from Aldrich, USA. The commercial grade enzymes AmanoPS (Amano Pharmaceuticals, Japan), CCL (Sigma, USA), PPL, PLE (Aldrich, USA), LIpozyme and Novozym-435 (Novo Nordisk, Denmark) were obatined as gift samples. PLAP and BLAP were generous gift samples from Prof. Basavaiah (University of Hyderabad). Light petroleum refers to the 60-80^{o}C boiling fraction of petroleum ether.

'Usual work-up' implies washing of the organic layer with water followed by brine, drying over anhd. sodium sulphate followed by removal of the solvent *in vacuo* using a rotavapor. TLC was performed on E-Merck pre-coated 60 F_{254} plates and the spots were rendered visible by exposing to UV light. Column chromatographic separations were carried out by gradient elution with light petroleum ether-ethyl acetate mixture, unless otherwise mentioned and silica gel (60-120 mesh /100-200 mesh). IR spectra were recorded in the solid state as nujol mull or KBr pellets and in $CHCl_3$ solution (conc.1µM). NMR spectra were recorded either on Bruker ACF 200 (200 MHz for ^{1}H) or MSL 300 (300 MHz for ^{1}H) spectrometers. Chemical shifts (δ) reported are referred to internal tetramethyl silane. Microanalytical data were obtained using a Carlo-Erba CHNS-0 EA 1108 Elemental Analyser. Elemental analyses observed for all the newly synthesized compounds were within the limits of accuracy. All the melting points reported are uncorrected and were recorded using an electro-thermal melting point apparatus. All the compounds previously known in the literature were characterized by comparison of their R_f values on TLC, IR and H^1 NMR spectra as well as melting point (in case of solids) with authentic samples.

Optical rotations were measured on Jasco DIP-181 digital polarimeter. Stereochemical assignments are based on the optical rotation of the known compounds. Enantiomeric excess were calculated by ^{1}H NMR ananlysis of the derivatzed compounds with *S*-(+)-MTPA-Cl (Moshers ester).

CONTENTS

CHAPTER 1

LIPASES IN BIOTRANSFORMATIONS

The universe is dissymmetrical; for if the whole of the bodies which compose the solar system were placed before a glass moving with their individual movements, the image in glass could not be superposed on reality_ _ _ Life is dominated by dissymmetrical actions. I can forsee that all living species are primordially, in their structure, in their external forms, functions of cosmic dissymmetry.

Louis Pasteur

1.1 INTRODUCTION

Chirality is a geometrical attribute. An object that is not superimposable on its mirror image is said to be chiral. The most common type of chiral organic molecule contains a tetrahedral carbon atom attached to four different groups. Such a carbon is said to be a stereogenic center and the molecule exists in two stereoisomeric forms. Chirality is not a prerequisite for bioactivity but in bioactive molecules where a stereogenic center is present, great differences are observed for the activity of the enantiomers. This is a general phenomenon and applies to all bioactive substances, such as drugs, insecticides, herbicides, flavors, fragrances, pheromones and food additives.

1.1.1 Receptor Theory and Pharmaceuticals:

Most drugs are specific and their action is usually explained on the basis of receptor theory. This concept for bioactive agents was introduced by Langely (1906),[1] in order to explain the observed effects of nicotine and curare on muscle tissues. Ehrlich[2] later coined the term chemoreceptors to describe such binding sites. The chemoreceptor proteins that have affinities to ligands are analogous to enzyme substrate binding, the binding triggers regulation of vital functions as blood pressure, muscle contraction, gastric acid secretion etc. The overwhelming majority of naturally occurring drugs are chiral molecules, existing as single active enantiomer, whereas for many years it was common practice to market the synthetic drugs as racemates. But trend is towards rational drug design to produce complex molecules and hence there is an increased probability that they are chiral. In case of certain drugs eg. antihypertensive agents, when administered as racemates the distomer (inactive isomer) displays no side-effects. But in some other cases the distomer may even exhibit toxic side-effects e.g., the (*S*)-isomer of ketamine has anaesthetic and analgesic activity, but (*R*)-isomer is an hallucinating agent. The most well-known example is that

of thalidomide, formerly used as sedative (also immunomodulator) where the distomer is a teratogenic agent and causes fetal abnormalities.[3] The grave side-effects due to the distomers have now made administering a single pure isomer very important. In short, different phamacodynamics and pharmacokinetics of the eutomer (active isomer) and the distomer in a racemic drug can lead to a variety of side-effects attributable to inactive isomers. Thus it becomes very important to synthesize and administer chiral molecules in enatiomerically pure form, and asymmetric synthesis, kinetic resolution of racemates, preferential/diasteromeric crystallization have hence gained strategic importance.

1.1.2 Asymmetrical Synthesis versus Kinetic Resolution:

There are two possible approaches for the preparation of optically active products by chemical transformation of optically inactive starting materials: (I) kinetic resolution/diastereomeric crystallization and (ii) asymmetric synthesis. A kinetic resolution depends on the fact that two enantiomers of a racemate react at different rates with a chiral reagent or catalyst, such as an enzyme. A more modern example of enzymatic kinetic resolution is acylase catalyzed L-specific hydrolysis of racemic N-acetyl amino acids commercialized by Tanabe company. In diastereomeric crystallization a solution of racemate is allowed to interact with a pure enantiomer (the resolving agent) thereby forming a mixture of diastereomers that can be separated by fractional crystallization. The most commonly used resolving agents are L-(+) tartaric acid, D (-) camphorsulfonic acid and various alkaloid bases. An asymmetric synthesis, on other hand, involves the creation of an asymmetric center by chiral auxiliary /reagent. Thus a kinetic resolution involves substrate selectivity while an asymmetric synthesis involves product selectivity.

A cursory appraisal of the relative economics of asymmetric synthesis versus kinetic resolution would seem to indicate a clear preference for the former since it has a

theoretical yield of 100% as compared to 50% for kinetic resolution. However, kinetic resolution has some advantage over asymmetric synthesis: (i) experimentally simple processes, (ii) tuning of the enantiomeric excess by adjusting the degree of conversion. The major disadvantage of kinetic resolution is that they require at least one extra step for racemization of the unwanted isomer for recycling to increase the yield. This can be circumvented if spontaneous *in-situ* racemization is carried, to attain kinetic dynamic resolution.

1.1.3 Enantiomeric Purity Determination:

The importance of optical purity in the context of biological activity has created a growing need for accurate unequivocal methods for the determination of the optical purities.

(i) Optical Rotation

This classical method involves measurement of specific optical rotation [α]

$$\text{Optical Purity} = \frac{[\alpha]}{[\alpha]_o} \text{ X } 100$$

where [α] = specific optical rotation of the mixture and $[\alpha]_o$ = specific optical rotation of the pure enantiomer.

$$\text{Enantiomeric excess (\% ee)} = \frac{R_{\text{Isomer}} - S_{\text{Isomer}}}{R_{\text{Isomer}} + S_{\text{Isomer}}} \text{X100}$$

where R and S are the relative proportions (ratio) of the two enantiomers. In practice, this may often lead to some confusion since the optical rotation is dependent upon various conditions of measurements such as solvents, temperature, purity etc. and ambiguity may exist if the compound is new or not well documented in literature.

(ii) HPLC Methods:

As enantiomers have the same adsorption properties, they are not amenable to direct chromatographic separation on achiral adsorbents. However, this can be accomplished *via* the formation of diastereomers either by derivatization of sample with suitable chiral reagent[4] or formation of transient diastereomers via the interaction of enantiomers with chiral stationary phase/mobile phase additive.[5]

(iii) GLC Methods:

For compounds that are readily vaporized without decomposition, gas chromatography on chiral stationary phase constitutes an accurate and reliable method for enantiomeric purity determination. The technique has inherent advantages of simplicity, speed, reproducibility and sensitivity.[6]

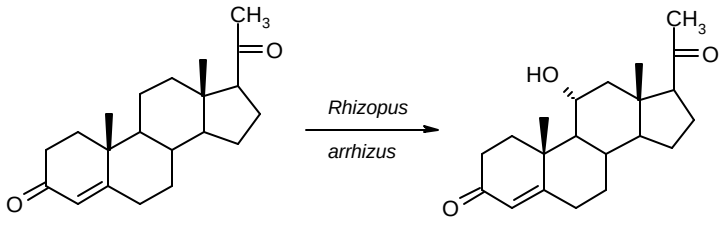
(iv) NMR Methods:

NMR is a widely used technique for enantiopurity determination and one well tested method involves conversion of mixture of enantiomers to a mixture of diastereomers by optically pure reagents eg. Mosher's reagent.[7] A closely related method employs the use of chiral Lanthanide shift reagents (LSRs),[8] having the property of shifting the NMR signals of substrates *via* diastereomeric complex formation.

1.2 BIOTRANSFORMATION

Biotransformations involve the use of biological methods to effect chemical reactions and form a bridge between chemistry and biology. Here biological systems are employed to consequent chemical changes on compounds that are not their natural substrates. This distinguishes biotransformation from biosynthesis, which involves action of biological systems in their normal habitat upon their natural substrates. Fermentation is a powerful technique for the production of alcohol, acids, antibiotics,

amino acids, and nucleic acid related compounds. In this technique, the product is the result of a complex metabolism of the microorganism, based on inexpensive carbon and nitrogen sources. In microbial transformations, it is not necessary to use active cells and the microorganism is akin to a bag of enzymes, requiring a suitable substrate instead of the carbon and nitrogen sources required for fermentation. In some biotransformations the synthetic substrate may resemble with the natural substrates while in some others (eg., xenobiotic transformation) it may be completely unrelated to the natural system. Whilst both may be used for synthetic purposes, the former can focus some light on structural and mechanistic features of biosynthesis. This type of study could be useful for the synthetic chemist in planning the retrosynthetic approaches. In biotransformations, isolated enzyme systems or intact whole organisms may also be used. Many biotransformations are not only chemo- and regioselective but are also enantioselective allowing the production of chiral materials from racemic mixtures. The conditions for biotransformations are mild and in majority of cases do not require the protection of other functional groups. Furthermore the features governing their regioselectivity differ from those controlling the chemical specificity and indeed it is possible to obtain biotransformations at centers that are chemically non-reactive (e.g. in Figure **1**, compound **1** is transformed to **2** by hydroxylation at the chemically



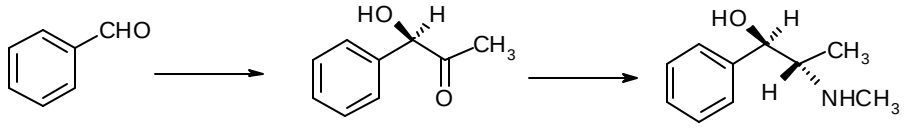
1 **2**

Figure 1

unreactive C-11 site). From a commercial point of view some biotransformations can be cheaper and more direct than their chemical analogues, whilst the transformations

proceed under the conditions that are normally regarded as “environmentally friendly”. There are many chemical reactions for which there are no equivalent biotransformation steps and the chemist should be versatile to use biotransformations in combination with the conventional chemical reagents in a synthesis. There is a vast amount of literature available on diverse aspects of biotransformation, a recent monograph by J. R. Hanson[9] gives an elegant overview of biotransformations in organic chemistry.

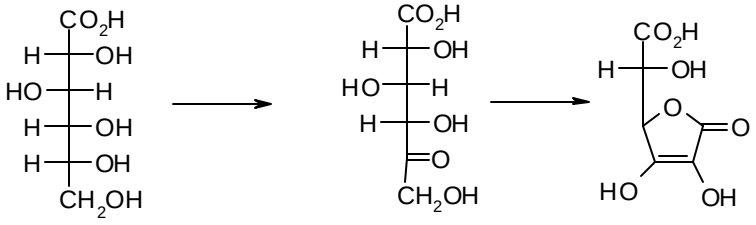
A particularly useful biotransformation early reported was the chiral acetoin condensation mediated by yeast. The addition of benzaldehyde (**3**) to the fermentation broth gave the ketol (**4**) by condensation with the acetaldehyde. This led in 1934 to a commercial synthesis of the alkaloid, ephedrine (**5**, Figure **2**).



3 **4** **5**

Figure 2

Another industrially useful application developed during the 1930's was the synthesis of vitamin C (**8**), which utilized the bacterial oxidation of D-sorbitol (**6**) to L-sorbose (**7**) by *Acetobacter suboxydans* (*Acetobacter xylinum*). The other chemical steps involve protection of L-sorbose as its bisacetonide, oxidation and deprotection to form 2-keto-L-gluconic acid, which is then converted to ascorbic acid (Figure **3**, vitamin C).



6 **7** **8**

Figure 3

A versatile bioprocess for hydration that has been scaled-up to operate at the tonnage level, is the hydrolysis of acrylonitrile (**9**, Figure **4**) to acrylamide (**10**) by the

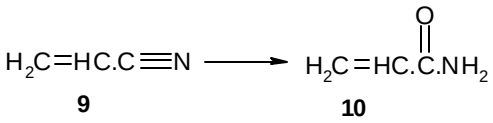


Figure 4

bacterium, *Rhodococcus rhodochorus*. The conditions are considerably milder than those required for the chemical hydrolysis, this material is one of the famous monomer used in polymer industry.

An interesting development with an impact on biotransformations involves the production of catalytic antibodies. An antibody possesses the property of molecular recognition by which it binds strongly to specific molecular structures - hapten and in fact antibodies are stimulated by the recognition of particular structural features in a macromolecule. This avidity of antibodies can be exploited to develop them for binding and stabilizing the transition state of a reaction to achieve catalysis. Since the transition states are unstable, the antibodies are raised to a stable transition state analog. Such antibodies in presence of suitable reactants exhibit catalytic activity and are termed "abzymes'. For example, a tetrahedral phosphonate (**11**, Figure **5**) mimics the

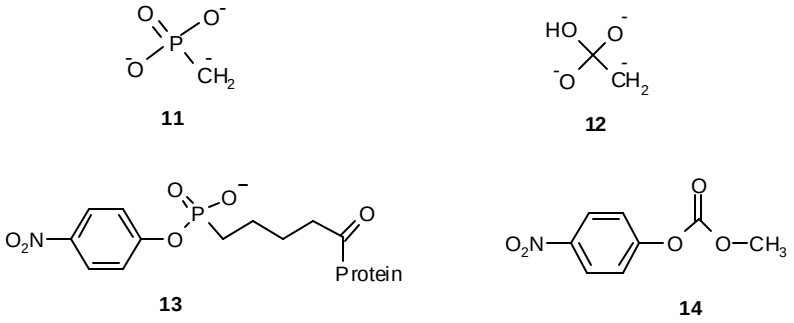


Figure 5

tetrahedral intermediate (**12**) in ester hydrolysis. The catalytic antibody elicited by exposure to (**13**) catalyzes the hydrolysis of the ester (**14**).

1.2.1 Classes of Enzymes

Enzymes are classified by rules prescribed by the commission on Enzymes of the International Union of Biochemistry, according to which each enzyme is designated by four numbers; the main class, the subclass, sub-subclass, and the serial number. There are six main classes as shown:

1. ***Oxidoreductases***: These enzymes mediate oxidation and reduction, including the insertion of oxygen to alkenes. This group also includes enzymes that are responsible for the addition or removal of hydrogen.
1. ***Transferases***: These enzymes are involved in the transfer of one group, such as an acyl or a sugar unit from one substrate to another.
2. ***Hydrolases***: This group includes the enzymes that mediate the hydrolysis or formation of amides, epoxides, esters and nitriles.
3. ***Lyases***: These are group of enzymes that fragment larger molecules with the elimination of smaller units.
4. ***Isomerases***: These enzymes are involved in epimerizaton, racemization and other isomerization reactions.
5. ***Ligases***: This group includes the enzymes responsible for the formation of C-C, C-O, C-S and C-N bonds.

Units: The international unit (I.U.) of any enzyme activity is described as μmoles of substrate utilized per minute or μmoles of product formed per minute and the specific activity is defined as activity per mg of enzyme.

1.2.2 Hydrolases

1.2.2a Esterases and Lipases

Hydrolysis of amides and esters by enzymatic methods (proteases, lipases, and esterases) are now routine biotransformations. Furthermore the general mechanisms by which some of these operate are quite well known and their scope has been

investigated to the extent that some predictive models are available. The molecular machinery of lipases is much like that of the serine protease's and consists of a catalytic triad of amino acids-serine, histidine, and aspartic (or glutamic) acid operating through a charge-relay system *via* hydrogen bonds (Figure **6**). The function of the

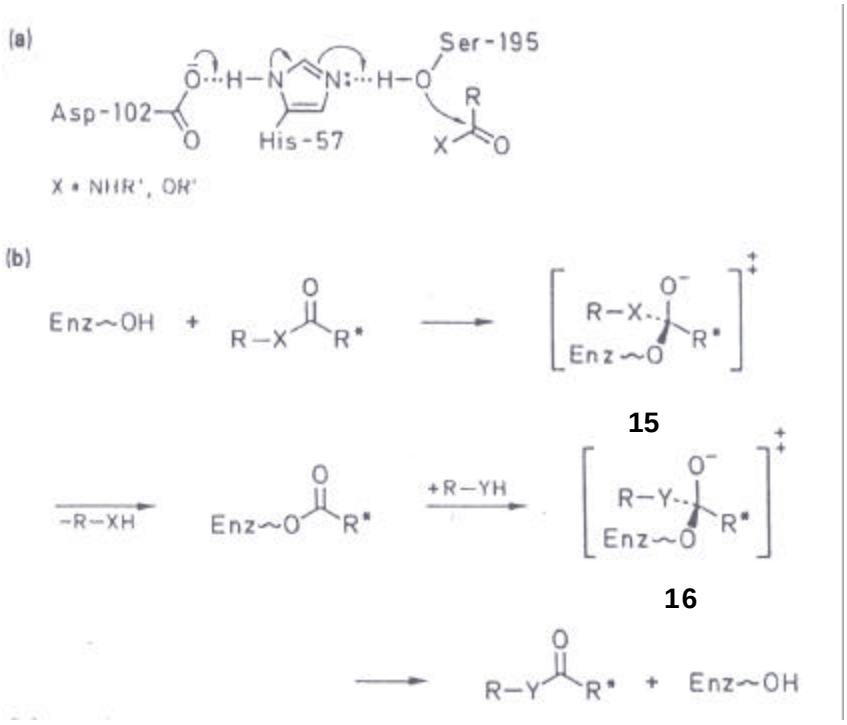


Figure 6: Mechanism of lipase/esterase and capture of acyl/enzyme complex with various nucleophiles.

buried aspartate group is to polarize the imidazole ring of histidine, since the remote negative charge induces a positive charge adjacent to it. This facilitates the proton transfer along the hydrogen bonds, and the hydroxy proton is finally bound to histidine. The active serine residue is now capable of attacking the scissile ester or peptide bond. The tetrahedral intermediate **15** is formed. After displacement of the leaving group R-XH by another nucleophile R-YH, histidine once again, at first deprotonates the new nucleophile with concomitant release of a proton to aspartate, and the activated species attacks the acylserine in the precisely reverse fashion as before. The resulting

tetrahedral intermediate **16** collapses to a new carbonyl compound and regenerates the serine-OH. The catalytic cycle may resume, enabling a variety of nucleophiles to participate in this process, suchas water resulting in hydrolysis, alcohol in esterification or transesterification, an amine offering amidation, hydrogen peroxide leading to formation of a peracid (which can epoxidize an available olefin). The structural shape of protein creates hydrophilic or lipophilic pockets within the enzyme active site, which leads to chemo-, regio-, enantio-, and diastereoselectivity.

In general, cells produce lipases to hydrolyze the extracellular fats and lipases are specially structured to act at water/organic interface. For this reason lipases appear to have optimum property among the enzymes to operate in organic solvents, in this case the interface is between the insoluble enzyme with its essential water of hydration and the organic solvent containing the acylating agent. Their broad synthetic potential is largely due to the fact that lipases, in contrast to most other enzymes, accept a wide range of substrates. They are stable in non-aqueous organic solvents and depending on the solvent system used, they can be employed for hydrolysis or esterification reactions. In addition, lipases can accommodate a wide range of substrates other than triglycerides such as aliphatic, alicyclic, bicyclic, and aromatic esters including the esters based on organometallic sandwich compounds. Lipases react with high regio- and enantioselectivity and although this view is clearly oversimplified and not valid for all types of esterases and lipases, it can explain the majority of the known applications of these enzymes in organic synthesis:

- Simple acylation and deacylation reactions under exceptionally mild conditions.
- Synthesis of amides and peptides.
- Tranesterification, diastereoselective esterifications or hydrolyses of lipids
- Regioselective reactions on polyfunctional compounds (polyols, sugars)

- Highly enantioselective synthesis of esters, half-esters, acids, lactones, and polyesters.
- Highly enantioselective synthesis of alocohols, diols, polyols, and amines.

In neat organic solvent, the enzymes retain the minimum amount of water which is necessary for their catalytic activity.[10] Molecules of hydrophilic solvents can enter the inner core of the macromolecule and eventually destroy the functional structure.[11] Water-immiscible solvents, containing water below the solubility limit (ca. 0.02 to10% by weight) are suitable for dry enzymes.[12] In other cases, more polar solvents or co-solvents improve the success of transformation. The use of organic solvents is seen to enhance the enantioselectivity[13] and thermostability[14] of the enzymes, probably due to restricted conformational flexibility. Conversely, some enzymes can loose or alter their enantioselectivity in organic media[15] indicating, that the conformation of the enzymes is dependent on the polarity of the medium and that individually optimized reaction conditions have to be developed for each transformation. From all the above discussion it is evident that in an adequate organic solvent, the catalytic activity can be of comparable order in aqueous medium with low water media,[10a] but overall, the rates of such (in organic media) reactions are slow

However, irrespective of the aforementioned complications, the use of organic solvents is of great importance, since this adds a new perspective to the applicability of the enzymes for organic synthesis. The advantages are obvious: (i) high solubility of most organic substrates, (ii) transformations of water-sensitive substrates in organic solvents, (iii) choice of a wide range of nucleophiles (Figure **7**, HY-R) for esterifications, aminolysis, oximolysis, or thiolysis etc. (iv) suppression of product or substrate inhibition when they can be retained in organic phase, (v) greater stability of the biocatalyst, (vi) ease of operation and easy removal of the insoluble catalyst.

1.2.2b Occurrence and Preparation

Lipases are ubiquitous enzymes and have been found in most organisms from the microbial,[16-18] plant[19,20] and animal kingdom.[21,22] They are prepared either by extraction from animal or plant tissue, or by cultivation of microorganisms. Usually lipases are just one member of a "hydrolytic enzyme cocktail" elaborated by an organism with the objective to sustain its growth. Often the lipases must be separated from other enzymes such as estrases and proteases occurring in the crude preparations. Purification protocols are often laborious as the affinity of lipases is high not only in the oil/water boundary, but at any interphase of lower polarity than water (e.g. water-immiscible organic solvents, glass and plastic surfaces, and air bubbles); lipases may irreversibly adsorb and denature at such interphases.[23]

1.2.2c Interaction of Lipases with Lipids

Since lipase catalytic action is strictly dependent upon the presence of a lipid interface, lipolytic enzymes provide a valuable model for studying protein-lipid interactions.[24] Most data dealing with the surface properties of lipases were obtained with the monolayer technique, by recording (either independently or simultaneously) the lipolytic activity, the amount of the protein adsorbed to the lipid monolayer, and the variations in surface pressure following protein adsorption. Several non-enzymatic proteins were used as controls in order to determine how lipase behavior differs from that of other proteins.

1.2.2d Structure and Mechanism

In 1958, Sarda and Desnuelle[25] defined lipase in kinetic terms, based on the phenomenon of interfacial activation. They suggested that the activity of lipases is low on monomeric substrates but strongly enhanced once an aggregated "supersubstrate" (such as an emulsion or micellar solution for instance) is formed above its saturation limit. This property is different from the usual esterases acting on water-soluble

carboxylic ester molecules, and for long time lipases were considered as a special category of esterases which are highly efficient at hydrolyzing molecules aggregated in water.

The first two lipase structures were solved in 1990 by X-ray crystallography which revealed a unique mechanism, unlike that of any other enzyme. Their three dimensional structures suggested that interfacial activation is due to the presence of an amphiphilic peptidic loop covering the active site of the enzyme in solution, just like a lid or a flap.[26] From the X-ray structure of cocrystals between lipases and substrate analogue, there is a strong evidence that, upon contact with a lipid/water interface, the lid undergoes a conformational rearrangement which renders the activation site accessible to the substrate (Figure **7**).[27]

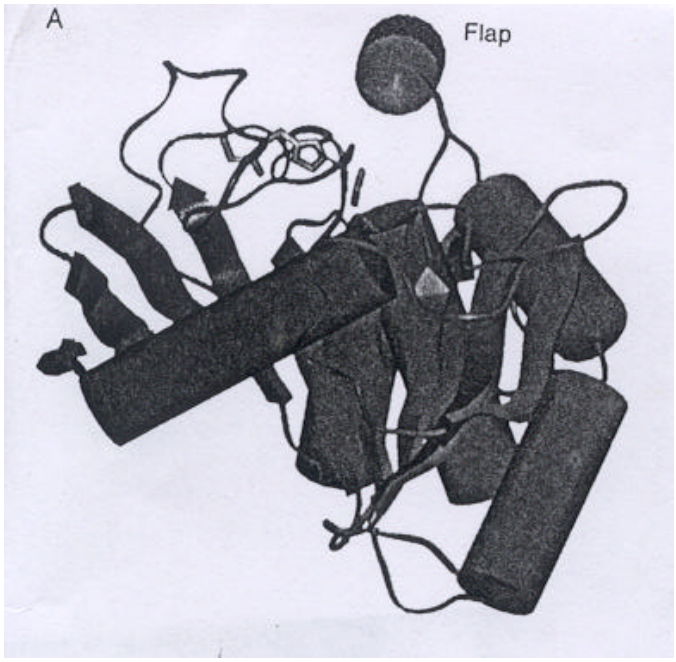


Figure 7 Structure of *Mucor miehei* lipase showing the catalytic triad and the α/β-hydrolase fold (lid).

There seems to be some evidence which suggests that not all lipases subscribe to the phenomenon of interfacial activation.[28] The lipases from *P.glummae*[29] and *C.antartica* (type B),[30] whose tertiary structure is known, have an amphiphilic lid

covering the active site but do not show interfacial activation. The lipase of the guinea pig enzyme features a mini-lid composing of only five amino acid residues and it shows no interfacial activation.[31] Thus, neither the phenomenon of interfacial activation nor the presence of a lid domain are appropriate criteria to classify an esterase into lipase subfamily. For classifying an esterase as a lipase, the safest experimental evidence is to find out whether or not it can hydrolyze long-chain acyl glycerols.[32] Till date, 12 lipases whose structure have been elucidated, are members of the "α/β hydrolase fold" family with a common architecture composed of a specific sequence of α-helices and β-strands.[26,27]

In spite of these similarities, subtle variations in the architecture of the substrate-binding site may have strong effect on the temperature and the stability of lipase in a solvent and hence its catalytic properties. These differences are of paramount importance for the selection of the individual lipase for a desired application.

1.3 LIPASES AS BIOCATALYSTS IN ORGANIC SYNTHESIS

Considering their specific and limited function in metabolism, one should expect lipases to be of limited interest for the organic chemist. However, chemists have discovered lipases to be one of the most versatile classes of biocatalysts in organic synthesis[33] for a few simple reasons:

1. Owing to the large enzyme structural domains required for the acyl group binding and the unpronounced structural features of acyl chains, lipases can accommodate a wide variety of synthetic substrates, while still showing chemo-, regio, and/or stereoselectivity.

2. Lipases act at the water/lipid boundary, which exhibits high interfacial energy. To withstand the denaturing effect of the interface, lipases have evolved unusually stable structures that may survive even the effect of organic solvents.

3. The free energy of fat hydrolysis is close to 0 kJ mol^{-1}.[34] As a result, thermodynamic equilibria are largely governed by the reactant concentrations, and lipase catalyzed ester hydrolysis in water can easily be reversed, in non-aqueous media, into ester synthesis or transesterification.

4. The acyl lipase formed in the first step of the enzymatic reaction can formally be considered as an acylating agent. The wide substrate specificity of this enzyme class allows acylation of nucleophiles other than those with hydroxyl groups, for example hydroperoxides, thiols and amines.

As a result of this unique combination of properties, chemists have used lipases for a plethora of synthetic reactions.[35] Following a thorough survey of the literature on chiral resolutions with lipases from *Candida rugosa* (CRL) and *Pseudomonas cepacia* (PCL), Kazlauskas et al. put forward tentative rules for the enantiopreference of these two enzymes based on the spatial requirements of the substituents on the reagent.[36] The basics of "Kazlauskas rules", are presented in Figure **8** and the literature has

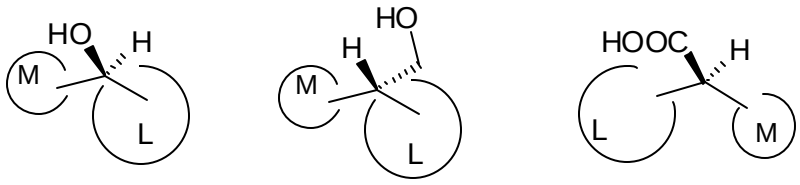


Figure 8 "Kazlauskas rules" (M = medium-sized substituent, L = large substituent)

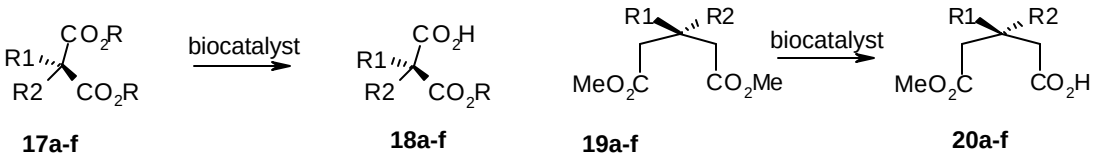
shown that the rule is highly predictive for lipase action on secondary alcohols, but less accurate for lipase-catalyzed transformations of primary alcohols and acids.

1.3.1 Transformations of Prochiral and *meso* Substrates ("Meso Trick")

In kinetic resoutions, theoretical yields are limited to 50%. Yields of up to 100% are possible with *meso* –diesters or diol substrates, which undergo enantioselective hydrolysis or transesterification through enantiotopic group differentiation.

a) Synthesis of Chiral Dicarboxylic Acid Monoesters

Meso compounds have to fit in and accommodate the active center in an optimum manner and only then the catalytical process creates a chiral molecule.[41,42]



17a-f **18a-f** **19a-f** **20a-f**

Enantioselective Hydrolysis of Malonate Diesters Enantioselective Hydrolysis of Glutarate Diesters

Figure 9

The result is that, at least theoretically, an achiral compound can be quantitatively converted into an optically pure product. This concept has been realized with a great number of acyclic, alicyclic and heterocyclic substrates. Most of the work on acyclic compounds has been devoted to derivatives of malonic (Figure **9**, Table **1**) and glutaric acids (Figure **9**, Table **2**) for the synthesis of their respective monoesters, which find use as valuable intermediates of amino acids and barbiturates.

Table 1 Enantioselective Hydrolysis of Malonate Diesters

Sub	R1	R2	Product	yield	% ee	Ref.
17a	alkyl	alkyl	**18a**	-	38-90	37
17b	Me	OH	**18b**	82	46	38
17c	CH_2OMe	Me	**18c**	86	21	39
17e	F	H, alkyl	**18e**	-	46-93	40

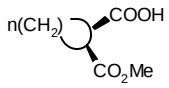
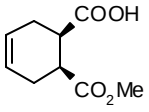
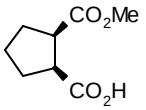
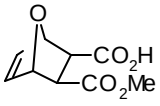
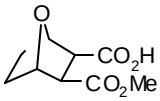
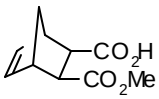
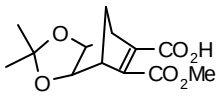
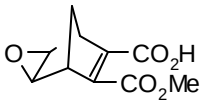
Table 2 Enantioselective Hydrolysis of Glutarate Diesters

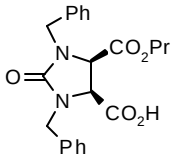
Sub	R1	R2	Product	yield	% ee	Ref.
19a	H	Me	**20a**	-	90	43
19b	OH	Me	**20b**	82	>99	46
19c	NHR3	H	**20c**	93	54-99	47

The enzyme catalyzed hydrolysis of cyclic *meso*-diesters has found broadest application in the preparation of synthetically useful chiral building blocks. Most of

these reactions are carried out with PLE, some of the representative examples are listed in Table **3**.

Table 3 Enantioselective Hydrolysis of Alicyclic and Heterocyclic *meso*-Diesters

Entry	Product	Yield (%)	ee (%)	Ref.
21		---	17-100	38
22		98	98	38
23		86	88	43
24		86	75	44
25		82	>98	44
26		85	<10	45
27		30	100	46
28		100	85	47

29		85	77	48

b) *Synthesis of Chiral Monoacyl Diols*

Instead of hydrolyzing a prochiral or *meso* diester, the corresponding diacetate or diol can be employed. In certain cases, the more readily available diols and diacetates are used and in consequence, the stereochemical complementarity of the synthetic or hydrolytic action of the enzymes can be exploited to the full extent. The 3-*O*-protected glycerols[49] or their diacetates,[50] can be transformed into optically active **32** and **33** (e.g. R = OBn, Figure **10**), this route provides an highly efficient access to chiral

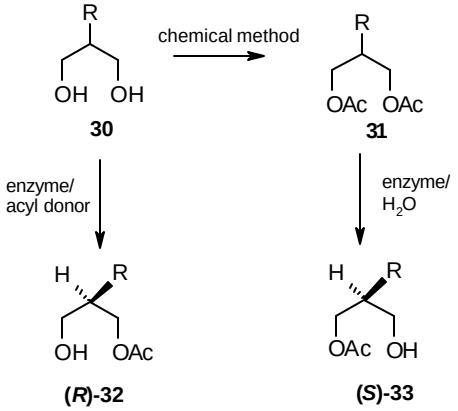


Figure 10

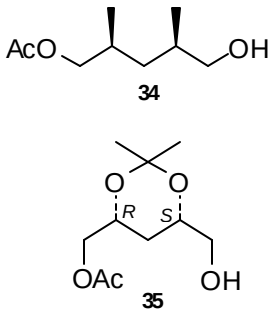
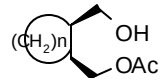
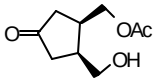
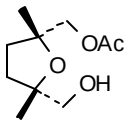
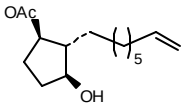
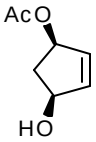
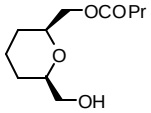
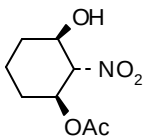


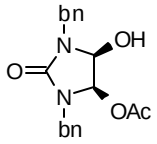
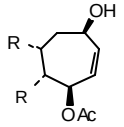
Figure 11

glycerol derivatives. Previous syntheses of these from carbohydrate precursors including laborious protective group technique are no longer required. These compounds are important intermediates in synthesis of many drugs, e.g. β-blockers, platelet activating factor (PAF), and many other biologically important compounds. Monoacyl diols of (Figure **11**) **34** and **35** were obtained in excellent optical purity and high yields with various enzymes as PFL, PPL or PLE.[50] Some examples of the different chiral alicyclic and other heterocyclic diols/diacetates that have been prepared

are cited in the Table**4**. In synthetic mode, the reactions are carried in anhydrous inert solvents e.g. toluene, diisopropyl ether, or ethyl acetate, the later serving as solvent and acyl donor. The different types of acyl donors used are vinyl acetate, propenyl acetate, or anhydrides. The concept has been realized with various 2 -alkypropane-1,3-diols **30** (R = alkyl, aryl, etc) as shown in the scheme above.

Table 4: Enzymatic Synthesis of Chiral Alicyclic and Heterocyclic Monoacetates

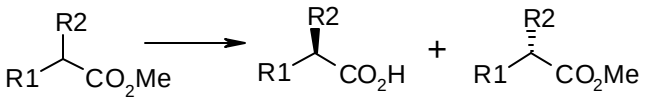
Entry	Product	Biocatalyst	Yield (%)	ee (%)	Reference
36	 n= 1-5	SAM-II, PPL	20-90	80-95	51
37		PPL	55-94	50-96	51
38		PLE, PPL, CCL, LPF	23-84	33-99	52
39		RDL	69	99	53
40		PLE, PPL, CCL, others	~90	50-100	54
41		PPL	84	55	44
42		PLE	50-80	>98	55

43		PLE	76	92	56
44		EECE, CCL	39-61	100	57

1.3.2 Kinetically Controlled Transformations of Racemic Substrates

a) *Synthesis of optically Active Acids and Esters via Hydrolysis*

The essential requirements for the successful separation of a racemate by esterases and lipases are sufficiently different rate constants for the transformations of the enantiomers, either in the synthetic direction, or driven in the reverse, towards



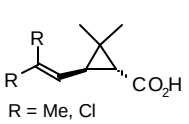
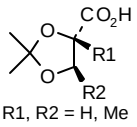
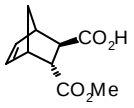
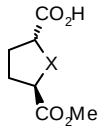
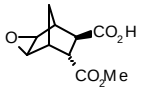
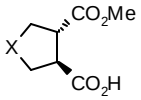
45 **46** **47**

Figure 12

hydrolysis (Figure **12**). Under optimum conditions, i.e., at complete enantiospecificity of the enzyme, the reaction will stop at 50% conversion. If the unwanted enantiomer cannot be recycled by racemization, 50% of the product gets discarded. On the other hand, compared to the classical approaches via diastereomers or tedious chromatographic procedures, the enzymatic approach has a great advantage that only a small amount of the biocatalyst may be needed instead of molar quantities of a chiral reagent. Table **5** gives an overall view of the enantioselective hydrolysis of the representative alicyclic and heterocyclic compounds. Using PLE, out of a mixture of diastereomeric, racemic chrysanthemum esters (R = Me) the *trans*-acid **48** has been obtained with high diastereselectivity. When pure *trans*-chrysanthemum esters were subjected for hydrolysis (50% conversion), the (1*R*, 2*R*)-**48** (70-80% ee) were obtained.

The enzymatic route to norborane esters and half esters **50** and **52** provides a very simple and effective alternative to asymmetric Diels-Alder reaction.

Table -5: Enantioselective Hydrolysis of Alicyclic and Heterocyclic Esters

Entry	Product	Biocatalyst	Yield (%)	ee (%)	Reference
48	 R = Me, Cl	PLE	~50	70	58
49	 R1, R2 = H, Me	CCL, PPL	19-47	32-95	59
50		PLE	33-66	23-59	60
51	 X = N, S, CH_2	PLE	48-71	5-23	60
52		PLE	45	73	61
53	 X = N, S, CH_2	PLE	45	82	61

b) *Cyclic and Aliphatic Monohydroxy Substrates*

The principal requirements for the resolution of the racemates have been mentioned in the previous section. However, in the case of simple secondary alcohols, an additional consequence of the kinetically controlled reaction namely, double resolution strategies, can be readily exploited. If the ester synthesis from a given alcohol and an appropriate acyl donor does not lead to a product of high % ee, this ester can be subjected to a second enzyme-catalyzed transesterification by an achiral

alcohol. Since, both reactions can be carried out in organic media, the conformation of the enzyme remains the same, and the faster reacting enantiomer in the synthetic direction will also be the faster released by transfer to an achiral alcohol. The strategy is outlined in the Figure **13**. Under suitable kinetic control in both directions, products of very high % ee can be obtained by this two-step procedure.

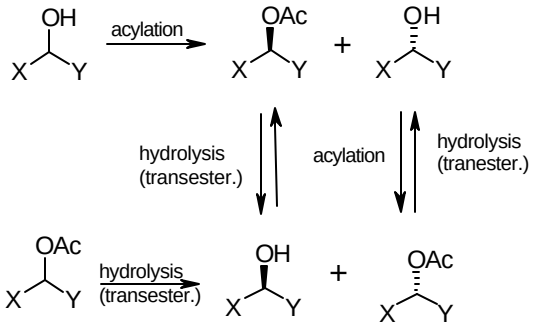


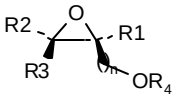
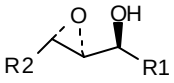
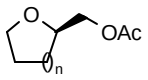
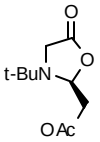
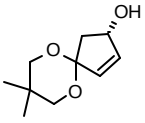
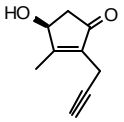
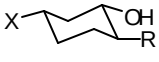
Figure 13

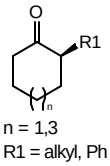
Aliphatic secondary alcohols constitute a class of industrially important aroma and flavor compounds. Hence much work has been devoted to explore optimum conditions for their preparation from chiral precursors. This includes the search for the best enzyme and acyl donor,[62] optimum conversion rates,[63] solvents,[62] and temperature.[64] The effect of temperature is interesting, since PPL produced esters, at 70 ^{o}C with higher % ee than at 40^{o}C.[65] Pheromones are semiochemicals and used for intraspecific communications, which have to be synthesized with very high % ee because of posssible interfering effects of other isomer.

Among the cyclic monohydroxy substrates as shown in Table **6** enantioselective preparation of 2,3-epoxy alcohols **54** is of great synthetic value.[66] Studies on variation of chain length,[59] co-solvents,[71] and temperatures with respect to efficiency of resolution[72] has been extensively done. Entries **58**, **59** and **60** are further examples for the utilities of the lipases in the field of cyclopentanoid precursors to natural products. The biocatalytic resolution of cyclohexanols **61** is an attractive route to chiral auxiliaries and reagents like menthol or phenylmenthol.[73] Entry (*S*)-**62** (n=1, R=Alkyl) deserves a

special attention, the substrate is an enol ester, hence hydrolysis of the ester moiety and transfer of the proton to the double bond have to occur from the same direction. The ketone *S*-(**62**) is obviously released without formation of an intermediary enol.[74]

Table -6: Enantioselctive Synthesis of Alicyclic Alcohols

Entry	Product	Biocatalyst	Yield (%)	ee (%)	Ref.
54	 n = 1,2 R^4 = H, acyl	PPL	30-60	30-95	59
55	 R1=R2 = alkyl	PPL	50	>98	65
56	 n = 1,2	PPL	50	0(n=1) >97 (n=2)	59
57		PL 266, others	30	93-95	67
58	 R = OAc, CO_2Et	Penicillin acylase	30-43	90	68
59		Lipase P	42	>99	69
60		*Arthrobacter* lipase	<50	>95	70
61	 X = alkyl, Ph, H R = Ph, Bn, OPh, OBn, alkyl	SAM-II, PLE, CCL	10-88	36-98	73

62		*Pichia miso*	60-98	41-94	74

1.3.3 Intra- and Intermolecular Transesterification of Hydroxy Acids

If the substrate is a hydroxy acid or a hydroxy ester such as **63**, the remote OH-group can act as an internal nucleophile onto the primarily formed acyl-enzyme. This reaction becomes feasible when water is replaced by an organic solvent as the reaction medium. Since hydroxy acids **63** (R = H, Figure **14**) are prone to spontaneous lactonization, the more stable methyl esters were subjected to enzymatic

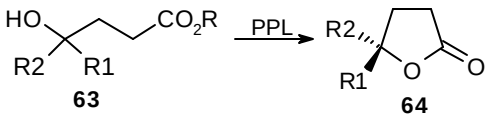
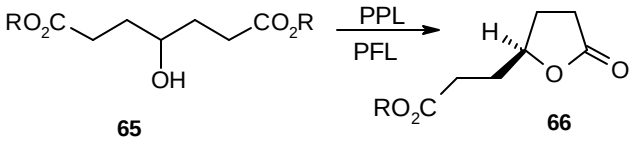


Figure 14

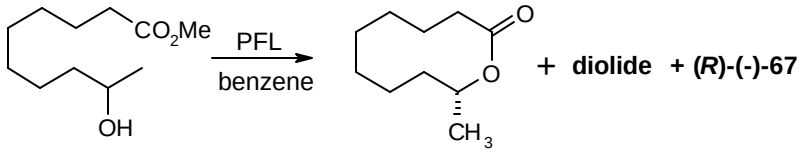
transesterification in diethylether or hexane. Using PPL, at low conversion rates (36%) for example (*S*)-(-)-γ-methylbutyrolactone **64** (R^1 = Me, R^2 = H) is obtained with high optical purity (97% ee). At high conversion rates (60%) the recovered (*S*)-4-OH ester **63** is of high optical purity (97% ee), and the corresponding R -(+)-lactone is available by acid treatment.[75] Up to τ-dodecalactone high optical purities (82-98% ee) have been obtained[76] whereas the α-substituted derivatives of the esters **63** are poor substrates. PFL yields γ-lactones **64** with opposite configuration, but their % ee is low (10-45%). Enzymatic (PPL or PFL) intramolecular transesterification of the symmetrical hydroxy diester (*meso*-substrate, Figure **16**) **65** leads to (*S*)-(-)-**66** (>98% ee, 100% conversion)[76] in an enantioconvergent manner.

If the hydroxy group is located at more remote positions, only very few lipases are able to catalyze lactonization, and intermolecular oligomerization becomes the preferred pathway. In general, if a ring containing five annular atoms can be formed,



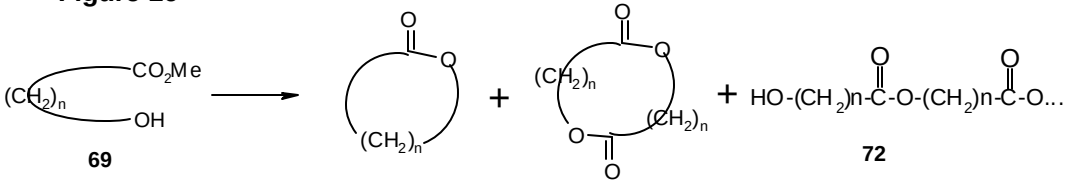
65 **66**

Figure 15



67 **68** + **diolide** + (***R***)-(-)-**67**

Figure 16



69 **70** **71** **72**

Figure 17

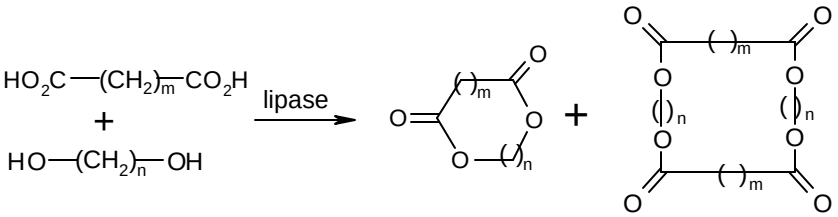


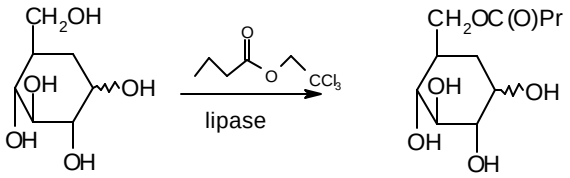
Figure 18 **73** **74**

this is the exclusive product; if of six or seven atoms, either the ring or the chain polymer is formed – in some instances both.[77] For example, methyl 9-hydroxydecanoate (**67**) Figure **16** is converted to the corresponding macrocyclic lactone (*S*)-**68** (100% ee) only by PFL;[78] others catalyze almost exclusively the formation of cyclic dimers of type **71**[79] and acyclic oligomers of type **72** (Figure **17**), respectively. However, careful optimization of the reaction conditions provided the whole series of C(12) to C(16) lactones **70** [70-80% yield for C(16) and C(15)] from the

corresponding ω-hydroxy acids.[78] With shorter chain hydroxy acids the formation of oligomers **72** is the preferred reaction.[77] With α,ω-diacids (m= 2-12) and α,ω-dialcohols (n= 5-16) and lipases from *Candida cylindracea* (OF-360) or *Pseudomonas* sp. (AK and K-10) the intermolecular transesterification leads to macrocyclic mono-and dilactones **73** and **74** (Figure **18**) in isooctane at 65 °C. Lower temperatures favor the production of linear oligomers[80] with the varying ratio of **73**/**74** depending on the enzyme used.

1.3.4 Enzyme Catalyzed Acylation and Deacylation of Polyhydroxy Compounds

The selective manipulation of individual hydroxy groups in complex sugar moieties is still a challenging problem. Up to now, the selective introduction of acid or base labile blocking groups into the polyfunctional molecules, often in cumbersome multisteps, is the most commonly employed strategy. Recent advances using lipases



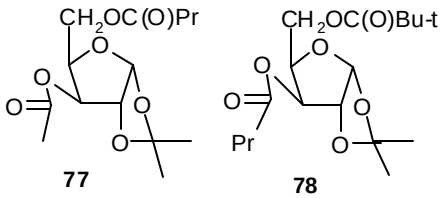
75 **76**

Figure 19

and activated esters of organic acids as acylating agents provide a new solution for this unsatisfactorily solved problem. Thus, for example, treatment of glucose **75**, galactose, or mannose, in dry pyridine with PPL in the presence of 2,2,2-trichloroethyl butyrate or acetate resulted in almost exclusive acyl transfer to the primary hydroxyl group and gave the 6-*O*-butyryl (acetyl) derivatives **76** (Figure **19**) of the corresponding sugars in 50-91% yield.[81] In the case of fructose, the two primary hydroxyl groups are not well distinguished and a mixture of 1-*O*-acetyl- (71%) and 6-*O*-acetylfructose (29%) was obtained. PPL also accepts triolein as an acyl donor. Similar results were reported with

medium and long chain fatty acids (C_{10} to C_{18} acids) and sugars or glucosides using lipases from *Candida antartica*[82] (up to 90% yield) and many other microorganisms. The resulting 6-*O*-monoesters represent an industrially important class of nonionic surfactants and emulsifiers. Since the embedding of substrates into the active center of many lipases is identical in aqueous and nonaqueous media, it follows, that CCL leaves preferentially the primary function of fully acylated sugars.[83]

Protected sugars (acetonides) can also be selectively deacylated with CCL or PPL. For example, CCL hydrolyzes a secondary butyrate from diacetone D-glucose or liberates the 5-OH of (Figure **20**) **77**. If the 5-OH is blocked by a pivolate, both



77 **78**

Figure 20

enzymes are able to hydrolyze the secondary butyrate[83] **78**. The regioselective hydrolysis of peracetylated furanose to 5-OH sugars (40-96% yield) is possible with CCL and ANL.[84]). Tetraocta(penta)noates of methyl D-glucosides, galactosides or mannosides react similarly yielding the 6-OH products, irrespective of the α or β-anomers used. PPL releases the 1-OH of pentaacetates of glucose and mannose with a significant preference for the α-anomer. Under the same conditions CCL cleaved the 4- and 6-*O*-acetyl groups. Acylation of methyl furanosides or pyranosides occur at the primary position using trifluoroethyl acetate as the acyl donor. When this position is already blocked (enzymatically acylated or chemically alkylated), CVL selectively acylates the 3-OH of the above substrates, while PPL has an overwhelming preference for the 2-OH.[81]

Glycals **79** and **80** and, in particular, their esters are interesting chiral intermediates.[85] CCL readily acylates the primary hydroxy group yielding (Figure **21**)

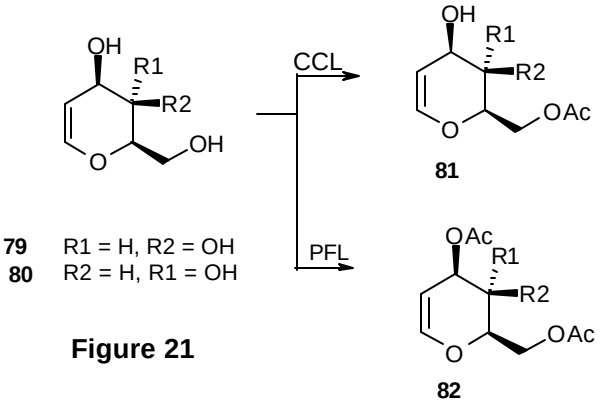


Figure 21

81, but PFL in combination with enol acetates leads to 3,6-di-*O*-acetyl derivatives[86] **82**. The PFL-catalyzed hydrolysis of the corresponding triacetate makes the 4.6-di-*O*-acetyl D-glucal available.[86] Optically active glycerols have been prepared by α-monobenzoylation via lipase-catalyzed tranesterifiction, similarly diastereo- and enantioselective esterification of butane-2,3-diol has been reported with Amano PS (91% de, 98% ee). The full differentiation of all hydroxy groups is possible by using this biocatalytic approach.

1.3.5 Nitrogen Containing Substrates; Formation of Peptide Bonds

The hydrolytic activity of lipases and esterases can be exploited for the preparation of optically active amino acids. Two major approaches, namely the

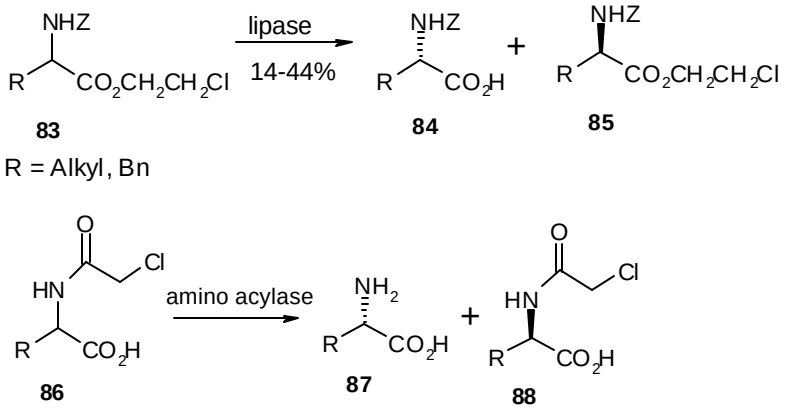


Figure 22

hydrolysis of esters of N-protected amino acids with lipases/esterases (**83** to **84**, Figure **22**) or the cleavage of amide bond of N-acetyl amino acids by proteases (**86** to **87**) are the most commonly employed. Conversion efficiencies with ANL or PFL were low to good (14-44%), yielding the corresponding amino acids **84** with acceptable optical purities (85-96% ee).[87] This process is particularly well suited for the synthesis of unnatural amino acids. Some examples of enantioselective hydrolysis of diesters are the synthesis of glutamic acid half-esters (PFL 100% ee), the preparation of optically

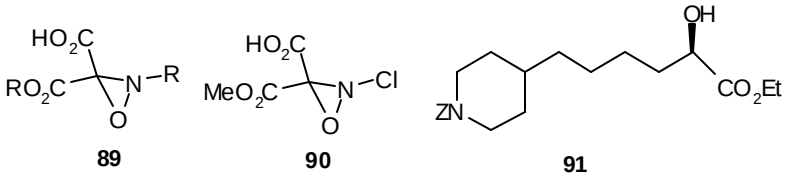


Figure 23

active oxaziridine[88] (*R*)-**89** (45-87% ee) and N-chloroazirine **90** with various enzymes.[89] The (2*R*) hydroxy ester **91** (93% ee) which is the building block for angiotensin converting enzyme inhibitor CV-5975, has been obtined with 86% conversion from its racemic ethyl ester with lipase PN from *Phycomyces nitens*.[90] Another interesting reaction carried out in organic media, is the lipase –catalyzed alcoholysis of the oxazol-5(4H)-one **92**. When **92** is treated with butanol in presence of MML, (*S*)-**94** is obtained (57% ee at 45% conversion) together with the ureacted azalactone **93** (Figure **24**).

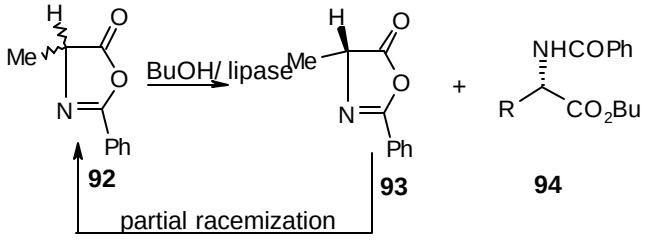


Figure 24

Since the latter showed no optical rotation, *in situ* racemization of **93** might have occurred.[91] Once optimized, this could be a promising, alternative, high yielding route to

chiral amino acids. If amines act as nucleophiles onto the intermediary acyl-enzyme, the synthesis of amides becomes possible in low water media. A simple application of this kind is the enantioselective synthesis o f a number of (*S*)-amides from racemic ethyl 2-chloropropionate catalyzed by CCL. The procedure works well with primary amides, while the secondary amides do not react (38-81% yield, 30-95%).[92]

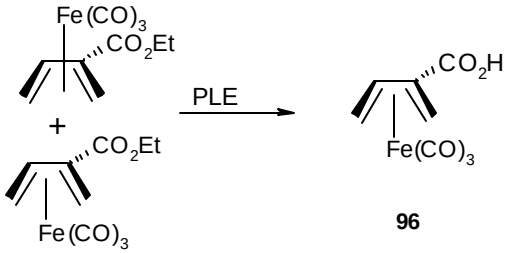
Unlike the solid phase synthesis of peptides, a scale-up of enzyme-catalyzed reactions is more readily achieved at least for small peptides. Further, the synthetic activity of many lipases in anhydrous media is greater than that of the proteases and amidases. Peptide synthesis can be also carried out with PPL, PLE o r CCL in ether or ethyl acetate, containing pyridine or 5-10% aqueous buffer.[82] Lipases entrapped in Amberlite XAD-8 in combination with glycerol esters of *N*-protected amino acids as acyl donors reduce the long reaction times.

1.3.6 Organometallic Substrates

The number of biotrasformations on organometallic substrates is rather limited. An interesting observation has been made, when tributylstannyl ethers were used in transeserification reactions.

$$R^1CO_2Et + R_2OSnBu_3 \longrightarrow R^1CO_2R^2 + EtOSnBu_3$$

In this case, CV lipase and PPL are not only able to utilize the organometallic ethers, but in addition they proved to be even more active than the free alcohols (e.g. hexanol).[93] The reason for this enhanced reactivity has not been fully elucidated. The first example of successful resolution of a racemic organometallic substrate is provided



95 **96**

Figure 25

by the kinetically controlled enantoselective hydrolysis of (±)-**95** (Figure **25**) into the chiral crystalline acid **96** (85% ee at 40% conversion).

Another example of PFL-catalyzed enantioselective esterfication is the resolution of the racemic mixture of **97** (Figure **26**) to get the air and light sensitive (*S*)-alcohol **98** in optically pure (100 ee, 47% yield) form using, isoprenyl acetate as the acyl donor.[94]

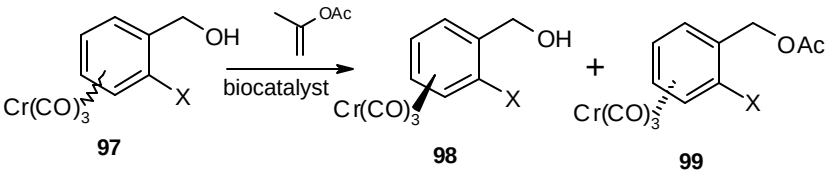


Figure 26

1.4 INDUSTRIAL APPLICATIONS OF LIPASES

1.4.1 Food Industry

The bulk of fats and oils produced every year are mostly used in food and renewable chemical feedstock and for non-food related applications. Many lipases exihibit *sn*-1,3 specificity, and can therefore be used to regioselectively interesterify positions 1 and 3 of a natural glyceride: however, the tendency towards acyl migration from *sn*-2 to the *sn*-1 or *sn*-3 positions must be suppressed. Some companies in Japan are operating on enzyme based processes for the preparation of highly pure unsaturated fatty acids, using lipase from Candida rugosa.[95]

Transesterified Triglycerides: The melting point of an oil can be modulated by the degree of catalytic hydrogenation of double bonds in unsaturated fatty acids, *viz*: for the preparation of margarines and shortenings of plant oils. Alternatively, the desired melting behaviour can be achieved through interesterification of suitable

triglyceride mixtures with the use of *sn*-1,3 specific lipases or a combination of both procedures.[96]

Improved Spreadability: Since the melting point of cocoa butter is around the human body temperature (37 ^{o}C), cocoa butter is well suited as a matrix for suppositories. The main application is in the production of chocolates, where the rapid melting conveys a desirable “mouth feel”. Cocoa-butter can be prepared either chemically or by lipase catalysis through interesetrification of suitable natural triglycerides.

Monoglycerides: Monoglycerides are mild emulsisfiers (HLB value 3.4, HLB= hydrophiolic lipophilic balance) permitted for use as food additives. Industrial applications include emulsifications in food, cosmetics, and drug preparations.[97] Many reports deal with the preparation of monoglycerides through lipase catalysis, but the key problem remains the formation of mixtures of mono- and diglycerides.[98]

Dairy Industry: Rennet paste, isolated from the stomach of ruminating animals such as cows or goats, is an example of an enzyme mixture traditionally used for the preparation of cheese.[84] The active component of rennet is chymosin, an aspartate protease involved in the clotting of milk through the hydrolysis of κ-caesin; however, lipases and esterases contained in rennet also contribute to cheese ripening. Depending on the chain-length specificity of a given lipase, its addition to a milk product may enhance the flavor of the cheese, accelerate the cheese ripening, or assist in the preparation of “enzyme modified cheese” (EMC). EMC is produced from cheese curd by the addition of the lipases at elevated temperatures, increasing the content of free fatty acids about tenfold.

1.4.2 Lipases in Detergents

Lipases are known to remove fat stains when used in detergent formulations and also to generate peracid bleach by perhydrolysis. Standard wash components contain anionic and nonionic surfactants, oxidants, and complexing agents at a pH of about 10 and temperatures around 50 ^{o}C, which is a rather hostile environment for enzymes. As a result, screening for lipases under these conditions have been carried out. The product 'Lipolase', a recombinant fungal lipase from *Humicola lanuginosa* expressed in a host strain of A*spergillus oryzae*, was produced on industrial scale.[99] This was probably the first genetically engineered protein to obtain permission by regulatory bodies in Europe to be used by consumer products and be discharged in the environment after use.

Soaps and Fatty Acids: A major application of triglycerides is in the preparation of soaps (from fats and oils), by enzyme-based processes for obtaining highly pure unsaturated fatty acids, using lipase from *Candida rugosa*.[95]

1.4.3 Lipases in Paper Manufacture

Nihon Seishi Co., a paper manufacturer in Japan, has developed a process by which the triglycerides contained in raw lumber are hydrloyzed by the addition of lipase. This resulted in better pitch control, that is, an easier processing of the lumber to low-grade paper. The process is carried out at a scale of several hundred tons of lumber per day, and other paper manufacturers are now using the similar procedures.

1.4.4 Lipases in Medicine

Substitution Therapy: Exocrine pancreatic insufficiency, often found in cystic fibrosis patients, results in two major problems: malnutrition and steatorrhea. These problems can be partly solved by administration of porcine pancreatic lipase extracts as a replacement therapy. In the past such preparations were far from satisfactory, since a

large proportion of the enzyme administered were denatured in the stomach due to acidity and gastric juice. With the advent of genetic engineering techniques, human gastric lipase (HGL) cDNA have been synthesized to overcome these shortcomings.

Lipase Inhibitors as Antiobesity Agents: Conventional treatment for obesity has focused largely on strategies to control energy intake. Under clinical circumstances, the use of an inhibitor of digestive lipase, which reduces dietary fat adsorption, holds great promise as an antiobesity agent. Tetrahydrolipstatin, derived from lipstatin produced by *Strptomyces toxytricini*, acts in vitro as a potent inhibitor of pancreatic and gastric lipases as well as cholesterol ester hydrolase. THL inhibits hydrolysis and thus the adsorption of triacylglycerols in the duodenum; it is being developed as an antiobesity agent by Hoffmann - La Roche. It could be demonstrated that an intraduodenal infusion of THL in humans strongly reduces the activity of pancreatic lipase, both by reducing its catalytic activity and pancreatic secretion.

1.5 RECENT TRENDS AND FUTURE IN LIPASE RESEARCH AND APPLICATIONS

1.5.1 Immobilization and Modification of the Biocatalyst

For practical and economic reasons it is often advantageous to use enzymes immobilized on solid supports. Such catalysts are particularly easy to handle and can be easily recovered at any time of the solutions. Furthermore, many of the immobilized enzymes can be repeatedly used without considerable loss of activity. Especially for large-scale preparations, the increased lifetime and enhanced stability of the modified proteins compensate for the lowered conversion rates. Sometimes the enantioselectivity may be enhanced.[100] Approved systems are, for example, PLE on Eupergit C,[100] α-chymotrypsin on silica, *Rhizopus japonicus* lipase or CCL on anion exchange resins (Duolite A-568 Dowex MWA-1) or graft-polymerized lipoprotein lipase

with *N*-vinylpyrrolidone or polystyrene. Other useful supports include methacrylic polymers containing hydroxy groups, Celite, PVC, chitosan, chitin, agarose, sepharose, hollow fibres, respectively. CCL was covalently bound to several surface-treated ceramic supports. Some of these systems can be enclosed in continuous flow reactors where they are capable of ester synthesis, hydrolysis or transesterification. A recent technique uses ferromagnetic-modified lipase from *Pseudomonas fragi* which can be recovered from the colloidal solution by application of strong magnetic field.[101] For the use of enzymes in organic solvents, the catalysts can be made soluble by several procedures. Coating of lipase from *P. fragi* with a synthetic dialkyl amphiphile resulted in lipase-lipid complex which is freely soluble in *iso*-octane. Most commonly employed modification of the enzymes is that with polyethylene glycol, resulting in powders that are soluble in benzene or chlorinated hydrocarbons. This concept has been extended to soluble magnetic lipases.[102] Other useful techniques are microencapsulation, or enclosure of enzymes into dialysis membranes which is recommended as an operationally simple and effective procedure. Other techniques, which overcome problems connected with low solubility of substrates, are the use of surfactants and reversed micelles.

1.5.2 Highly Pure Lipases

Lipases hold considerable promise in synthetic organic chemistry and have already found practical applications in detergents, oleochemistry, cheese production, medical therapy, and industrial synthesis of speciality chemicals. By now, lipases from over 30 biological sources have been cloned, sequenced, and expressed in host organisms. Pure recombinant lipases from *Humicola languinosa, Pseudomonas pseudoalcligenes, Pseudomonas aeruginosa, Mucor miehi, Candida antartica* (type B), *Bacillus thermocatenulatus*, and other sources are now commercially available in free or immobilized form or as part of a screening set (e.g. Chirazyme from Boehringer

Mannheim). Even cross-linked crystals of *Candida rugosa* and *Psedomonas cepacia* lipase (ChiroCLEC) are commercially available from Altus (Cambridge, MA). The tertiary structures of twelve lipases have been resolved and this now allows a more rational approach to modify lipases for specific applications.

1.5.3 Protein Engineering of Lipases

With increasing knowledge on lipase structure and function, it has become clear that substrate-binding domains vary greatly from one lipase to another. As detergents are still the commercially most important field of lipase applications, most pertinent patents deal with enhancing lipase stability and activity in a household detergent matrix.[103] The chain-length specificity observed in *Rhizopus delemar* lipase is related to the steric effects involved in the binding of acyl groups. Indeed, site-directed mutagenesis of lipase leading to sterically demanding points of acyl chain led to a significant shift in the preference of the muatant lipase for the hydrolysis of medium-chain triglycerides.[103]

1.5.4 Combinatorial Designs

In view of their broad applications and the rapidly advancing knowledge about their structure and function, lipases are interesting candidates for combinatorial approaches to modify their stability and substrate specificity. The esterase activity of *Rhizomucor miehei* lipase could already be enhanced with this technique.[104] Recently, evolutionary screening of proteases led to an enhanced solvent stability[105] and modified substrate specificity.[104] It is safe to predict that before long lipases will become an eminent example of those enzymes which have been modified by rational and combinatorial design for use in industrial processes.

1.6 SUMMARY

As described in this chapter, lipolytic and esterolytic enzymes have found broadest application in almost any area of organic synthesis. Besides finding new applications and new enzymes from different organisms, in the forthcoming years organic chemist has to become familiar with enzyme kinetics and their routine exploitation for the production of highly optically active products. Another promising field of research will be the improvement of the disposable biocatalyst by the enantioselective inhibition or genetic manipulations of the producing organism. However the most important aspects are: (i) the availability of more exact three dimensional protein structures, (ii) the detailed elucidation of the catalytic processes which have to be correlated to functional, three dimensional models useful for providing the chemist with reliable predictions on the stereochemical course of an intended reaction. Furthermore, new approaches via catalytic antibodies (abzymes), together with the methods of site-directed mutagenesis of proteins are a new and promising route towards tailor-made enzymes for specific synthetic problems.

1.7 PRESENT WORK

This chapter presented an overview of biocatalysts in organic synthesis with special reference to lipase-catalyzed reactions and their applications.

Chapter 2 demonstrates (i) an efficient AmanoPS catalyzed resolution of (±)-methyl O-acetylmandelates in high yields (45-50%) and optical purities (90-99% ee) and (ii) an exceptional chemo-, regio-, and enanatioselctive lipase catalyzed (AmanoPS and PLAP) hydrolysis of (±)-*threo*-ethyl 3-(4-methoxyphenyl)-2,3-diacetoxy propionate to yield the optically pure diols in good yields (83-85%) and enantiopurity (86-98 %ee).

Chapter 3 demonstrates a first simple method to correlate the actual and observed regioselectivity/enantioselectivity in enzymatic hydrolysis of unsymmetrical/ *meso* diacetates. By suitable manipulation of reaction conditions (enzyme source, pH, solvent, temp etc) it may be possible to obtain either of the isomers by completely preventing or forcing the acyl migration. This method will be also useful in assessing the actual and observed regioselectivities in polyacylated systems including sugars.

Chapter 4 deals with a facile two-step synthesis of Ras Farnesyl-Protein Transferase Inhibitor, chaetomellic acid A with 89% overall yield and preparation of its racemic analogues. This is a new convenient and efficient method to model a variety of other dialkyl-substituted maleic anhydride derivatives. The results on biological screenings of these novel analogues are awaited.

1.8 REFERENCES

1. Langley, J. N. *Proc. Roy. Soc., B* **1906**, *78*, 170.
2. Ehrlich, P. *Brit. Med. J. II* **1913**, 353.
3. De Camp, W. H. *Chirality* **1989**, *1*, 2.
4. Buck. R. H.; Krummen, K. *J. Chromatogr.* **1984**, *315*, 279.
5. Däppen, R.; Arm, H.; Meyer, V. R. *J. Chromatogr*. **1986**, *373*, 1-20.
6. Schürig, V.; Nowotny, H. P.; *Angew. Chem. Int. Ed. Engl.* **1990**, *29*, 939.
7. (a) Dale, J. A.; Dull, D. L.; Mosher, H. S. *J. Org. Chem.* **1969**, *34*, 2543. (b) Dale, J. A.; Mosher, H. S. *J. Am. Chem. Soc.* **1973**, *95*, 512.
8. (a) Whitesides, G. M.; Lewis, D. W.; *J. Am. Chem. Soc.* **1970**, *92*, 6979. (b) Baldwin, J. J.; Abrams, W. B. *Drug Stereochemistry. Analytical Methods and Pharmacology*, (Eds. Wainer, I. W.; Drayer, D. E.), Marcel Dekker, New York, **1998**, p-311.
9. Hanson, J. R. *An Introduction to Biotransformations in Organic Chemistry* (Ed Mann, J.), W. H. Freeman, New York, **1995**.
10. (a) Zaks, A.; Klibanov, A. M. *Proc. Natl. Acad. Sci. USA* **1985**, *82*, 3192. (b) Klibanov, A. M. *CHEMTECH* **1986**, 354.
11. Rodionova, M. V.; Belova, A. B.; Mozhaev, V. V.; Martinek, K.; Berezin, I. V. *Dokl. Akad. Nauk. USSR* **1987**, *292*, 913; *C. A.* **1987**, *106*, 171823.
12. Chen, C. -S.; Sih, C. J. *Angew. Chem. Int. Ed. Engl.* **1989**, *28*, 695.
13. Chen, C. -S.; Wu, S. –H.; Girdaukas, G. Sih, C. J. *J. Am. Chem. Soc.* **1987**, *109*, 2813.
14. Zaks, A.; Klibanov, A. M. *Science* **1984**, *224*, 1249.
15. (a) Sakurai, T.; Margolin, A. L.; Russel, A. J.; Klibanov, A. M. *J. Am. Chem. Soc.* **1988**, *110*, 72. (b) Wong, C. –H. *Science* **1989**, *244*, 1145.
16. Jaeger K. E.; Ransac, S.; Dijkstra, B. W.; Colson, C.; Heuvel, M. van.; Misset, O. *FEMS Microbiol Rev.* **1994**, *15*, 29.
17. Gilbert, E. J. *Enzym. Microb. Technol.* **1993**, *15*, 634.
18. Wohlfahrt, S.; Jaeger, K. E. *BioEngineering* **1993**, *9*, 39.
19. Huang, A. H. C. *Lipases*, Elsevier, Amsterdam, **1984**, p-419.

20. Mukerjee, K. D.; Hills, M. J. *Lipases: Their Structure, Biochemistry and Application*, Cambridge University Press, Cambridge, **1994**, p-49.

21. Carriere, F.; Gargouri, Y.; Moreau, H.; Ransac, S.; Rogalska, E.; Verger, R. *Lipases: Their Structure, Biochemistry and Application*, Cambridge University Press, Cambridge, **1994**, p-181.

22. Carriere, F.; Bezzine, S, Verger, R. *J. Mol. Catal. B* **1997**, *3*, 55.

23. Aires-Barros, M. R.; Taipa, M. A.; Cabral, J. M. S. *Isolation and Purification of Lipases* (Eds.: Wolley, P., Petersen, S. B.) Cambridge University Press, Cambridge, **1994**, p-243.

24. De la Fournière, L.; Ivanova, M. G.; Blond, J. P.; Carriere, F.; Verger, R. *Colloids Surf. B* **1994**, *2*, 585.

25. Sarda, L.; Desnuelle, P. *Biochim. Biophys. Acta.* **1958**, *30*, 513.

26. (a) Winkler, F. K.; D' Arcy, A.; Hunziker, W. *Nature* **1990**, *343*, 771. (b) Brady, L..; Brzozowski, Z. S.; Derewenda, E.; Dodson, E.; Dodson, G.; Tolley, S.; Turkenberg, J. P.; Christiansen, L.; Huge-Jensen, B.; Norskov, L.; Thim, L.; Menge, U. *Nature* **1990,** *343*, 767.

27. Brzozowski, A. M.; Derewenda, U.; Derewenda, Z. S.; Dodson, G. G.; Lawson, D. M.; Turkenburg, J. P.; Bjorkling, F.; Huge-Jensen, B.; Patkar, S. A.; Thim, L. *Nature* **1991**, *351*, 491.

28. Nieuwenhuis, B. *The CEC Bridge Lipase Project* Eds.: Alberghina, L.; Schmid, R. D.; Verger, R.), VCH, Weinheim, **1990**, p-3.

29. Noble, M. E. M.; Cleasby, A.; Johnson, L. N.; Frenken , L. G. J.; Egmond, M. R. *FEBS Lett.* **1993**, *331*, 123.

30. Uppenberg, J.; Patkar, M. T.; Hansen, S.; Jones, A. *Structure* **1994**, *2*, 293.

31. Hjorth, A.; Carriere, F.; Cudrey, C.; Wöldike, H.; Boel, D.; Lawson, D. M.; Ferrato, F.; Cambillau, C.; Dodson G. G.; Thim, L.; Verger, R. *Biochemistry* **1993**, *32*, 4702.

32. Verger, R. *Trends Biotechnol.* **1997**, *15*, 32

33. (a) Faber, K. *Biotransformations in Organic Chemistry*, Springer, Heidelberg, **1995**. (b) Sheldon, R. *Chirotechnology: Industrial Synthesis of Optically Active Compounds*, Marcel Dekker, New York, **1993**.

34. Kaufmann, H. P.; Keller, M. C. *Fette Siefen* **1937**, *44*, 105.

35. Theil, F. *Chem. Rev.* **1995**, *95*, 2203.

36. Kazlauskas, R.; Weissfloch, A. N. E.; Rappaport, A. T.; Cuccia, L. A. *J. Org. Chem.* **1991**, *56*, 2656.

37. Milkovic, D.; Canadi, D.; Petrovic, J.; Stankovic, S.; Ribár, B.; Argay, G.; Kálmán, A. *Tetrahedron* **1984**, *25*, 1403.

38. Mohr, P.; Waespe-Sarcevic, N.; Tamm, C.; Gawronska, K.; Gawronski, J. K. *Helv. Chim. Acta* **1983**, *66*, 2501.

39. Luyten, M.; Müller, S.; Herzog, B.; Kreese R. *Helv. Chim. Acta* **1987**, *70*, 1250.

40. Kitazume, T.; Sato, T.; Ishikawa, N. *Chem. Lett.* **1984**, 1811.

41. Schneider, M. P.; Engel, N.; Boensmann, H. *Angew. Chem. Int. Ed. Engl.* **1984**, *23*, 67.

42. Kurihara, M.; Kamiyama, K.; Kobayashi, S.; Ohno, M. *Tetrahedron Lett.* **1985**, *26*, 5831.

43. Nagao, Y.; Kume, M.; Wakabayashi, R. C.; Nakamura, T.; Ochiai, M. *Chem. Lett.* **1989**, 2139. (b) Gais, H. -J.; Bulow, G.; Zatorski, A.; Jentsch, M.; Maidonis, P.; Hemmerele, H.; *J. Org. Chem.* **1989**, *54*, 5115.

44. Jones, J. B.; Francis, C. J. *Can. J. Chem.* **1984**, *62*, 2578.

45. Bloch, R.; Guibe-Jample, E.; Girard, C. *Tetrahedron Lett.* **1985**, *26*, 4087.

46. Ito, Y.; Shibata, T.; Arita, M.; Sawai, H.; Ohno, M. *J. Am. Chem. Soc.* **1981**, *103*, 6739.

47. Niwayama, S.; Kobayashi, S.; Ohno, M. *Tetrahedron Lett.* **1988**, *29*, 6313.

48. Iriuchijima, S.; Hasegawa, K.; Tsuchihashi, G. I. *Agric. Biol. Chem.* **1982**, *46*, 1907.

49. Wang, Y. –F.; Wong, C. –H. *J. Org. Chem.* **1988**, *53*, 3127.

50. Breitgoff, D.; Laumen, K.; Schneider, M. P. *J. Chem. Soc., Chem. Commun.* **1986**, 1523.

51. Hemmerle, H.; Gais, H. –J. *Tetrahedron Lett.* **1987**, *28*, 3471.

52. Naemura, K.; Takahashi, N.; Chikamatsu, H.; *Chem. Lett.* **1988**, 1717.

53. Suemune, H.; Mizuhara, Y.; Akita, H.; Sakai, K. *Chem. Pharm. Bull.* **1987**, *35*, 1741.

54. Laumen, K.; Schneider, M. P. *Tetrahedron Lett.* **1985**, *26*, 2073.

55. Seebach, D.; Eberle, M. *Chimia* **1986**, *40*, 315.

56. Kirk, D. N. *Natl. Prod. Rep.* **1984**, *1*, 209.

57. Pearson, A. J.; Bansal, H. S.; Lai, Y. –S. *J. Chem. Soc., Chem. Commun.* **1987**, 519.

58. Schneider, M. P.; Engel, N.; Honicke, P.; Heinemann, G.; Gorisch, H. *Angew. Chem. Int. Ed. Engl.* **1984**, *23*, 66.

59. Ladner, W. E.; Whitesides, G. M. *J. Am. Chem. Soc.* **1984**, *106*, 7250.

60. Morimoto, Y.; Terao, Y. Achiwa, K. *Chem. Pharm. Bull.* **1987**, *35*, 2266.

61. Klunder, A. J. H.; van Gastel, F. J. C.; Zwannenburg, B. *Tetrahedron Lett.* **1988**, *29*, 2697.

62. Sonnet, P. E.; Moore, G. G. *Lipids.* **1988**, *23*, 955.

63. Engel, K. H.; Bohnen, M.; Tressl, R. *Z. Lebensm. Unters. Forch.* **1989**, *188*, 144.

64. Kirchner, G.; Scollar, M. P.; Klibanov, A. M. *J. Am. Chem. Soc.*, **1985**, *107*, 7072.

65. Gerlach, D.; Lutz, D.; Mey, B.; Schreier, P. *Z. Lebensm. Unters. Forch.* **1989**, *189*, 141.

66. Marples, B. A.; Roger-Evans, M.; *Tetrahedron Lett.* **1989**, *30*, 261.

67. Hamaguchi, S. Hasegawa, J.; Kawahadra, H.; Watanabe, K.; *Agric. Biol. Chem.* **1984**, *48*, 2055.

68. Fuganti, C.; Graselli, P.; Servi, S.; Lazzarini, A.; Casati, P. *Tetrahedron* **1988**, 44, 2575.

69. Crout, D. H. G.; Gaudet, V. S. B.; Laumen, K.; Schneider, M. P. *J. Chem. Soc., Chem. Commun.* **1986**, 808.

70. Washausen, P. Grebe, H.; Kieslich, K.; Winterfield, E. *Tetrahedron Lett.* **1989**, *30*, 3777.

71. Bianchi, D.; Cabri, W.; Cesti, P.; Francalani, F.; Rama, F. *Tetrahedron Lett.* **1988**, *29*, 2455.

72. Jones, J. B. *Tetrahedron* **1986**, *42*, 3351.

73. Laumen, K.; Breitgoff, D.; Seemayer, R.; Schneider, M. P.; *J. Chem. Soc., Chem. Commun.* **1989**, 148

74. Ohta, H.; Matsumoto, Tsutsumi, S.; Ihori, T. *J. Chem. Soc., Chem. Commun.* **1989**, 485.

75. Gutman, A. L.; Zuobi, K.; Boltansky, A. *Tetrahedron Lett.* **1987**, *28*, 3861.

76. Gutman, A. L.; Bravdo, T. *J. Org. Chem.* **1989**, *54*, 4263.

77. Gutman, A. L.; Oren, D.; Boltanski, A.; Bravdo, T. *Tetrahedron Lett.* **1987**, *28*, 5367.

78. Yamamoto, Y.; Yamamoto, K.; Nishioka, T.; Oda, J. *Agric. Biol. Chem.* **1988**, *52*, 3087.

79. Ngooi, T. K.; Scilimati, A.; Guo, Z. –W.; Sih, C. –H. *J. Org. Chem.* **1989**, *54*, 911.

80. Margolin, A. L.; Crenne, J. –Y.; Klibanov, A. M. *Tetrahedron Lett.* **1987**, *28*, 1607.

81. Therisod, M.; Klibanov, A. M. *J. Am. Chem. Soc.* **1987**, *109*, 3977.

82. Kirk, O.; Bjoerkling, F.; Godtfredsen, S. E. *J. Chem. Soc., Chem. Commun.* **1989**, 934.

83. Kloosterman, M.; Mosmuller, E. W. J.; Schoemaker, H. E.; Meijer, K. M. *Tetrahedron Lett.* **1987**, *28*, 2989.

84. Hennen, W. J.; Sweers, H. M.; Wang, Y. –F.; Wong, C. –H. *J. Org. Chem.* **1988**, *53*, 4939.

85. Inch, T. D. *Tetrahedron* **1984**, *40*, 3161.

86. Holla, E. W. *Angew. Chem. Int. Ed. Engl.* **1989**, 1057.

87. Miazawa, T.; Takitani, T.; Ueji, S.; Yamada, T.; Kuwata, S. *J. Chem. Soc., Chem. Commun.* **1988**, 1214.

88. Bucciarelli, M.; Forni, A.; Moretti, I.; Prati, F. *J. Chem. Soc., Chem. Commun.* **1988**, 1614.

89. Bucciarelli, M.; Forni, A.; Moretti, I.; Prati, F. *Tetrahedron Asymmetry* **1990**, *1*, 5.

90. Kori, M.; Itoh, K.; Sugihara, H. *Chem. Pharm. Bull.* **1987**, *35*, 2319.

91. Bevinkatti, H. S.; Newadkar, R. *Tetrahedron Asymmetry* **1990**, *1*, 583.

92. Gotor, V.; Brieva, R.; Rebolledo, F. *Tetrahedron Lett.* **1988**, *29*, 6973.

93. Therisod, M. *J. Organomet. Chem.* **1989**, *361*, C8.

94. Nakamura, K. Ishihara, K.; Ohno, A.; Uemura, M.; Nishimura, H.; Hayashi, Y. *Tetrahedron Lett.* **1989**, 746.

95. Hashizume, R.; Tanaka, Y.; Funada, T. (Nippon Oil and Fats Co.), JP-A 03019693 A2, **1991**,[*Chem. Abstr.* **1991**, *115*, 90690]

96. (a) Holemans, P. M. J.; Schijf, K. P. A. M.; van Putte, de Mans, T. (Unilever, N.V.; Uniliver PLC), EP-B 209176 A1, **1987**, [*Chem. Abstr.* **1987**, *106*, 118502]. (b) Moore, H (Unilever PLC), GB-B 2185990 A1, **1987**, [*Chem. Abstr.* **1987**, *107*, 174780]

97. a) Baumann, H.; Bühler, M.; Fochem, H.; Hirsinger, F.; Zoeblein, H.; Falbe, J. *Angew. Chem. Int. Ed. Engl.* **1988**, *27*, 41. (b) Sonntag, N. O. V.; *J. Am. Oil Soc.* **1982**, *59*, 795A.

98. (a) Moore, S.; Quinlan, P. *INFORM*, **1993**, *4*, 579. (b) Partali, V.; Kvittengen, L.; Sliwka, H. –R.; Anthonson, T. *Angew. Chem. Int. Ed. Engl.***1996**, *35*, 329.

99. Boel, E.; Christensen.; T.; Woldike, H. (Novo Nordisk AS), US-A 5536661, **1996**, [*Chem. Abstr.* **1996**, *125*, 160364].

100. Pottie, M.; Van der Eycken, J.; Vandewalle, M.; Dewanckele, J. M.; Roper, H. *Tetrahedron Lett.* **1989**, *30*, 5319.

101. Tamaura, Y.; Takahashi, K.; Kodera, Y.; Mihama, Y.; Saito, Y.; Yuji, I.; Inanda, Y. *Biochem. Biophys. Res. Commun.* **1987**, *142*, 291.

102. Takahashi, K.; Tamaura, Y.; Kodera, Y.; Saito, Y.; Inanda, Y.; Sweers, H. M.; Wong, C. –H. *J. Am. Chem. Soc.* **1986**, *108*, 6431.

103. Wolf. A. M.; Showell, M. S. *Enzymes in Detergency* (Eds.: Van Ee, J. H.; Misset, O.; Baas, E. J.), Marcel Dekker, New York, **1997**, p-107.

104. Gaskin, D. J. H.; Bovanget, A. H.; Turner, N. A.; Vulfson, E. N. *Bichem. Soc. Trans.* **1997**, *25*, 128.

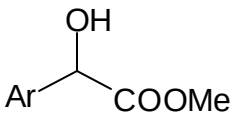
105. Moore, J. C.; Arnold, F. H. *Nature Biotechnol.* **1996**, *14*, 458.

CHAPTER 2

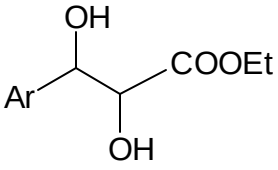
LIPASE CATALYZED CHEMO-/ REGIO- AND ENANTIOSELECTIVE HYDROLYSIS OF (±)-METHYL *O*-ACETYLMANDELATES AND (±)-*threo*-ETHYL 2,3-DIACETOXY-3-ARYLPROPIONATES.

2.1 INTRODUCTION

This chapter deals with the lipase-mediated resolution of compounds (±)-methyl mandelates **I** and (±)-*threo*-ethyl 2,3-dihydroxy-3-arylpropionates **II** *via* their respective acetates. The purpose was to study the chemoselectivity of the enzymes for the acetate/diacetate hydrolysis in the presence of carbmethoxy/ethoxy functions. The utility of enantiopure mandelates is well proven, while the enantiopure diols **IIa**/**b** are of pharmaceutical interest. Both the enantiomers of **IIa** are precursors for side chain of the anti-cancer agent taxol and both the enantiomers of **IIb** are the precursors of a clinically used antihypertensive drug, (+)-diltiazem.



I a/b



II a/b

a = phenyl b = *p*-anisyl

2.2 STRATEGY

The enzymatic (source: pig liver acetone powder) hydrolysis of (±)-methyl *O*-acetylmandelate has been shown to be chemo- and enantio-selective, with the *O*-acetate hydrolyzed in the presence of methyl ester in moderate enantioselectivity.[1] In pursuit of a better enantioselectivity we investigated the hydrolysis with a different lipase.

We have also investigated selectivity in hydrolysis of (±)-*threo*-ethyl 2,3-diacetoxy-3-arylpropionates, which provides an example with additional hydroxy group as in a glycol system, compared to (±)-methyl *O*-acetylmandelate. This would help us in a better understanding of the chemoselectivity obtained and to examine whether there is any

regioselectivity as well. Further, this substrate would be a good example to demonstrate the three different types of selectivities, *viz*: chemo-, regio-, and enantioselectivity in enzyme reactions. It was rationalized that by directing the lipase hydrolysis on the diacetoxy function of (±)-*threo*-ethyl 2,3-acetoxy-3-arylpropionates, the enzyme might have a better enantioselectivity and hence we attempted the enzymatic hydrolysis on (±)-*threo*-ethyl 2,3-diacetoxy-3-aryl propionates. This would be particularly interesting since the literature reports on the lipase catalyzed hydrolysis of (±)-*threo*-methyl 2,3-dihydroxy-3-arylpropionates indicate that the hydrolysis occurs on the carbmethoxy moiety. While the optical purities obtained in one of the case is very poor,[2] % ee values have not been mentioned in the second report.[3]

Section A deals with the resolution of (±)-methyl *O*-acetylmandelates using AmanoPS lipase and Section B describes lipase catalyzed resolution of (±)-*threo*-ethyl 2,3-dihydroxy-3-arylpropionates along with the results and discussion.

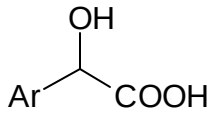
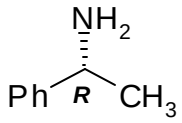
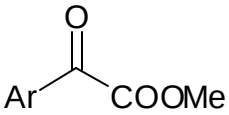
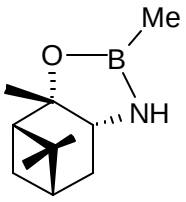
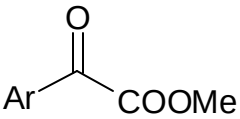
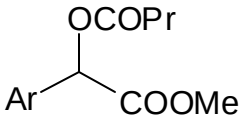
SECTION A

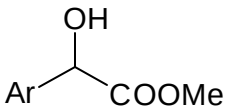
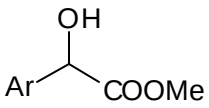
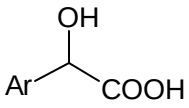
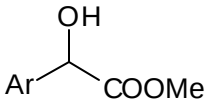
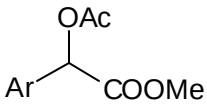
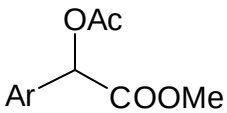
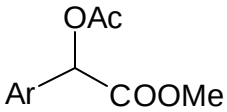
2.3 METHYLMANDELATES: BACKGROUND

The enantiopure methylmandelates have been prepared by conventional resolution (entry 1, Table **1**), asymmetric reduction (entry 2, Table **1**), or by microbial/enzymatic conversions (entries 4-11, Table **1**). In microbial resolutions, one of the isomers in a racemate is utilized by the organism leaving the other isomer in high % ee and the yields are often less than 50%, with one of the isomer consumed. Enzymatic esterification on the hydroxy group is known to be low yielding process with a moderate to high optical purity (entry 6, Table **1**) or high yielding process accompanied by good optical purity (entry 5, Table **1**). Satisfactory to modest results have been obtained in lipase catalyzed

transesterification on the esters of the hydroxy function of methylmandelates (entry 4, Table **1**). Lipase catalyzed hydrolysis targeted on the acetoxy function of (±)-methyl *O*-acetylmandelate by PLAP (pig liver acetone powder) is known to give moderate optical purity (entry 9, Table **1**). The enzymatic reduction of benzoylformic acid derivative involves the use of costly cofactor requirements. Table **1** depicts a few examples on enzymatic/microbial conversions, known to yield enantiopure derivatives of mandelic acid. The literature on the methods to offer optically pure mandelates is very vast and no pretension has been claimed in completeness of this aspect.

Table 1: Enzymatic/ Microbial Conversions Yielding Chiral Derivatives of Mandelic Acid.

No.	Starting material	Method	Yield, % ee, product	Ref.
1		 (Classical resolution)	~50%,very high, *R*(+)-mandelic acid	4
2		 (Asymmetric reduction)	55-65%, 60%ee, *S*-methyl mandelate	5
3		bacteria *L. kefir* (Asymmetric enzymatic reduction).	94-99%, NA, *S*-methyl mandelate	6
4		a. Lipase catalyzed hydrolysis b. Lipase catalyzed tranesterification	a. 40%, 99%ee, *S*-isomer b. 60%, 99%ee, *R*-isomer	7

5		Immobilized lipoprotein lipase (tranesterification)	53%, 82:93 of *S*-acetate: *R*-hydroxy]	8
6		CLEC (cross linked enzyme crystal catalyzed hydrolysis)	24%, 30:96 *R*-ester : *S*-acid	9
7		a. *A. bronchosepticus* (oxidation of only *S*-isomer to keto-form) b. *S.faecalis* (reduction keto-form to *R*-isomer)	85-90%, 99%, R-isomer	10
8		Penicillin-G acylase (hydrolysis)	50%,15-20%ee, *R*-acid	11
9		PLAP (hydrolysis on -OAc group)	44:56, 57:44 of *S*-hydroxy : *R*-acetate	1
10		*Rhizopus nigricans* (hydrolysis on -OAc group)	24%, 59%ee *S*-methyl mandelate	12
11		*Bacillus subtilis* var *niger* (hydrolysis on -OAc group)	Low conversion 73 : 47 of *R* :*S*-isomer	13

In light of these literature methods, we have employed AmanoPS catalyzed resolution of (±)-methyl *O*-acetylmandelate (**2**) as discussed below.

2.4 PRESENT WORK

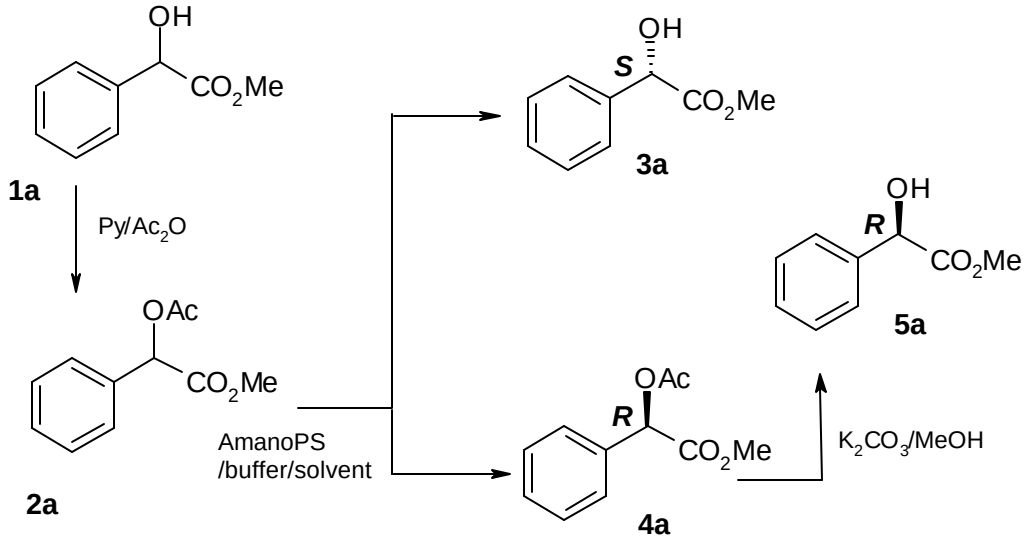
AmanoPS is a powdered form of lipase, isolated from *Pseudomonas cepacia*. The powder is not immobilized, and can be filtered from the reaction mixture and reused. It is one of the most versatile catalyst in chiral synthesis, especially on secondary alcohols. There is a vast literature on resolution of carboxylic esters, *O*-acetates, and on secondary alcohols (either by hydrolysis or *via* transesterification). The enzyme has optimum pH of 7.0, and is active in the range of pH 6-9. The optimum temperature is within the range of ambient temperature to 50 ^{o}C. This form of the enzyme was used in all the experiments in this thesis.

2.4.1 Results and Discussion: Methylmandelates

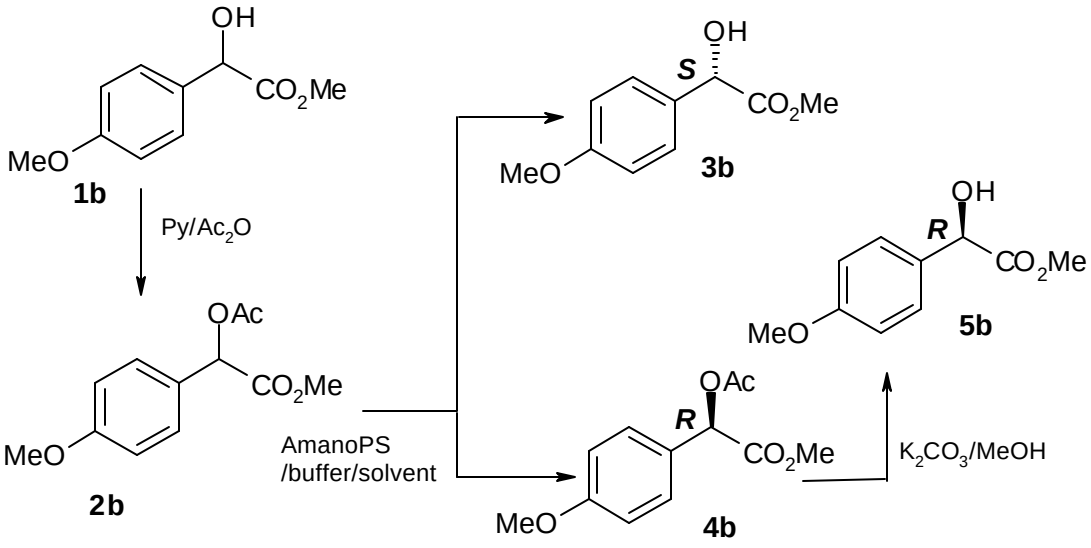
The (±)-methylmandelate on treatment with Ac_2O/Py for 24 h at rt furnished (±)-methyl *O*-acetylmandelate (**2a**) in 98% yield. The AmanoPS catalyzed biphasic enzymatic hydrolysis of **2a** in benzene plus petroleum ether (1:2) and 5 mM sodium phosphate buffer of pH 7.0 at rt yielded a mixture of methyl mandelate (**3a**) and methyl *O*-acetylmandelate (**4a**) in quantitative yield. The mixture on silica gel column chromatographic separation gave pure **3a** and **4a** in 47% and 50% yields respectively. In the ^{1}H NMR spectrum of the hydroxy ester **3a**, carbmethoxy peak was seen to be intact by the presence of the methyl signal at δ 3.50 while no peak at δ 2.20 was seen for the methyl group of the acetyl moiety. *The hydrolysis is hence ~100% chemoselective, the O-acetate gets hydrolysed rather than carbmethoxy group (-COOMe)*. The optical rotations measured for both the products when compared with the literature values helped in assigning the configuration at C-2 of methyl mandelate (**3a**) to be *S*-isomer and that methyl *O*-acetylmandelate (**4a**) to be *R*-isomer. High optical purities, 98-99% ee for the *S*-hydroxy

ester **3a** and 90-92% ee for the *R*-acetate **4a** were observed. The optical purity of **3a** was further established by ^{1}H NMR of its MTPA derivative[14] (98-99% ee), revealing a very high enantioselectivity in the enzymatic hydrolysis of **2a**. The compound **4a** was deacylated in presence of catalytic amount of K_2CO_3 in methanol at room temperature in 1 h to obtain **5a** in 95-96% yield. The optical purity of **5a** was determined by specific rotation and ^{1}H NMR of its MTPA-derivative (90-92% ee).

Scheme 1a



Scheme1b



Similarly the enzymatic hydrolysis of **2b** (phenyl is substituted with *p*-anisyl) was performed to obtain **3b** and **5b** (*via* **4b**) and the results obtained are summarized in Table **2**. It is seen that the presence of *p*-methoxy group on the aromatic ring does not show any noticeable change in the course of the AmanoPS catalyzed hydrolysis of both the (±)-methyl *O*-acetylmandelates.

Principle of MTPA derivatization to obtain the enantiomeric excess: Diastereomeric esters and amides have been prepared from optically pure *S*- or *R*-α-methoxy-α-trifluoromethylphenylacetyl chloride and various secondary alcohols/amines. The NMR spectra of the esters prepared from racemic alcohol/amine is recorded and the proton/fluorine signals of the resulting diastereomers of such a racemate are assigned. The relative integral ratios of the signals corresponding to the two diastereomers would then be 1:1. Similarly the enantiopure compound whose % ee is to be measured, is also derivatized to obtain the diastereomers along with diasteromer of the unwanted isomer. The relative integral ratios of the diasteromers signals are calculated to obtain the enantiomeric excess of the enantiopure compound.[14] Depiction of this concept is shown in the Figure **1** and for spectral dataof the racemic methylmandelate (page) and with our results on *S*-methylmandelate (page).

$$\%\ ee = \frac{Isomer_{major} - Isomer_{minor}}{Isomer_{major} + Isomer_{minor}} X100$$

Figure **1**

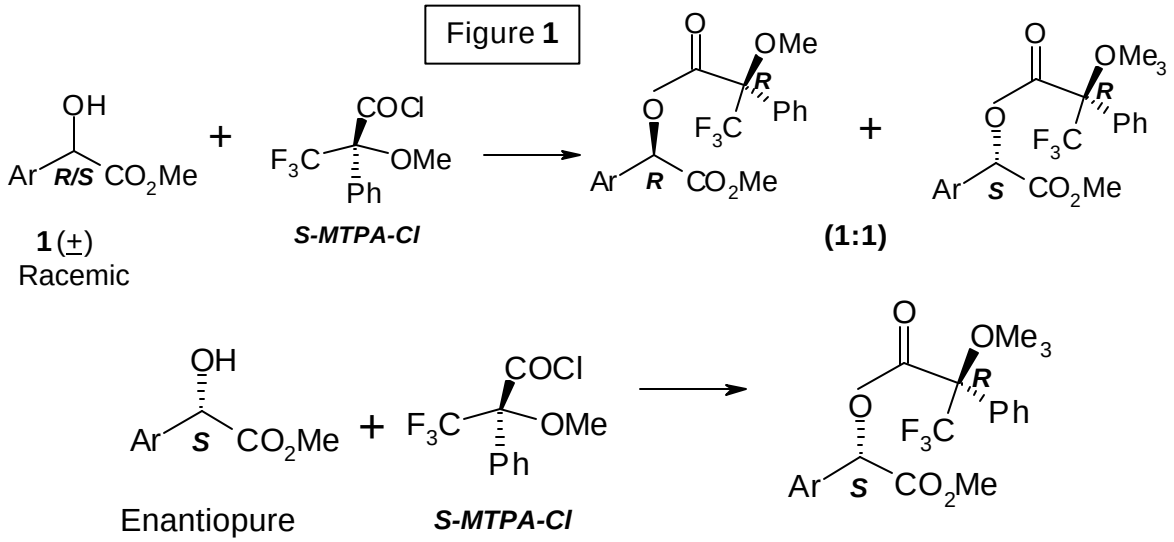


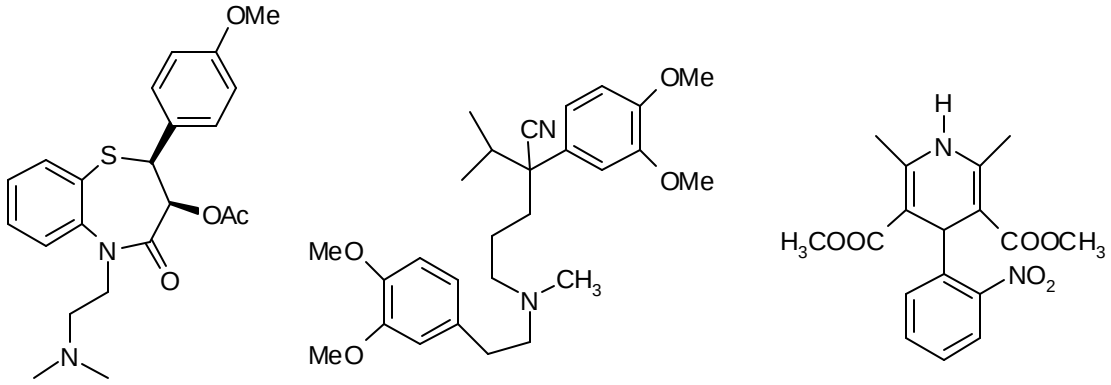
Table 2: Biphasic Amano PS Catalyzed Hydrolysis of Methyl *O*-Acetylmandelates

No	Solvent system (1:2)	Time (h)	(*S*)-methyl mandelate **- 3**			(*R*)-methyl *O*-acetylmandelate - **4**		
			%yield	$[\alpha]^{D}_{25}$	% ee	%yield	$[\alpha]^{D}_{25}$	% ee
1a	C_6H_6 : Pet. ether	144	47	$+144^{o}$ (a)	98 -99	50	-146^{o} (b)	90 - 92
	C_6H_6 : *iso*-octane	168	46	$+144^{o}$ (a)	98 -99	50	-145^{o} (b)	90 - 92
1b	C_6H_6 : Pet. ether	144	42	$+139^{o}$ (c)	96- 97	55	-124^{o} (b)	72 - 74
	C_6H_6 : *iso*-octane	168	44	$+140^{o}$ (c)	97 -98	54	-135^{o} (b)	78 - 80

(a) Optical rotation in methanol (c=1.0). (b) Optical rotation in benzene (c=1.9). (c) Optical rotation in ethanol (c=1).

SECTION B

2.5 (+)-DILTIAZEM: BACKGROUND

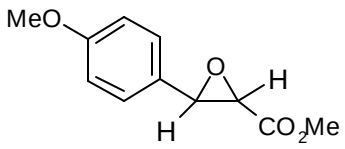


A **B** **C**

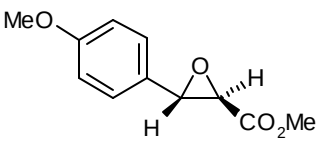
There are three major groups of calcium channel blockers, identified by structural types and represented here (**A**) diltiazem (**B**) verapamil (**C**) nifedipene. Diltiazem, (+)-*cis*-(2*S*,3*S*)-3-acetoxy-5-[2-(dimethylamino)-ethyl]-2,3-dihydro-2-(4-methoxyphenyl)-1,5-benzothiazepin-4(5H)-one (**A**) is one of the most potent calcium channel blockers[15] in

clinical use in more than 100 countries. At present the commercially used chemical route consists of condensation of a key intermediate, (±)-*trans*-*p*-methoxyphenylglycidic acid (PGA) methyl ester[16] with *o*-aminothiophenol (*o*-ATP), followed by late stage-resolution.[17] Since only the (2*S*, 3*S*)-isomer elicits the desired biological effect, diltiazem is marketed as a single isomer. The success of diltiazem has stimulated significant activity in related chemical synthesis, directed both to enantiomerically pure compund (EPC) syntheses[18] and also for their analogues.[19]

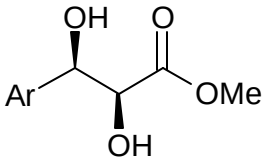
There are various routes known in the literature for the synthesis of (+)-diltiazem, and the essence of these indicate the following as the starting materials/intermediates for the same.



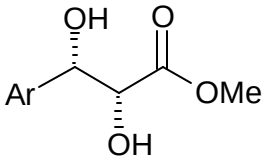
(±)-PGA Ester



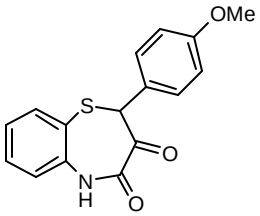
(2*R*,3*S*)-PGA-ester



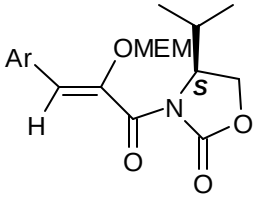
(2S,3R)-diol



(2*R*,3*S*)-diol



1,5-Benzothiazepine



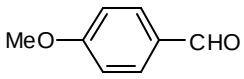
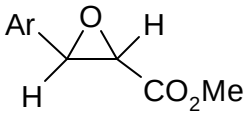
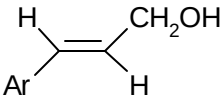
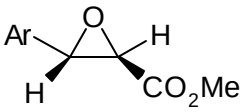
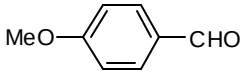
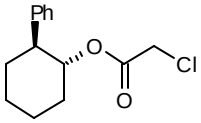
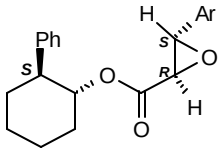
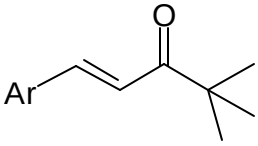
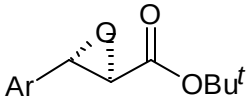
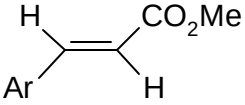
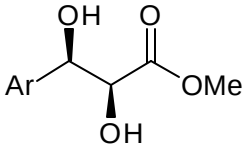
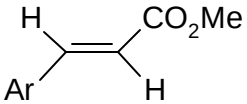
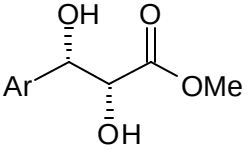
(Z)-α-Alkoxy Cinnamic Acid Derivative

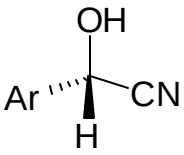
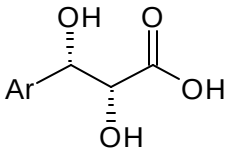
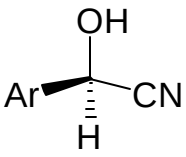
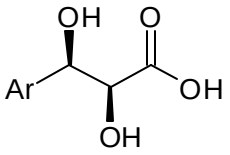
The methods for the synthesis of racemic/(2*R*, 3*S*)-PGA ester and the optically pure (2*R*, 3*S*)- and (2*S*, 3*R*)-diols are summarized in Table 3. Further the routes for the enzymatic conversions to obtain the chiral precursors to (+)-diltiazem are described in **Schemes 3-6**.

2.5.1 Synthetic Approaches for the (+)-Diltiazem Intermediates

The (±)-*trans*-*p*-methoxyphenylglycidic methyl ester (PGA-ester) or the optically pure (2*R*, 3*S*)-PGA-ester is used to condense with *o*-aminothiophenol (*o*-ATP) which is the key step and the later steps are as followed by Tanabe process.[15] The (±)-*trans*-*p*-methoxyphenylglycidic methyl ester (PGA-ester) is obtained by Darzens condensation of *p*-anisaldehyde with Me/Et-chloroacetate under basic conditions[20a] (entry 1, Table **3**). Similarly an asymmetric Darzen condensation should lead to the desired (2*R*, 3*S*)-PGA ester, but unfortunately this condensation using stoichiometric quantities of many expensive chiral bases showed very poor optical purity.[20b] Recently an improved procedure for Julia-Colonna asymmetric epoxidation of α,β-unsaturated ketones employing three-phase system comprising alkaline hydrogen peroxide, an organic solvent (toluene), and an insoluble polymer e.g. poly-L-leucine has been described to prepare (2*R*, 3*S*)-PGA ester (70% yield, 96 %ee entry 4, Table **3**) leading to diltiazem as single isomer (with high optical purities). While the preparation of optically pure (2*S*, 3*R*)- and (2*R*, 3*S*)-diols is accomplished by either Sharpless asymmetric dihydroxylation using OsO_4, dihydroquinine/dihydroquinidine on cinnamate ester derivative (entry 5/6, Table **3**) or alternatively from chiral cyanohydrin derived from *p*-anisaldehyde in the presence of Inoue dipeptide as a catalyst (entry 7/8, Table **3**). The optically pure diols are the alternate intermediates for the synthesis of (+)-diltiazem, the scheme from the same leading to (+)-diltiazem has been discussed at the end of this section.

Table 3: Asymmetric Methods Leading to Precursors of (+)-Diltiazem

No.	Starting material	Reaction Conditions	Product Yield (%ee/ de)	Ref
1		Darzen condensation $ClCO_2R$, base.		20a
2		Sharpless asymmetric epoxidation followed by oxidation.	 procedure patented	21
3		Asymmetric Darzen condensation. 	 54% yield ee/de not reported	22
4		Julia-Colonna epoxidation by poly-L-leucine, UHP, DBU, MCPBA.	 70% yield, >96% ee	23
5		Sharpless (ADH). OsO_4, Dihydroquinidine acetate, NMO	 80%, 98% ee	24
6		Sharpless (ADH). OsO_4, Dihydroquinine acetate, NMO	 80%, 97% ee	24

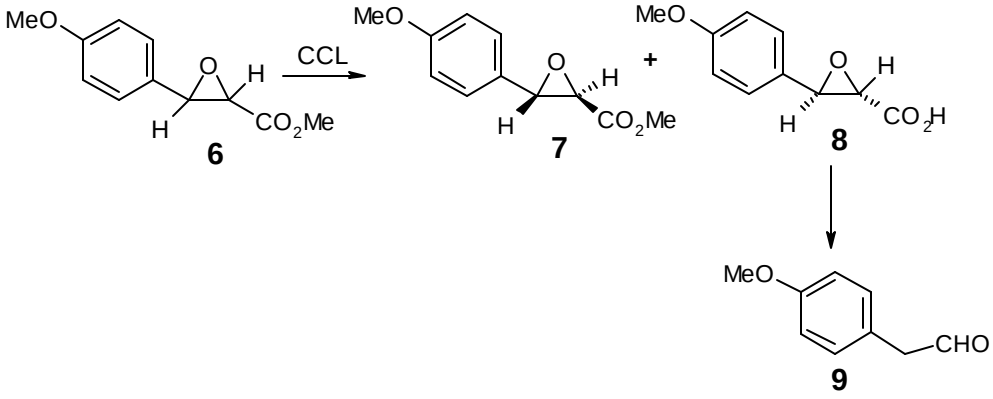
7		Inoue dipeptide catalyst 1. H_2/Ni, $NaHSO_3$ 2. $(OMe)_2CMe_2/H^+$ 3. KOH/H^+, H^+/MeOH	 % ee not reported	25
8		Inoue dipeptide catalyst 1. H_2/Ni, $NaHSO_3$ 2. $(OMe)_2CMe_2/H^+$ 3. KOH/H^+, H^+/MeOH	 % ee not reported	25

2.5.2 Enzymatic Routes

In recent years enzymatic reactions have been increasingly used for the optical resolution of several functionalized chiral molecules[26] as evident from chapter 1. There is a vast scope to obtain chiral precursors of (+)-diltiazem so as to get high enantiomeric purity under environmentally benign conditions. The literature report on enzymatically resolved precursors for (+)-diltiazem has been summarized below.

(i) An approach[27] discussed here uses hydrolysis of (±)-PGA methyl ester **6** in cyclohexane/water with CCL lipase in 1.5 h, where the (2*R*, 3*S*)-PGA ester **7** was found to be stable. The formed (2*S*, 3*R*)-PGA acid **8** decomposed to the corresponding aldehyde **9**

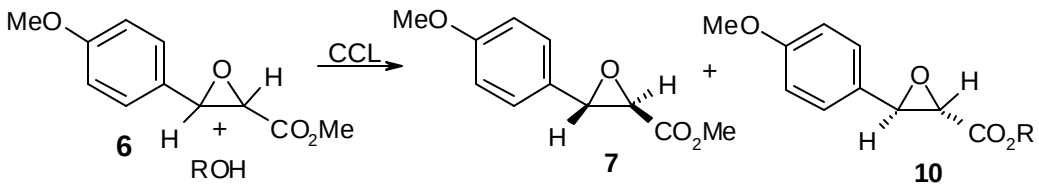
Scheme 2



which inactivated the enzyme. The unhydrolyzed isomer (2*R*, 3*S*)-PGA ester **7** (35% yield, 98% ee) is the one that is used for further synthetic route to diltiazem. This process is thus subtractive one wherein one of the isomer is not recovered.

(ii) Another report describes the resolution[28] of (±)-PGA methyl ester **6** aided by CCL catalyzed octanolysis in *tert*-pentyl alcohol as the solvent and octan-1-ol as the nucleophile. The reaction was over in 11 days giving the unreacted (2*R*, 3*S*)-PGA ester **7** in 37% yield with 88% ee and the transesterified (2*S*, 3*R*)-PGA ester **10**.

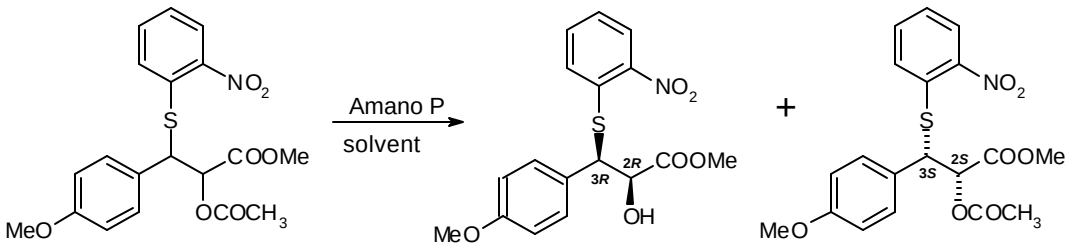
Scheme 3



(iii) Recently Tanabe Seiyaku Co. found an effective new route wherein the (2*R*, 3*S*)-PGA ester **7** obtained from lipase resolution (**Scheme 1/2**) was amidated to give the corresponding optically pure glycidamide[29] in 43% yield (99% ee), which was further condensed with *o*-ATP to give the key intermediate (95% 92:8 *threo:erythro*) of (+)-diltiazem.

(iv) Asymmetric hydrolysis[30] of water-insoluble *a*-acyloxy ester **11** [got from the condensation of (±)- (PGA-ester) with *o*-nitrothiophenol followed by acylation] was carried out using immobilized Amano P in water saturated organic solvent (*iso*octane : benzene) at 33 °C for 16 days. The reaction furnished both isomers (2*R*, 3*R*)-**12** in 44% yield with 98% ee and (2*S*, 3*S*)-**13** in 52% with 81% ee, of which **13** was used for the synthesis of (+)-diltiazem with known route. This strategy has the disadvantage of loosing 50% of the undesired isomer in a late stage resolution and hence is expensive.

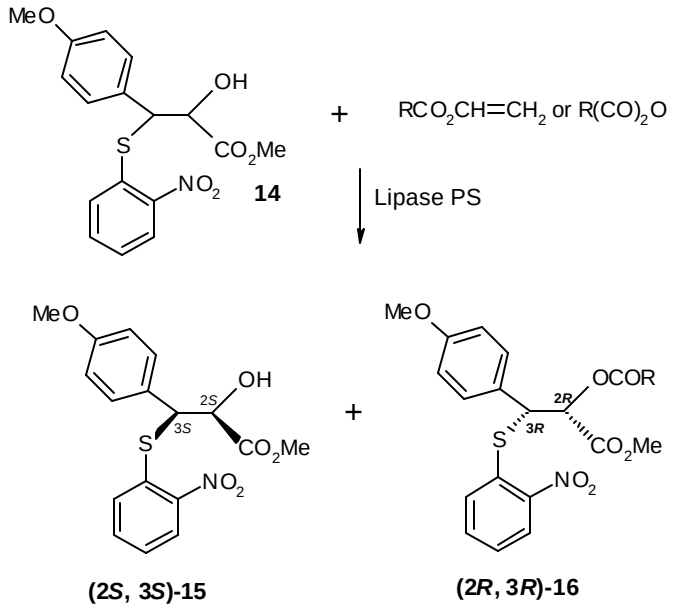
Scheme 4



11 **(2*R*, 3*R*)-12** **(2*S*, 3*S*)-13**

(v) Another method involves Lipase PS catalyzed acylation[28] of the (±)-hydroxy ester **14** by vinyl acetate and acid anhydrides in THF at 22 °C offering both the isomers (2*R*, 3*R*)-**15** with 95 % ee and (2*S*, 3*S*)-**16** with 95% ee in 50% yield for each, of which the later was used for the further synthetic route to (+)-diltiazem.

Scheme 5



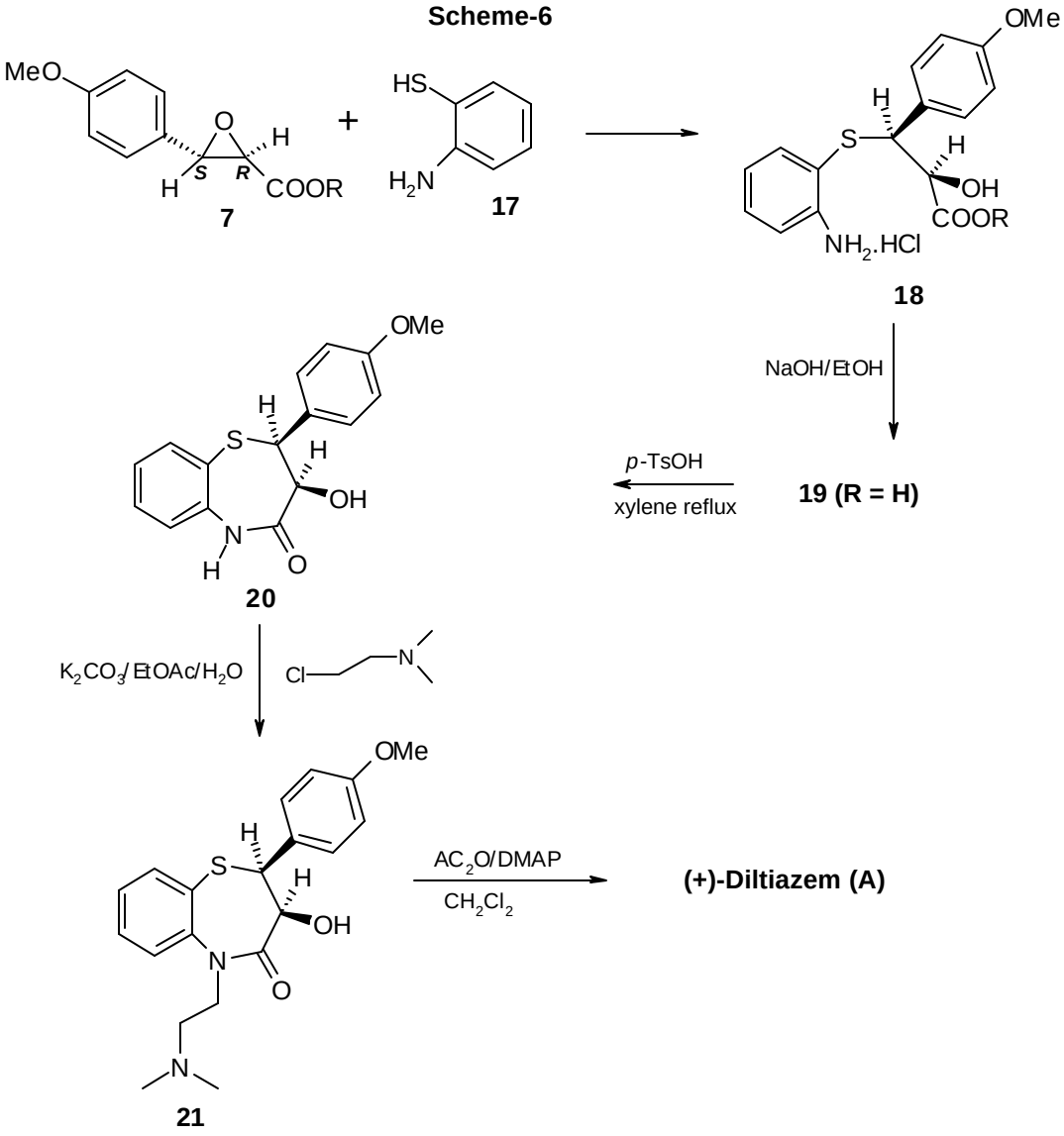
(2*S*, 3*S*)-15 **(2*R*, 3*R*)-16**

(vi) A new lipase from the culture of *Serratia marcescens* is known to enantioselectively hydrolyze[3] (±)-diol ester **IIb** and no information on yield and enantiomeric purities of the hydrolysed products is documented.

2.5.3 Conversion of (2*R*, 3*S*)-PGA Ester and (2*S*, 3*R*)- and (2*R*, 3*S*)-Diols to (+)-Diltiazem

As depicted in **Scheme 6** the (2*R*, 3*S*)-PGA ester **7** was condensed with *o*-ATP **17** (*o*-aminothiophenol) to give the adduct **18** with retention of configuration at the reacting

Scheme-6

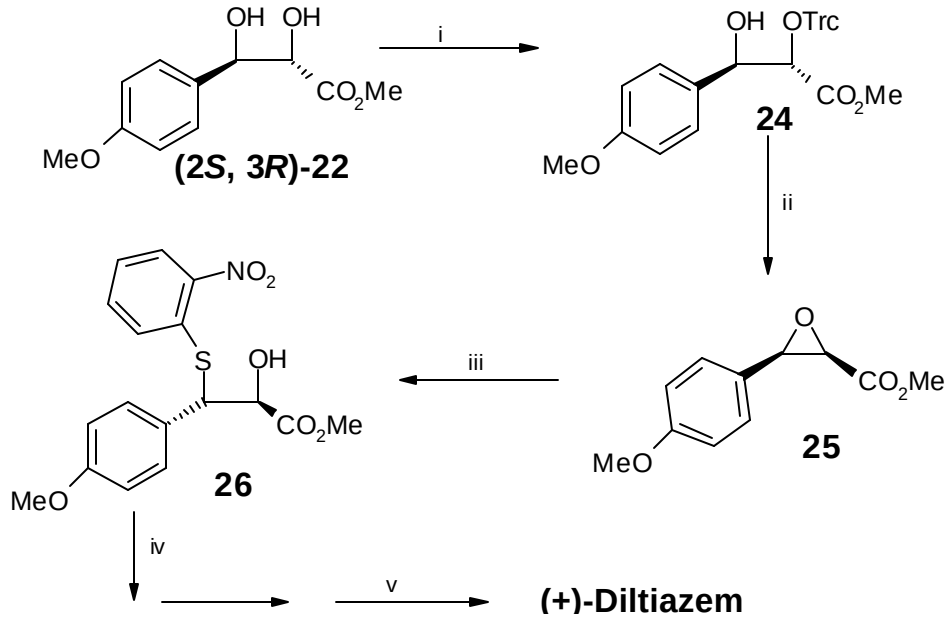


center. The process is perhaps thermally driven with the thiol acting as a weak acid, protonating the epoxide from the proximal side of the plane, leading to resonance-

stabilized *p*-quinone methide cation. If collapse of the resulting ion pair proceeds faster than migration of the thiol anion, the observed retention of configuration becomes comprehensible. The amine-ester **18** was hydrolyzed to give the corresponding acid which on further thermal cyclization gave the 1,5-benzothiazepine **20**, later N-alkylation followed by acylation of hydroxy function gave (+)-diltiazem.

An efficient synthesis of (+)-diltiazem by partially independent routes,[31] has been reported starting from enatiopure diols (2*S*, 3*R*)-**22** and (2*R*, 3*S*)-**23** which are synthesized either by Sharpless' asymmetric dihydroxylation[24] or by (*R*)- or (*S*)-4-methoxy benzaldehyde cyanohydrins in the presence of Inoue catalyst.[25]

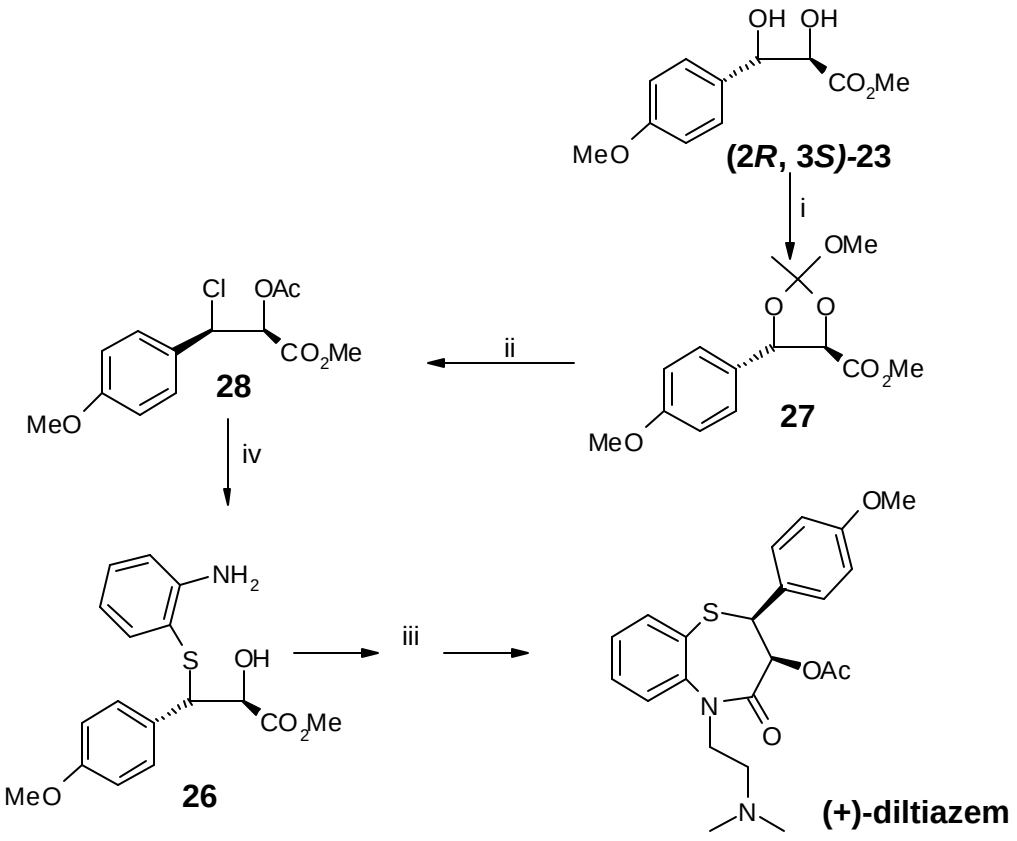
Scheme 7



Reagents and Conditions: i. Triscyl (Trc) chloride-pyridine, 95% ii. NaH-THF, 97%
iii. *o*-$O_2NC_6H_4SH$, $NaHCO_3$, EtOH, 60% iv. H_2, Pd-C, EtOAc: NaOH, EtOH H_2O, 95%
v. Ref. 22a, 81%

Conversion of (2*S*, 3*R*)-**22** into (*2S*, 3*S*)-thioether **26** involves reactions that lead to inversion of configuration at both the chiral centers (**Scheme 7**). Conversion of (2*R*, 3*S*)-**23** involves reactions leading to retention of configuration at both C-2 and C-3 centers (double inversion at C-3 center, **Scheme 8**).

Scheme 8



Reagents and Conditions: i. $MeCl(OMe)_3$, *p*-$MeC_6H_4SO_3H$, *ca.* 100% ii. Me_3SiCl, Et_3NHCl^- (trace) *ca.* 100% iii. *o*-$H_2NC_6H_4S^-K^+$ in DMF *ca.* 100% iv. ref 22a, 82%

2.6 RATIONALE FOR PRESENT WORK

From the above discussion it is evident that there is a scope for improving the resolution of the intermediates in diltiazem synthesis through approaches involving resolution at the earlier stages of synthesis for example, resolution of the diols [(±)-*threo*-

ethyl 2,3-dihydroxy-3-arylpropionates]. A major component of the work in this chapter addresses on this issue by attempting the preparation of optically pure diols (2*S*, 3*R*)-**22** and (2*R*, 3*S*)-**23** *via* enzymatic resolution of their racemic diacetyl derivatives using PLAP and AmanoPS lipase under various reaction conditions to screen the best conditions.

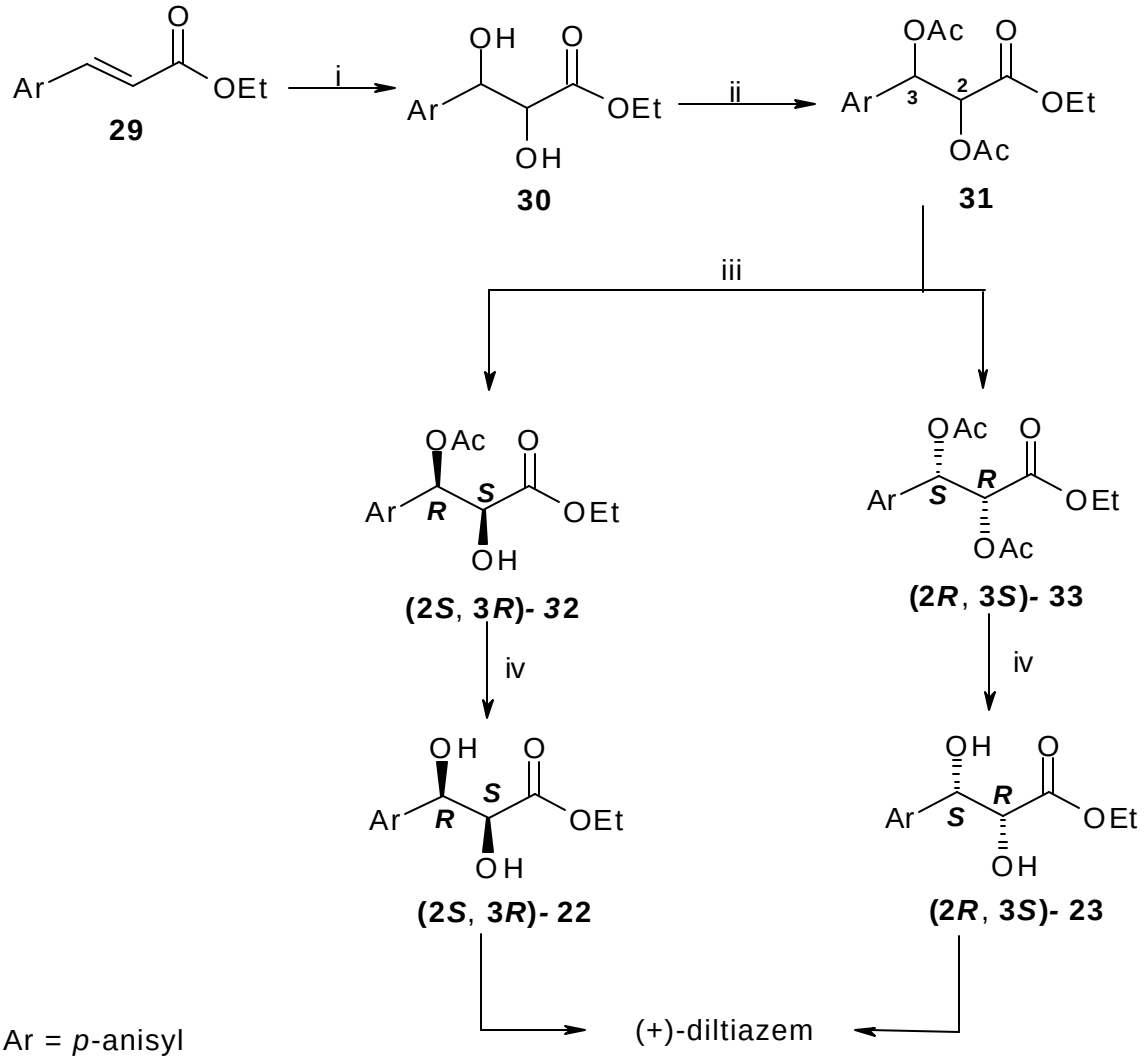
2.7 RESULTS

2.7.1 (±)-*threo*-Ethyl 2,3-Dihydroxy-3-(4-Methoxyphenyl)propionates

We launched a systematic program to study the enzymatic resolution of (±)-diacetate ethyl ester **31** using lipases from animal and microbial sources and herein, report a highly chemo-, regio-, and enantioselective lipase catalysed hydrolysis of (±)-**31** (**Scheme 9**) which leads to (+)-diltiazem precursors (2*S*, 3*R*)-diol **22** and (2*R*, 3*S*)-diol **23** in both high yield and optical purity. The (±)-diol ester **30** was prepared in 88% yield by dihydroxylation of (*E*)-ethyl 3-(4-methoxyphenyl)propeonate (**29**) with catalytic amount of osmium tetroxide[32] in presence of NMO in *t*-BuOH. Treatment of (±)-diol ester **30** with acetic anhydride/pyridine at rt. gave (±)-diacetate **31** in 92.0% yield. Considering the importance of the optically pure (2*S*, 3*R*)-(**22)** and (2*R*, 3*S*)-*threo*-ethyl 3-(4-methoxyphenyl)-2,3-dihydroxypropionate (**23)**, the diacetate was chosen for the enzymatic hydrolysis using enzymes from microbial and animal sources and in different solvents. The enzyme powders used were AmanoPS, PLAP, BLAP, PPL, Novozym-435 and Bioprotease-alk (all except PLAP and BLAP are commercial preparations). The biphasic enzymatic hydrolysis of (±)-diacetate **31** with enzymes Amano PS, pig liver acetone powder (PLAP) and pig pancreatic lipase (PPL) did take place, while the enzymes bovine liver acetone powder (BLAP), Novozym-435 and Bioprotease-alk were ineffective in hydrolysis. In control reactions without enzymes, the (±)-diacetate ester **31** remained

unreacted. In presence of the enzyme AmanoPS in phosphate buffer at pH 7.0 and *iso*-octane:benzene (2:1) solvent system, at 25 °C showed highest conversion (44.2%) of diacetate **31** to hydroxyacetate **32** along with unhydrolyzed diacetate **33** in 180 h as monitored by GC. The retention of the groups, ethyl ester and 3-acetoxy, in the

Scheme 9



hydrolysis product was indicated by the ^{1}H NMR spectrum of product mixture of **32** and **33**. The integration for the methyl (of *O*-acetyl) was less and also a new signal was observed

upfield to the proton of the C-2 hydrogen (from δ 5.32 to δ 4.37) suggesting the formation of hydroxyacetate **32**. The hydrolysis products were separated on a silica gel column to give hydroxyacetate **32** in 42% and the diacetate **33** in 51% isolated yields, indicating that the hydrolysis was both *chemo- and regioselective*. The optical purity of hydroxyacetate **32** was determined by measuring the (i) specific rotation, (ii) ^{1}H NMR of its complex with the chiral shift reagent, tris[3-(heptafluropropylhydroxymethylene)-(+)-camphorato]europium (III) and (iii) derivatization to corresponding MTPA ester with (*S*)-(+)-MTPA-Cl[14] and both the latter methods concurred to a 96% ee of the hydroxyacetate **32**. The C-2 H and C-3 H protons showed substantial shifts in the δ values, the integral ratios of these peaks were used to calculate the % enantiomeric excess.

The hydroxyacetate **32** was derivatized with MTPA-Cl (using pyridine as a base) in CH_2Cl_2 to give the resulting 2 diastereomers which were then recorded by ^{1}H NMR to calculate the % enantiomeric excess. The hydroxyacetate **32** and the diacetate **33** (obtained from Amano PS reaction in *iso*-octane-benzene) were individually hydrolyzed with potassium carbonate (catalytic amount) in dry ethanol to the corresponding diol **22** and diol **23** respectively, in 83-85% yield. The specific rotation for both the diols were measured and compared with the literature values. The diol **22** obtained from the solvolysis of hydroxyacetate **32** was assigned (*2S*, 3*R*) configuration and the diol **23** obtained from the solvolysis of diacetate **33** was assigned (*2R*, 3*S*) configuration. In order to check the optical purity of both the diols, the racemic diol **30** was derivatized with *S*-(+)-MTPA-Cl to give the two diastereomeric esters (2*S*, 3*R*)-bis-*R*-MTPA and (2*R*, 3*S*)-bis-*R*-MTPA. The optical purity of both diols as seen in ^{1}H NMR of their bis-MTPA derivatives, corresponded to 98% ee for **22** (page 94) and 86% ee for **23**. From these studies the hydrolysis of the diacetate **31** with both enzymes has been shown to give (2*S*, 3*R*)-

hydroxyacetate **32** and (2*R*, 3*S*)-diacetate **33**. The enzymes AmanoPS and PLAP not only show *chemo- and regioselectivity* but also *enantioselectivity* in the hydrolysis of (±)-diacetate **31**. The results are summarized in Table **4**.

Table 4: Biphasic Enzymatic Hydrolysis of (±)-Diacetate 31

No	Enzyme	Solvent (2:1)	Time (h)	(2*S-OH*,3*R-OAc*)- 32		(2*R*,3*S*)-diacetate 33	
				%conversion	*%ee*	*%conversion*	*%ee*
1	PPL	pet. ether/ benzene	140	11.5 (10.3)	92[a]	88.5 (83.2)	d
2	PLAP	pet. ether/ benzene	140	36.4 (35.7)	88[b]	63.5 (61.1)	51.3[c]
3	PLAP	diethyl ether	144	36.8 (31.9)	70[b]	61.5 (58.5)	d
4	PLAP	*iso*-octane/ benzene	156	20.0 (17.2)	96[a]	79.0 (77.1)	d
5	Amano PS	pet. ether/ benzene	100	40.0 (38.5)	98[b]	60.0 (57.1)	71.7[c]
6	Amano PS	*iso*-octane/ benzene	180	44.2 (42.0)	96-98[a-c]	55.7 (51.1)	86.0[c]

For entries 1-4, the reactions were carried out at 35^{o}C in 5mM sodium phosphate buffer at pH 8.0 and for entries 5, 6 reactions were done at 25^{o}C in 5mM sodium phosphate buffer at pH 7.0. % Conversion refers to estimation by GC and figures in bracket indicate isolated yield.

[a] by chiral shift reagent,

[b] by ^{1}H NMR of MTPA-derivative of hydroxyacetate **22**,

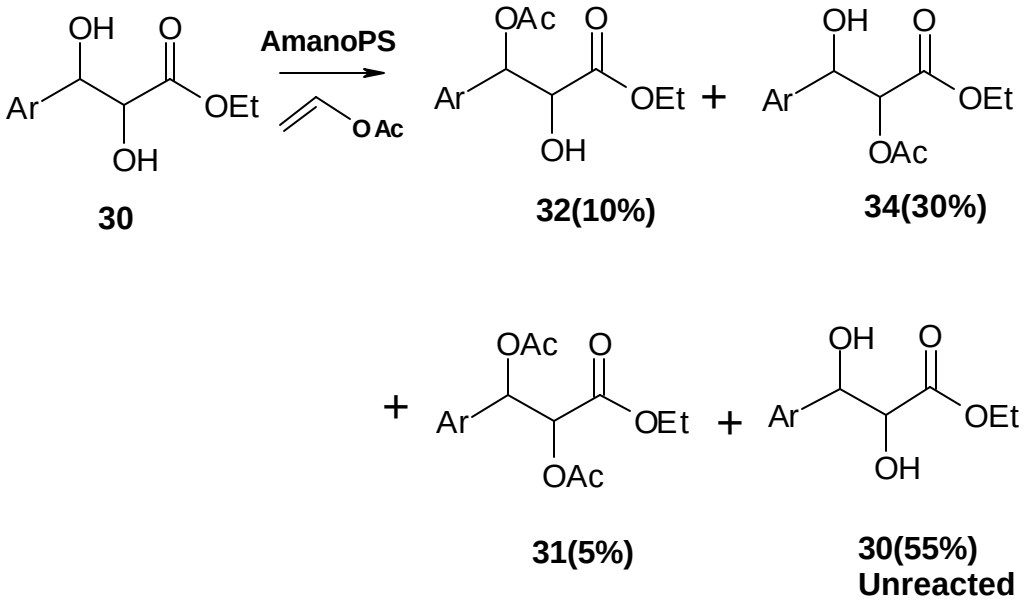
[c] by ^{1}H NMR of Bis-MTPA-derivative of corresponding diol

[d] not determined

2.7.2 Enzymatic Acylation of (±)-*threo*-Ethyl 3-(4-Methoxyphenyl)-2,3-dihydroxypropionate

Similarly Amano PS catalyzed acylation of (±)-**30** with vinyl acetate in anhydrous petroleum ether/benzene was also carried out to probe the selectivity of the enzyme for the esterification in anhydrous media. The enzyme catalyzed acylation of **30** with vinyl acetate in anhydrous pet.ether:benzene (2:1), at 25 °C was carried out and monitored for 96 h and upon 45% conversion gave a mixture of diacetate **31**, 2-hydroxy-3-acetoxy **32** and 2-acetoxy-3-hydroxy **34** derivatives in 5.2, 10.2 and 30.2 % respectively as estimated by ^{1}H NMR (page 95) indicating a poor regioselectivity.

Scheme 10



30 **32(10%)** **34(30%)**

31(5%) **30(55%) Unreacted**

2.7.3 (±)-*threo*-Ethyl 2,3-Dihydroxy-3-Phenylpropionates

The (±)-diol ester **30a** was prepared in 88% yield by dihydroxylation of (*E*)-ethyl 3-phenylpropeonate with catalytic amount of osmium tetroxide[32] in presence of NMO in *t*-BuOH. The treatment of (±)-diol ester **30a** with acetic anhydride/pyridine at room temperature gave the (±)-diacetate **31a** in 92% yield. Initially an enzymatic resolution of (±)-*threo*-ethyl 2,3-diacetoxyphenylpropionate **31a** in a biphasic system consisting of benzene : petroleum ether (1:2) and AmanoPS in 5 mM sodium phosphate buffer of pH 7.0 was attempted. The biphasic enzymatic hydrolysis of (±)-diacetate **31a** with enzymes Amano PS, pig liver acetone powder (PLAP) did take place while the enzymes pig pancreatic lipase (PPL), bovine liver acetone powder (BLAP), Novozym-435, *Candida cylindracea* lipase (CCL) and Bioprotease-alk were ineffective in hydrolysis. In control reactions without any enzymes, the (±)-diacetate ester **31a** remained unreacted proving absence of any chemical hydrolysis. The hydrolysis was monitored by tlc and GC. The reaction catalyzed by AmanoPS after 10 days showed just 10-12% conversion and similar observations were seen with PLAP. The reaction mixture on usual aqueous work-up and silica-gel column chromatographic purification gave *threo*-ethyl 2-hydroxy-3-acetoxyphenylpropionate **32a** (10%) and the diacetate **31a** (80%) whose structures were confirmed by ^{1}H NMR. Since the yield of the hydrolysis product was not encouraging, no efforts were made to determine the optical purity. Alternatively in attempts to increase the yield, other esters such as O-chloroacetate, O-butyrate, O-pivolate and O-*p*-nitrobenzoate were tried, but the enzymes AmanoPS and PLAP showed reluctance to hydrolyze these substrates. [For **30a**, **31a** and **32a** structures, see tabulated spectral data.]

2.8 DISCUSSION

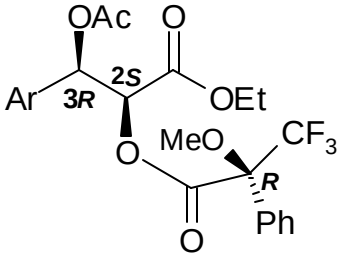
2.8.1 (±)-*threo*-Ethyl 2,3-Dihydroxy-3-(4-Methoxyphenyl)propionates

The biphasic enzymatic hydrolysis of (±)-diacetate **31** with enzymes Amano PS, pig liver acetone powder (PLAP) and PPL (pig pancreatic lipase) was successful, while the bovine liver acetone powder (BLAP), Novozym-435, *Candida cylindracea* lipase (CCL) and Bioprotease-alk were ineffective in hydrolysis. In control reactions without enzymes, the (±)-diacetate ester **31** remained unreacted, proving the absence of any accompanying chemical hydrolysis under the reaction conditions. The hydrolysis was monitored by GC and in order to achieve enantioselective kinetic resolution, it was necessary to arrest the hydrolysis before 50% conversion and so, with AmanoPS the hydrolysis was arrested at 44% conversion. By comparison with the assigned peaks of the racemic diol **30** in the literature, the ^{1}H chemical shifts for C 2-H and C 3-H in the diacetate **31** were assigned to be at δ 5.30 and 6.23 respectively (page 85, comparing with the corresponding peaks of the diol **30** as reported in the literature). The enzymatically hydrolyzed products of **31** after chromatographic purification and ^{1}H NMR spectral analysis were identified as hydroxyacetate **32** and diacetate **33.** On careful study of the spectra of the two compounds, it is evident that the signals corresponding to carbethoxy group (triplet and quartet) were intact in the hydrolyzed product hydroxyacetate **32**, suggesting *complete chemoselectivity in the hydrolysis of the diacetate **31** by both enzymes AmanoPS and PLAP*. Further, as seen from the ^{1}H NMR spectral analysis of the diacetate **31**, the C2 proton appears at δ 5.30 and C3 proton at δ 6.23 (page 85). In comparison with the diacetate ester **31**, a large upfield shift (δ 4.37, Δδ= 0.93) was noticed for the chemical shift of C2 proton upon formation of the hydroxyacetate **32**, relative to a minor difference observed in the shift of C3 proton (Δδ, 0.18). This gives a direct proof for the assigned

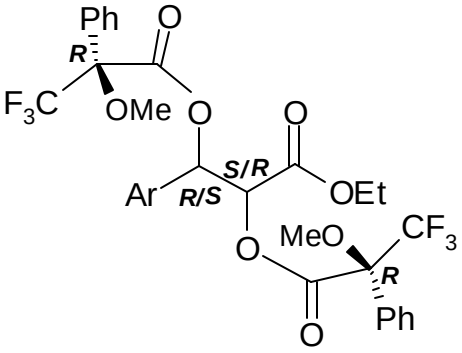
structure for **32**. *Thus the enzymatic hydrolysis was operative only on the 2-acetoxy group, amounting to a complete regioselectivity.* No established data is available in literature on the hydroxyacetate **32** and diacetate **33** to estimate their optical purity. Hence both compounds were subjected to solvolysis in EtOH/K_2CO_3 to yield the respective optically active diols **22** and **23,** which are known in the literature. [24c] The diol-**22** was *levo* rotatory and this resulted from the hydroxyacetate **32**, which confirms the stereochemistry for the hydroxyacetate as (2*S*, 3*R*)-3-(4-methoxyphenyl)-2-hydroxy-3-acetoxypropionate **(32)**. Similarly the diol-**23** resulting from the diacetate **33** was *dextro* rotatory, confirming the stereochemistry of the diacetate **33** as (2*R*, 3*S*)-3-(4-methoxyphenyl)-2.3-diacetoxypropionate. The optical purities of the hydroxyacetate **32**, diols **22** and **23** were confirmed by derivatization with *S*(+)-α-methoxy-α-trifluoromethylphenylaceticacid chloride (MTPA-Cl). The NMR spectrum of bis-MTPA derivative of racemic diol **30** was taken as the standard for assignments. The enantiomeric purity was first measured by specific rotation and on comparison with literature values it was found to be more than 95% for (2*S*, 3*R*)-diol **22** and about 90% for (2*R*, 3*S*)- diol **23**. The enantiomeric purity of the hydroxyacetate could not be ascertained by rotation and hence it was complexed with the chiral shift reagent, tris[3-(heptafluropropylhydroxymethylene)-(+)-camphorato]europium (III) for ^{1}H NMR study. In ^{1}H NMR spectrum of the complexed hydroxyacetate **32** the C2 and C3 protons of the diastereomers were clearly resolved and their relative integrations indicated 96% ee of the hydroxyacetate **32**. This was further confirmed by derivatizing **32** with *S*(+)-MTPA-Cl and in NMR of the resultant diastereomeric mixture, the protons at C2-H and C3-H are resolved (figure **2**). The relative integrations of C2 protons in the two diastereomers were then calculated to prove the enantiomeric excess to be 96%. Similarly the diols (±)-**30**, (2*S*, 3*R*)-**22** and (2*R*, 3*S*)- **23** with *S*-(+)-MTPA-Cl were derivatized to give respective

diastereomers as shown in the figure **2**. Apart from several other peaks, the C2 and C3 protons of the racemic bis-MTPA-ester of diol (±)-**30** split nicely to give peaks of equal integration (C3 proton: *S*-isomer,δ 6.48 and *R*-isomer, δ 6.52, C-2 proton: *S*-isomer, δ 5.41 and *R*-isomer, δ 5.47, see page 92 and 93). In the ^{1}H NMR spectrum of (2*S*, 3*R*)-bis-MTPA ester of diol **22**, the C3 proton in the complex for the 3(*S*)-H at δ 6.47 and 3(*R*)-H at δ 6.52 showed peaks with relative integral ratios 0.01:0.99 respectively to give 98% ee of (2*S*, 3*R*)-diol **22** (page 94). Similarly ^{1}H NMR spectrum of (2*R*, 3*S*)-bis-MTPA ester of diol **23** diastereomer 2(*R*)-H at δ 5.40 and 2(*S*)-H at δ 5.48 showed peaks with relative integral ratios 0.93:0.07 respectively to give 86% ee of (2*S*, 3*R*)-diol **23**.

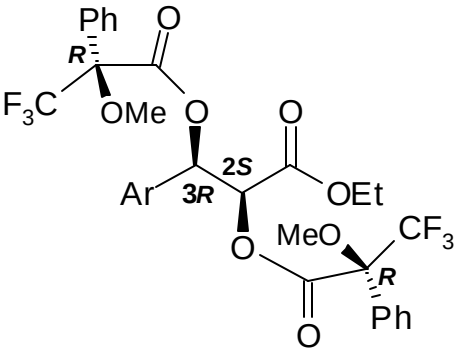
Figure **2**



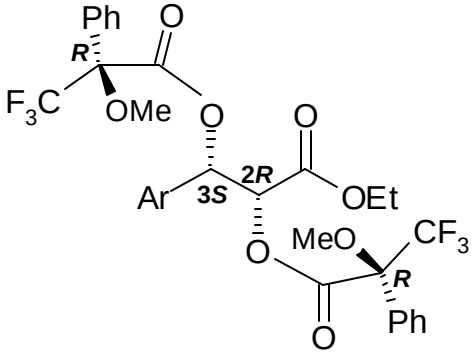
MTPA-derivative of 32



***Bis*-MTPA-derivative of 30**



MTPA-derivative of 22



MTPA-derivative of 23

2.8.2 (±)-*threo*-Ethyl 2,3-Dihydroxy-3-phenylpropionates

The biphasic enzymatic hydrolysis of (±)-diacetate **31a** with enzymes Amano PS, pig liver acetone powder (PLAP) did take place while the enzymes pig pancreatic lipase (PPL) bovine liver acetone powder (BLAP), Novozym-435, *Candida cylindracea* lipase (CCL) and Bioprotease-alk were ineffective in hydrolysis. In control reactions without enzymes, the (±)-diacetate ester **31a** remained unreacted, proving absence of any accompanying chemical hydrolysis under the reaction conditions. The hydrolysis was monitored by tlc and after 24-48 h a lower moving spot with respect to the diacetate was observed. The conversion was also monitored with GC. The reaction after 10 days showed just 10-12% conversion and similar observations were seen with PLAP. The reaction mixture was purified by silica-gel column chromatography and the structure of the hydrolyzed product was assigned by ^{1}H NMR. The chemical shifts of the C2-H and C3-H of the diacetate-**31a** appeared as doublets at δ 5.32 and δ 6.28. In the product, the doublet corresponding to δ 5.32 of the C2-H showed considerable upfield shift to δ 4.36-4.45 (dd, $\Delta\delta$ 0.90) whereas the doublet peak at 6.28 (C3-H) shifted to 6.10 ($\Delta\delta$ 0.18). This indicates that the *O*-acetyl group on the C2 carbon was regioselectively hydrolyzed, retaining the C3 acetate completely intact and the hydrolyzed product was identified as *threo*-ethyl 2-hydroxy-3-acetoxyphenylpropionate (**32a**). The reactions were not further studied, as the yields were poor (10-12%). The enzyme AmanoPS and PLAP are thus chemo-and regioselective in hydrolysis of the diacetate **31a,** while the enantioselectives were not determined.

2.9 SUMMARY

2.9.1 Methylmandelates

In summary we have demonstrated an enzymatic resolution of (±)-methyl *O*-acetylmandelates **2** using Amano PS to yield *S*-methylmandelates **3**, (44-47%; 97-99% ee) and *R*-methylmandelates **5** (50-54%;80-92% ee). [33]

2.9.2 (±)-*threo*-Ethyl 2,3-Dihydroxy-3- Arylpropionates

We have also demonstrated a convenient and efficient enzymatic strategy for resolution of racemic (±)-*threo*-ethyl 3-(4-methoxyphenyl)-2,3-acetoxypropionate (**31**) *via* selective hydrolysis of one of the vicinal diacetates rather than the well known enantioselective hydrolysis of the terminal ester (carbethoxy) function. The specificity in hydrolysis is remarkable since it is not only chemoselective (*O*-acetate rather than ester), but also accompanied by regio- (2-*O*-acetate is hydrolyzed and not 3-*O*--acetate) and high enantioselectivity (hydrolysis of only 2*S* *O*-acetate).In this above example given a multiple choice of ester functions in a single molecule, the enzyme prefers to hydrolyze a specific one; the selective hydrolysis of the central (C2) O-acetyl ester is perhaps favored due to electronic assistance from the oxygens of the neighboring ester groups. Enzymatic resolution as reported here, followed by solvolysis, enables access to a pair of enantiomerically pure diols (**22** and **23**),[34] both of which can be processed by partially separate routes to obtain (+)-diltiazem.[31] Enzymatic acylation of (±)-*threo*-Ethyl 3-(4-methoxyphenyl)-2,3-dihydroxypropionate **30** with vinyl acetate catalyzed by AmanoPS and PLAP showed poor regioselectivity, though the time required is much less with respect to the hydrolysis of its diacetate. The results on the enzymatic hydrolysis of (±)-*threo*-Ethyl

2,3-diacetoxyphenylpropionate **31a** under various conditions of solvents, enzyme powders, ester derivatives were not encouraging and the studies were not further pursued.

2.10 EXPERIMENTAL SECTION

The enzymatic hydrolysis was monitored by TLC (ethyl acetate:pet ether 40:60) and GC (HP-I, 3m megabore column). Chiral shift reagent (Aldrich) and (+)-MTPA (Sigma) were used for NMR estimation of % ee. The enzymes used were PPL (Sigma), Amano PS (Amano Pharmaceuticals), Novozym-435 (Novo Nordisk Fermentation Ltd.), Bioprotease-alk (Biocon India Ltd.) and PLAP, BLAP refer to crude extracts of lipases (from pig and bovine source respectively). The enzyme AmanoPS (800-1420U) was obtained from Amano pharmaceuticals. [1 Unit corresponding to micromoles of butyric acid (estimation by GC) liberated from glyceryl tributyrate per minute per milligram of enzyme powder].

(±)- Methyl *O*-acetylmandelate (2a): To a solution of methyl mandelate **1a** (2.0 g) in pyridine (10 mL) was added acetic anhydride (5 mL), and the reaction mixture was kept in dark for 24 h at rt, later it was concentrated in vacuo. The oily residue was dissolved in ether (75 mL) and the organic layer was washed with water, aqueous bicarbonate, brine and dried over Na_2SO_4. Concentration in vacuo followed by column purification gave pure **2a** as a thick oil in 98% yield.

AmanoPS catalyzed hydrolysis of (±) methyl *O*-acetylmandelate (2a): A solution of **2a** (208 mg, 1 mmol) in benzene:pet ether / benzene : isooctane (1 : 2) mixture (20 mL) was added to a suspension of AmanoPS lipase (250 mg) in 5 mM aqueous sodium phosphate buffer of pH 7.0 (7 mL). The reaction mixture was stirred at 25^0C for 144 / 168 h and filtered through celite and extracted with ethyl acetate (15 mL x 3). The combined organic layer was washed with water, aqueous bicarbonate, brine, dried over Na_2SO_4 and concentration under vacuo gave an oily residue, which was subjected to column

chromatography. Elution with ethyl acetate:pet ether gave **4a** as a thick oil, 104 mg (50% yield) and **3a**, 78mg (47% yield) mp 56-58 ^{0}C. Similarly **2b** on AmanoPS catalyzed hydrolysis furnished **4b** as a thick oil (44% yield) and **3b** (54% yield) mp 63-65 ^{0}C.

(*R*)- Methyl mandelate (5a): To a solution of **4a** (100 mg) in dry methanol (3 mL) was added anhydrous K_2CO_3 (2-3 mg) and the reaction mixture was stirred at rt for 1 h, then it was filtered through celite and washed with methanol. The combined methanol solution on concentration in vacuo gave a thick oil which was dissolved in ethyl acetate (15 mL) and dried over Na_2SO_4. Removal of ethyl acetate in vacuo followed silica gel column chromatographic purification gave **5a**, 76 mg (95% yield) mp 56-58 ^{0}C. Similarly **5b** was obtained from **4b** (95- 96% yield) mp 63-65 ^{0}C.

MTPA-Ester of (±)-Methyl mandelate 1a: To a solution of methylmandelate **1a** (17 mg, 0.1 mmol) in pyridine (0.4 mL) was added 0.5 M solution of (*S*)-(+)-MTPA-Cl in EDC (0.4 mL) at 0 ^{0}C and the reaction mixture was further stirred for 4 h at rt. Usual aqueous work-up followed by ether extraction (10 mL x 2), aqueous bicarbonate wash, brine wash, Na_2SO_4 drying and concentration in vacuo furnished corressponding MTPA derivative as thick oil.

MTPA-Ester of (*S*)-Methylmandelate 3a: To a solution of (*S*)-methylmandelate **3a** (17 mg, 0.1 mmol) in pyridine (0.4 mL) was added 0.5 M solution of (*S*)-(+)-MTPA-Cl in EDC (0.4 mL) at 0 ^{0}C and the reaction mixture was further stirred for 4h at rt. Usual aqueous work-up followed by ether extraction (10 mL x 2), aqueous bicarbonate wash, brine wash, Na_2SO_4 drying and concentration in vacuo furnished

MTPA-Ester of (*R*)-Methylmandelate 5a: To a solution of (*R*)-methylmandelate **5a** (17 mg, 0.1 mmol) in pyridine (0.4 mL) was added 0.5 M solution of (*S*)-(+)-MTPA-Cl in EDC (0.4 mL) at 0 ^{0}C and the reaction mixture was further stirred for 4 h at rt. Usual aqueous

work-up followed by ether extraction (10 mL x 2), aqueous bicarbonate wash, brine wash , Na_2SO_4 drying and concentration in vacuo furnished

MTPA-Ester of (±)-Methyl-μ-hydroxy-μ-(4-methoxyphenyl)acetate 1b was prepared similarly.

MTPA-Ester of Methyl (*S*)-μ -hydroxy-μ-(4-methoxyphenyl)acetate 3b: was prepared similarly.

MTPA-Ester of Methyl (*R*)- μ-hydroxy-μ-(4-methoxyphenyl)acetate 5b: was prepared similarly.

(±)-*threo*-Ethyl 3-(4-methoxyphenyl)-2,3-dihydroxypropionate (**30**). To a stirred solution of **29** (2.06 g, 10 mmol) in *t*-butanol (25 mL) was added 60% aqueous NMO (15 mL) solution and the reaction mixture was cooled to 0 °C. To this mixture, was added dropwise a solution of OsO_4 (40 mg, 0.02 mmol) in *t*-butanol (1 mL). The reaction mixture was stirred at rt. for 24 h, a fresh lot of NMO solution (10 mL) was added and stirred at rt. for further 24 h and reaction was quenched with an aqueous solution of sodium sulphite (25 mL, 20%). The mixture was stirred at rt. for 1 h, filtered through celite, concentrated in vacuo and extracted with ethyl acetate (50 mL x 3). The organic layer upon usual work-up gave a residue which was chromatographed on silica gel to obtain pure diol **30**, 2.1 g (88% yield).

(±)-*threo*-Ethyl 3-(4-methoxyphenyl)-2,3-diacetoxypropionate (**31**). To a stirred solution of diol **30** (1.92 g, 8 mmol) in pyridine (20 mL) was added acetic anhydride (15 mL) and the reaction mixture was kept in dark for 48 h at rt. On usual aqueous work-up, ether extraction followed by concentration and column purification gave pure diacetate **31**, 2.38 g (92% yield).

Amano PS Lipase catalyzed hydrolysis of (±)-*threo*-ethyl 3-(4-methoxyphenyl)-2,3-diacetoxy propionate (31). A solution of (±)-diacetate **31** (324 mg, 1 mmol) in *iso*-octane: benzene (2:1) mixture (20 mL) was added to a suspension of Amano PS lipase (275 mg) in 5 mM aq. sodium phosphate (10 mL) at pH 7.0. The reaction mixture was stirred at 25 ^{o}C for 180 h after which it was filtered through celite and extracted with ethyl acetate (25 mL x 3). The combined organic layer after usual work-up and concentration in vacuo gave an oily residue which was subjected to column chromatography. Elution with 12% ethyl acetate:pet ether gave ethyl (2*R*,3*S*)-3-(4-methyoxyphenyl)-2,3-diacetoxypropionate (**33**), 165 mg (51% yield):[$\alpha]^{25}_{D}$ = + 26.4^{o} (c 2.0, $CHCl_3$) and with 18% ethyl acetate:pet ether gave ethyl (2*S*,3*R*)-3-(4-methoxyphenyl)-2-hydroxy-3-acetoxypropionate (**32**) 118 mg (42% yield).

Hydrolyses reactions with other enzymes were similarly carried out on (±)-diacetate **31** (1 mmol) in organic solvent (20 mL) and buffer solution (10 mL) using PPL (500 mg), PLAP (500 mg), Amano PS (275 mg), Novozym-435 (300 mg), BLAP (500 mg) and Bioprotease-alk (275 mg). For details see Table **4**.

PLAP Lipase catalyzed hydrolysis of (±)-*threo*-ethyl 3-(4-methoxyphenyl)-2,3-diacetoxypropionate (31). A solution of (±)-diacetate **31** (324 mg, 1 mmol) in *iso*-octane: benzene (2:1) mixture (20 mL) was added to a suspension of Amano PS lipase (275 mg) in 5 mM aq. sodium phosphate (10 mL) at pH 8.0. The reaction mixture was stirred at 37 ^{o}C for 140 h after which it was filtered through celite and extracted with ethyl acetate (25 mL x 3). The combined organic layer after usual work-up and concentration in vacuo gave an oily residue, which was subjected to column chromatography. Elution with 12% ethyl acetate:pet ether gave ethyl (2*R*, 3*S*)-3-(4-methyoxyphenyl)-2,3-diacetoxy propionate (**33**), 180 mg 61% yield):[$\alpha]^{25}_{D}$ = + 26.2^{o} (c 2.0, $CHCl_3$) and with 18% ethyl acetate:pet

ether gave ethyl (2*S*, 3*R*)-3-(4-methoxyphenyl)-2-hydroxy-3-acetoxypropionate (**32**) 91 mg (36% yield).

MTPA-Ester of (2*S*, 3*R*)-hydroxyacetate 32. To a solution hydroxyacetate **32** (14 mg, 0.05 mmol) in pyridine (0.1 mL) was added 0.5 M solution of (*S*)-(+)-MTPA-Cl[14] in EDC (0.2 mL) at 0 °C and the reaction mixture was stirred at rt. for 12 h. On usual aqueous work-up, ether extraction and concentration in vacuo furnished MTPA-derivative of **32** as thick oil. The ^{1}H NMR spectrum of (2*S*, 3*R*)-hydroxyacetate **32** (10 mg) in $CDCl_3$ (0.5 mL) with chiral shift reagent, tris [3-(heptafluropropylhydroxymethylene)-(+)-camphorato] europium (III) (10 mg) in $CDCl_3$ (0.2 mL) indicated 96% ee [as seen by signal ratio (0.02 :0.98) for benzylic protons at δ 6.67 and 6.77 respectively].

Ethyl (2*S*, 3*R*)-2,3-dihydroxy-3-(4-methoxyphenyl) propionate (22). To an ice-cold solution of hydroxyacetate **32** (100 mg) in dry ethanol (2 mL) was added anhyd. K_2CO_3 (5 mg) and the reaction mixture was stirred at rt. for 3 h after which it was filtered through celite, and washed with ethanol. The combined ethanol solution on concentration in vacuo gave a thick oil which was dissolved in ethyl acetate (15 mL), washed successively with water, brine and dried over Na_2SO_4. Removal of ethyl acetate in vacuo followed by silica gel column chromatographic purification gave (2*S*, 3*R*) diol **22**, 73 mg (85.8% yield): mp 87-89 °C; $[\alpha]^{24c}_D$ = - 4.8° (c 2.0, $CHCl_3$).

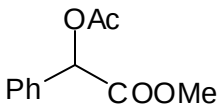
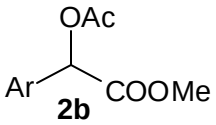
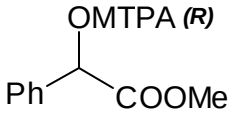
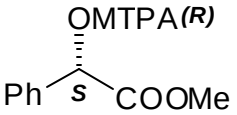
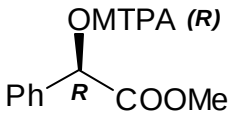
Ethyl (2*R*, 3*S*)-2,3-dihydroxy-3-(4-methoxyphenyl)propionate (23). The reaction of diacetate **33** (100 mg) with anhyd. K_2CO_3 (5 mg) in dry ethanol (2 mL) for 16 h similarly furnished (2*R*,3*S*) diol **23**, 62 mg (83.7% yield): mp 88-90 °C; $[\alpha]^{25}_D$ = + 4.3° (c 2, $CHCl_3$). [lit.[24c] + 5.0° (c 1.08, $CHCl_3$)]

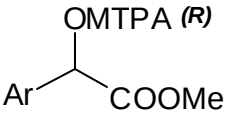
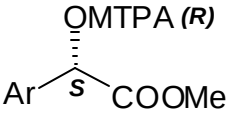
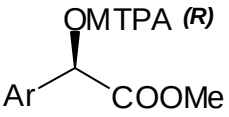
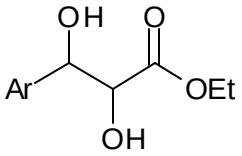
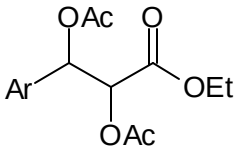
Bis-MTPA-Esters of (±)-diol 30, (2*S*,3*R*)-diol 22 and (2*R*,3*S*)-diol 23. To a solution of diol (12 mg, 0.05 mmol) in pyridine (0.1 mL) was added 0.5 M solution of (*S*)-(+)-MTPA-Cl[14] in EDC (0.3 mL) at 0 °C and the reaction mixture was further stirred for 8 h at 0 °C. Usual work-up furnished corresponding bis-MTPA-derivatives as thick oil, for analysis by ^{1}H NMR.

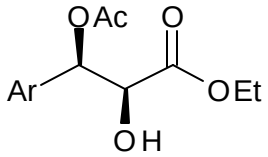
(±)-*threo*-Ethyl 2,3-dihydroxyphenylpropionate (30a): Dihydroxylation of ethyl cinnamate was carried as described earlier for (±)-diol **30** with similar yields.

(±)-*threo*-Ethyl 2,3-diacetoxyphenylpropionate (31a): Diacetylation of (±)-diol **30a** was done similar to preparation of (±)-diacetate **31** with similar yields.

(±)-*threo*-Ethyl 2-hydroxy, 3-acetoxyphenylpropionate (32a): A solution of (±)-diacetate **31a** (294 mg, 1 mmole) in *iso*-octane: benzene (2:1) mixture (20 mL) was added to a suspension of Amano PS lipase (275 mg) in 5 mM aq. sodium phosphate (10 mL) at pH 7.0. The reaction mixture was stirred at 25°C for 10 d after which it was filtered through celite and extracted with ethyl acetate (25 mL x 3). The combined organic layer after usual work-up and concentration in vacuo gave an oily residue, the conversions never exceeded 12-15% and hence further characterization was not continued.

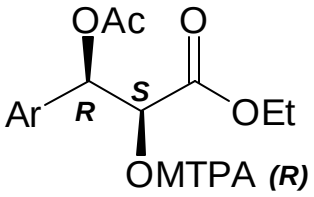
Structure (No.)	Ir (cm^{-1}) PMR (d) CMR (d) and Mass spectral data
 **2a**	**2a** as a thick oil. Ir (neat):ν_{max} 1740, 1725 cm^{-1}. ^{1}H NMR ($CDCl_3$, 200 MHz): δ = 2.20 (s, 3H), 3.78 (s, 3H), 5.95 (s, 1H), 7.25-7.50 (m, 5H). ^{13}C NMR ($CDCl_3$, 50 MHz): δ= 20.3, 52.2, 74.3, 127.6, 128.7, 129.1, 133.8, 169.1, 170.0.
 **2b** Ar = *p*-anisyl	**2b** also obtained as thick oil. Ir (neat): ν_{max} 1740, 1725 cm^{-1}. ^{1}H NMR ($CDCl_3$, 200 MHz) : δ = 2.18 (s, 3H), 3.76 (s, 3H), 3.85 (s, 3H) 5.87 (s, 1H), 6.95 (d, *J* = 10 Hz, 2H), 7.40 (d, *J* = 10 Hz, 2H).
	**MTPA-Ester of (±)-Methyl mandelate (1a)**: ^{1}H NMR ($CDCl_3$, 200 MHz) : δ = 3.58 (bs, 3H), 3.72 (bs, 3H), 3.75 (s, 3H), 3.79 (s, 3H), 6.10 (s, 1H), 6.13 (s, 1H,), 7.30-7.70 (m, 20H).
	**MTPA-Ester of *(S)*-Methylmandelate (3a)**: ^{1}H NMR ($CDCl_3$, 200 MHz): δ= 6.10 (s, *R*-isomer), 6.30 (s, *S*-isomer). The integral ratios of these protons indicated 98-99% ee.
	**MTPA-Ester of *(R)*-Methylmandelate (5a)** : ^{1}H NMR ($CDCl_3$, 200 MHz): δ = 6.10 (s, *R*-isomer), 6.30 (s, *S*-isomer). The integral ratios of these protons indicated 90-92% ee of **5a**.

 Ar = *p*-anisyl	**MTPA-Ester of (±)-Methyl-μ-hydroxy-μ-(4-methoxyphenyl)acetate (1b)**: ^{1}H NMR ($CDCl_3$, 200 MHz): δ 3.35 (s, 3H), 3.68 (s, 3H), 3.72 (s, 3H), 3.76 (s, 3H), 3.78 (s, 3H), 3.80 (s, 3H), 6.06 (s, 1H), 6.08 (s, 1H), 6.80-7.80 (m, 18H).
 Ar = *p*-anisyl	**MTPA-Ester of Methyl (*S*)-μ -hydroxy-μ-(4-methoxyphenyl)acetate 3b** : ^{1}H NMR ($CDCl_3$,200 MHz): δ= 6.06 (s, *R*-isomer), 6.08 (s, *S*-isomer). The integral ratios of these protons indicated 97-98 %ee of **17b**.
 Ar = *p*-anisyl	**MTPA-Ester of Methyl (*R*)- μ -hydroxy- μ-(4-methoxyphenyl)acetate 5b** : ^{1}H NMR ($CDCl_3$, 200 MHz): δ= 6.06 (s, *R*-isomer), 6.08 (s, *S*-isomer). The integral ratios of these protons indicated 78-80 %ee.
 **30** Ar = *p*-anisyl	**30:** mp 67-69 °C; ^{1}H NMR ($CDCl_3$, 200 MHz), δ 1.25 (t, *J* = 7.3 Hz, 3H), 3.05 (bs, 1H), 3.35 (bs, 1H), 3.85 (s, 3H), 4.15-4.30 (q, *J* = 7.3 Hz, 2H), 4.33 (bs, 1H), 4.95 (bs, 1H), 6.90 (d, *J* = 9.8 Hz, 2H), 7.34 (d, *J* = 9.8 Hz, 2H). IR (nujol) ν_{max} 3460, 1700, 1610 cm^{-1}.
 **31** Ar = *p*-anisyl	**31**: mp 56-58 °C, ^{1}H NMR ($CDCl_3$, 200 MHz) δ 1.25 (t, *J* = 7. 3 Hz, 3H), 2.10 (s, 3H), 2.14 (s, 3H), 3.80 (s, 3H), 4.15 (q, *J* = 4.8 Hz, 2H), 5.30 (d, *J* = 4.8 Hz, 1H), 6.23 (d, *J* = 4.8 Hz, 1H), 6.88 (d, *J* = 9.8 Hz, 2H), 7.42 (d, *J* = 9.8 Hz, 2H); ^{13}C NMR ($CDCl_3$, 50 MHz) 13.7, 20.0, 20.4, 54.9, 61.4, 73.3, 74.1, 113.6, 127.4, 128.2, 159.7, 166.8, 169.0, 169.5; MS (m/e) 324, 264, 222, 206, 193, 179, 161, 150, 137, 121, 109, 91, 77; IR (nujol) ν_{max} 1740, 1730, 1720, 1610 cm^{-1}.

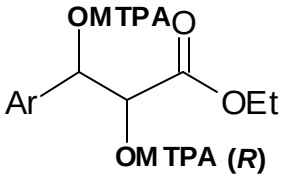


(2*S*, 3*R*)-32

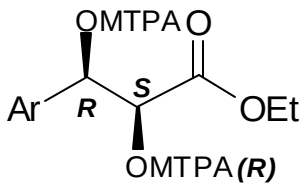
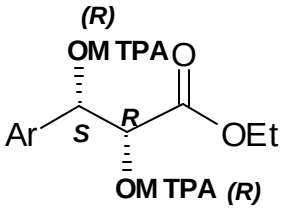
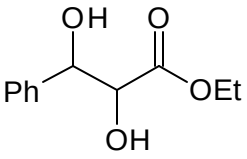
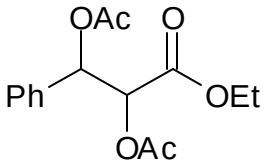
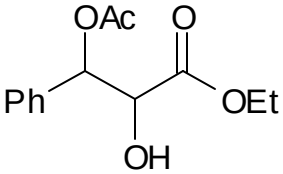
32: mp 48-50 °C, $[\alpha]^{25}_{D}$ = - 37.1° (c 2.0, $CHCl_3$) ; ^{1}H NMR ($CDCl_3$, 200 MHz) δ 1.30 (t, *J* = 8.1 Hz, 3H), 2.12 (s, 3H), 3.02 (bs, 1H), 3.85 (s, 3H), 4.25 (q, *J* = 8.1 Hz, 2H), 4.37 (bs, 1H), 6.05 (d, *J* = 2.7 Hz, 1H), 6.90 (d, *J* = 10.8 Hz, 2H),7.35 (d, *J* =10.8 Hz, 2H); ^{13}C NMR ($CDCl_3$, 50 MHz) δ 14.2, 21.0, 55.4, 62.3, 73.8, 75.5, 114.0, 128.5-129.0 (2-carbons), 159.9, 169.9, 172.1. MS (m/e) 282, 223, 206, 179, 161, 149, 137, 121, 109, 104, 94, 77, 65. IR nujol ν_{max} 3420, 1730, 1720, 1610 cm^{-1}.



MTPA-Ester of 32: ^{1}H NMR ($CDCl_3$, 200 MHz) δ 1.22 (t, *J* = 7.3 Hz, 3H), 2.10 (s, 3H), 3.48 (s, 3H), 3.83 (s, 3H), 4.20 (q, *J* = 7.3 Hz, 2H), 5.35 (d, *J* = 4.6 Hz), 5.43 (d, *J* = 4.2 Hz), 6.23 (d, *J* = 4.6 Hz), 6.33 (d, *J* = 4.2 Hz), 6.88 (d, *J* = 9.8 Hz, 2H), 7.28 (d, *J* = 9.8 Hz, 2H), 7.35-7.45 (m, 5H). On expansion, the signals at δ 6.23 and 6.33 were in a ratio of 0.02:0.98, indicating 96% ee. The ^{1}H NMR spectrum of (2*S*,3*R*)-hydroxyacetate **32** (10 mg) in $CDCl_3$ (0.5 mL) with chiral shift reagent, tris [3-(heptafluropropylhydroxymethylene)-(+)-camphorato] europium (III) (10 mg) in $CDCl_3$ (0.2 mL) indicated 96% ee [as seen by signal ratio (0.02 :0.98) for benzylic protons at δ 6.67 and 6.77 respectively].



***Bis*-MTPA-Ester of 30 :** ^{1}H NMR ($CDCl_3$, 200 MHz) δ 1.16-1.30 (m, 6H), 3.40 (bs, 6H), 3.62 (bs, 6H), 3.82 (s, 3H), 3.87 (s, 3H), 4.12-4.25 (m, 4H), 5.41 (d, *J* = 2.9 Hz, 1H), 5.47 (d, *J* = 3.5 Hz, 1H), 6.50 (d, *J* = 2.9 Hz, 1H), 6.52 (d, *J* = 3.5 Hz, 1H), 6.62 (d, *J* = 9.7 Hz, 2H), 6.84 (d, *J* = 9.7 Hz, 2H), 7.11 (d, *J* = 9.7 Hz, 2H), 7.21 (d, *J* = 9.7 Hz, 2H), 7.30-7.52 (m, 20H).

	***Bis*-MTPA-Ester of 22:** ^{1}H NMR ($CDCl_3$, 200 MHz) δ 2(*R*)-H, 5.40 (d, *J* = 2.9 Hz), 2(*S*)-H, 5.47(d, *J* = 3.5 Hz), the integral ratios of these protons indicated 98% ee of (2*S*, 3*R*)-diol **22**.
	***Bis*-MTPA-Ester of 23**: ^{1}H NMR ($CDCl_3$, 200 MHz), δ 2(*R*)-H 5.40 (d, *J* = 2.9 Hz), 2(*S*)-H 5.48 (d, *J* = 3.5 Hz), the integral ratios correspond to 86% ee of (2*R*,3*S*)-diol **23**.
 **30a**	**30a:** ^{1}H NMR ($CDCl_3$, 200 MHz): δ 1.28 (t, *J* = 7 Hz, 3H), 3.00 (bs, 1H), 3.30 (bs, 1H), 4.27 (q, *J* = 7 Hz, 2H), 4.37 (bs, 1H), 5.00 (d, *J* = 4 Hz, 1H), 7.25-7.50 (m, 5H); IR (neat) 3400, 1730 cm^{-1}.
 **31a**	**31a:** ^{1}H NMR ($CDCl_3$, 200 MHz): δ 1.20 (t, *J* = 8Hz, 3H), 2.10 (s, 3H), 2.15 (s, 3H), 4.16 (q, *J* = 8 Hz, 2H), 5.35 (d, *J* = 5 Hz, 1H), 6.28 (d, *J* = 5 Hz, 1H), 7.30-7.45 (m, 5H); IR (neat) 1750, 1745, 1730 cm^{-1}.
 **32a**	**32a:** ^{1}H NMR ($CDCl_3$, 200 MHz): δ 1.28 (t, *J* = 7 Hz, 3H), 2.13 (s, 3H), 3.05 (dd, *J* = 9 & 4 Hz, 1H), 4.25 (bq, *J* = 8 Hz, 2H), 4.42 (dd, *J* = 9 & 4 Hz, 1H), 6.10 (d, *J* = 4 Hz, 1H), 7.30-7.45 (m, 5H).

2.11 REFERENCES

1. Basavaiah, D.; Krishna, P. R. *Tetrahedron* **1995**, *51*, 2403.

2. Matsumae, H.; Shibatani, T. J. *Ferment. Bioeng*. **1994,** *77*, 152.

3. Du, J. F.; Zhou, J.; Huang, L. *Chin. Chem. Lett.* **1992**, *3*, 95.

4. Kirk-Othmer, *Encyclopedia of Chemical Technology*, (4^{th} edition), 1996, Vol-18, p-519.

5. Masai, M.; Shiori, T. *Synlett* **1996**, 49.

6. Bradshaw, C. W.; Humel, W.; Wong, C. H. *J. Org. Chem.* **1992**, *57*, 1532.

7. Miyazawa, T.; Kurita, S.; Ueji, S.; Yamada, T.; Kuwata, S. *J. Chem. Soc., Perkin Trans. 1* **1992**, 2253.

8. Hsu, S. H.; Wu, S.S.; Wang, Y. F.; Wong, C. H. *Tetrahedron Lett*. **1990**, *31*, 6403.

9. Lalonde, J. J.; Govardhan, C.; Khalaf, N.; Martinez, A. G.; Visari, K.; Margolin, L. *J. Am. Chem. Soc.* **1995**, *117*, 6845.

10. Tsuchiya, S.; Miyamoto, K.; Ohta, H. *Biotech. Lett.* **1992**, *14*, 1137

11. Fuganti, C.; Rosell, C. L.; Servi, S.; Tagilani, A.; Terreni, M. *Tetrahedron Asymm.* **1992**, *3*, 383.

12. Ziffer, H.; Kawai, K.; Kasai, M.; Imuta, M.; Froussios, C. *J. Org. Chem.* **1983**, *54*, 3017.

13. Mori, K.; Akao, H. *Tetrahedron* **1980**, *36*, 91.

14. (a) Dale, J. A.; Dull, D. L.; Mosher, H. S. *J. Org. Chem.* **1969**, *34*, 2543. (b) Dale, J. A.; Mosher, H. S. *J. Am. Chem. Soc.* 1973, *95*, 512.

15. Abe, K.; Inoue, H.; Nagao, T. *Yakugaku Zasshi*. **1988**, *108*, 716 .

16. Hashiama ,T.; Inoue, H.; Takeda, M.; Aoe, K.; Kotera, K. *J. Chem. Soc., Perkin Trans. 1* **1985,** 421.

17. Senuma, M.; Shibazaki, M.; Nishimoto, S.; Shibata, K.; Okamura, K.; Date, T. *Chem. Pharm. Bull*. **1989,** *37*, 3204. (b) For late stage enzymatic resolution, see; Akita, H.; Enoti, Y.; Yamada, H.; Oishi, T. *Chem. Pharm. Bull.* **1989**, *37*, 2876.

18. Shionogi, and Co. Ltd. U. S. Patent 4, 552, 695, 1985. (b) Fuji Chemical, Jpn. Patent 61-268663, 1986.

19. (a) Floyd, D. M.; Moquin, R. M.; Atwal, K. S.; Ahmed, S. Z.; Spergel, S. H.; Gougotas, J. Z.; Maley, M. F. *J. Org. Chem.* **1990**, *55*, 5572. (b) Atwal, K. S.; Bergey, J. L.; Hedberg, A.; Moreland, S. *J. Med. Chem.* **1987**, *30*, 635. (c) Carson, J. R U. S. Patent 4, 729, 994, 1988.

20. Palmer J. T.; European Patent Appl., 0.342.904 (1889) to Marion Laboratories. (b) Coffen D. L.; Madan, P. B.; Schwartz, A. European Patent Appl., 0.343.474 (1988) to Hoffmann La Roche.

21. (a) Shionogi and Co. Ltd. U.S. Patent 4,552, 695, 1985. (b) Igarashi, H.; Konma, T. Ger. Pat. 3,415,035, 1984.

22. (a) Schwartz, A.; Madan, P. B.; Mohacsi, E.; O'Brien, J. P.; Todaro, L. J.; Coffen, D. L. *J. Org. Chem.* **1992**, *57*, 851. (b) Sanada, K.; Inamoto, Y.; Kawai, S.; Endo, T. Jap. Pat. 61/268663 A2 [86268663], 1986.

23. Adger, B. M.; Barkley, J. V.; Bergeron, S.; Cappi, M. W.; Flowerdew, B. E.; Jackson, M. P.; McCague, R.; Nugent, T. C.; Robetrs, S. M. *J. Chem. Soc., Perkins Trans 1* **1997**, 3501.

24. (a) Wai, J. S. M.; Marko, I.; Svendsen, J. S.; Finn, M. G.; Jacobsen, E. N.; Sharpless, K. B. *J. Am. Chem. Soc.* **1989***, 111,* 1123. (b) Jacobsen, E. N.; Marko, I.; Mungall, W.

S.; Schroder, G.; Sharpless, K. B*. J. Am. Chem. Soc*. **1988**, *110*, 1968. (c) Lohray, B. B; Jayachandran, B.; Bhushan, V.; Nandan, E.; Ravindranathan, T. *J. Org. Chem.* **1995**, *60*, 5983.

25. Watson, K. G.; Fung, Y. M.; Gredley, M.; Bird, G. J.; Jackson, W. R.; Gountzos, H.; Matthews, B. R*. Tetrahedron Lett.* **1989**, *30*, 5157.

26. Theil, F. *Chem. Rev*. **1995**, *95*, 2203. (b) Mori, K. *Synlett* **1995**, 1097.

27. Gentile, A.; Giordano, C.; Fuganti, C.; Ghirotto, L.; Servi, S. *J. Org. Chem.* **1992**, *57*, 6635.

28. Kanerva, L. T.; Sundholm, O. *J. Chem. Soc., Perkin Trans 1* **1993,** 1385.

29. Yamada, S.; Tsujioka, I.; Shibatani, T.; Yoshioka, R. *Chem. Pharm. Bull.* **1999**, *47*, 146.

30. Akita, H.; Enoti, Y.; Yamada, H.; Oishi, T. *Chem. Pharm. Bull.* **1989**, *37*, 2876.

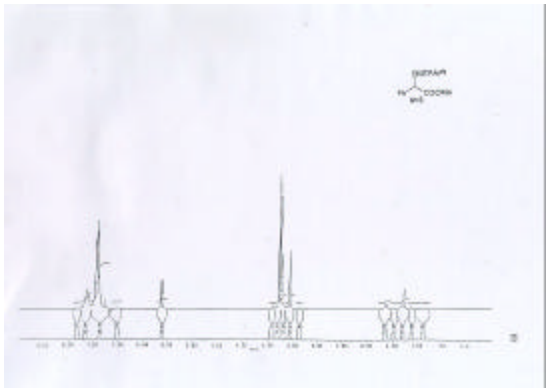
(Kirk-Othmer, *Encyclopedia of Chemical Technology*, (4th edition), 1996, Vol-18, p-519.

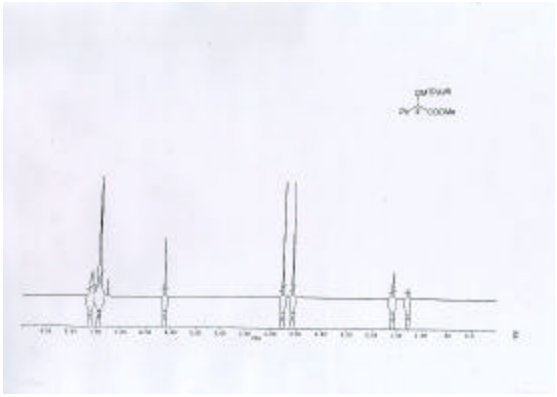
31. Watson, K. G.; Fung, Y. M.; Gredley, M.; Bird, G. J.; Jackson, W. R.; Gountzos, H.; Matthews, B. R*. J. Chem. Soc., Chem. Commun.* **1990**, 1018.

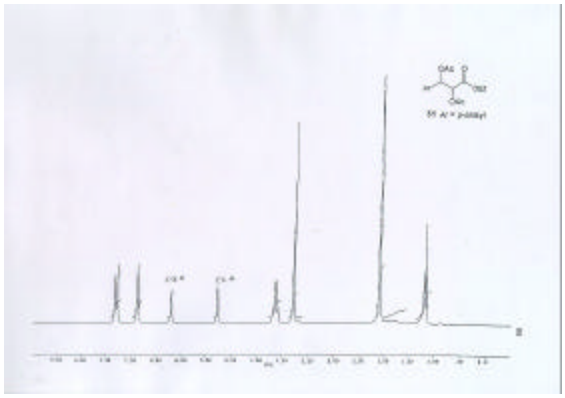
32. Crispina, G. A.; Jeong, K. S.; Kolb, H. C.; Wang, Z. M.; Xu, D.; Sharpless, K. B. *J. Org. Chem.* **1993***, 58,* 3785*.*

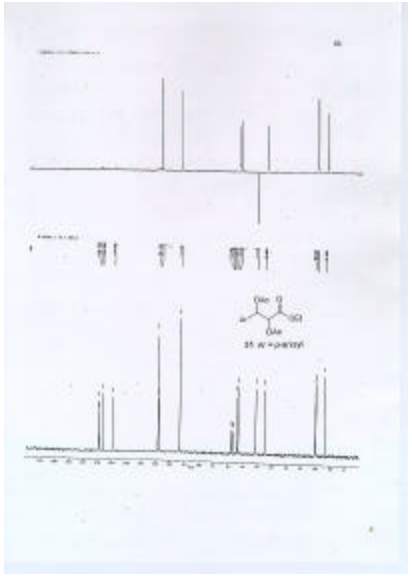
33. Desai, S. B.; Ganesh, K. N.; Argade, N. P. *Indian J. Chem.* **1998**, *37B*, 918.

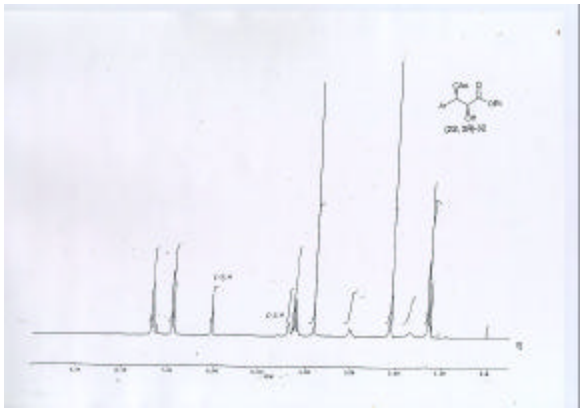
34. Desai, S. B.; Argade, N. P.; Ganesh, K. N. *J. Org. Chem.* **1996**, *61*, 6730.

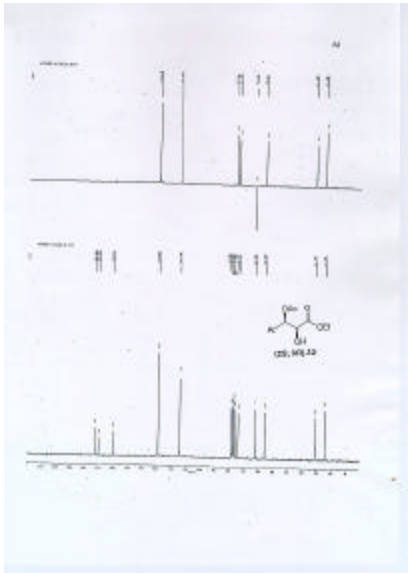


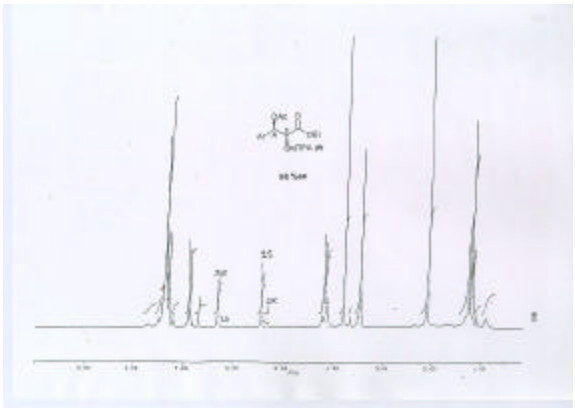


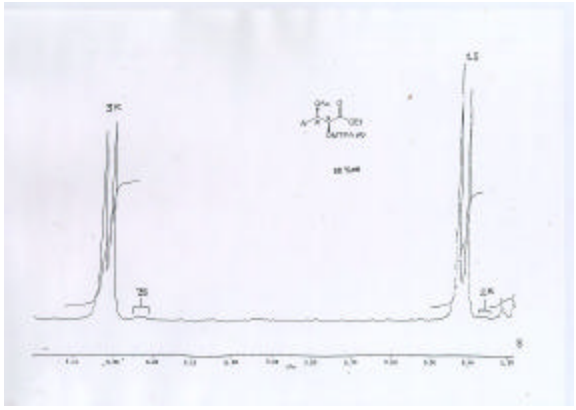


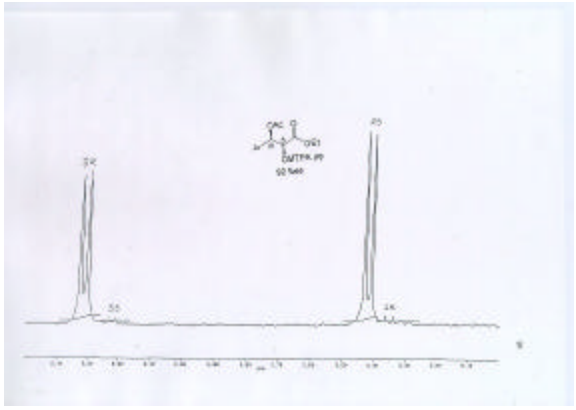


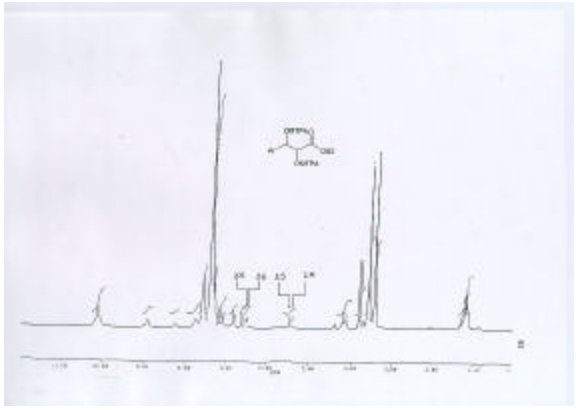


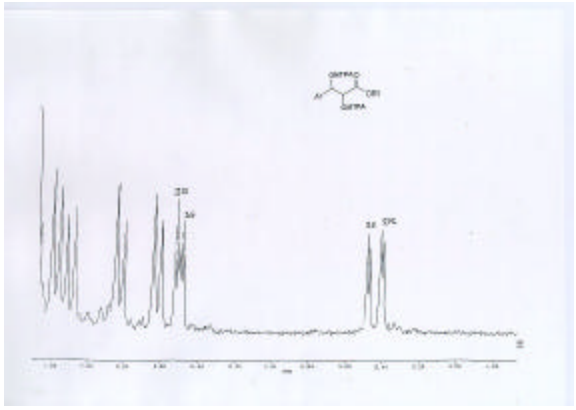


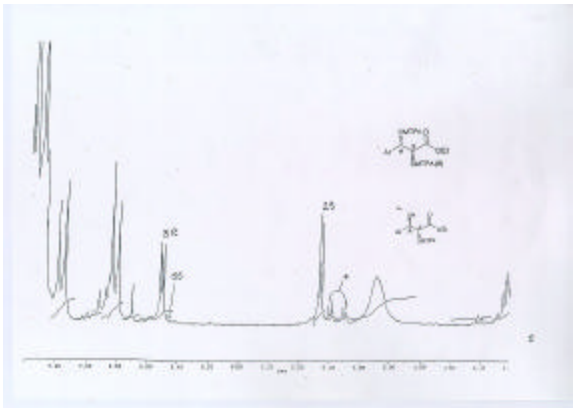


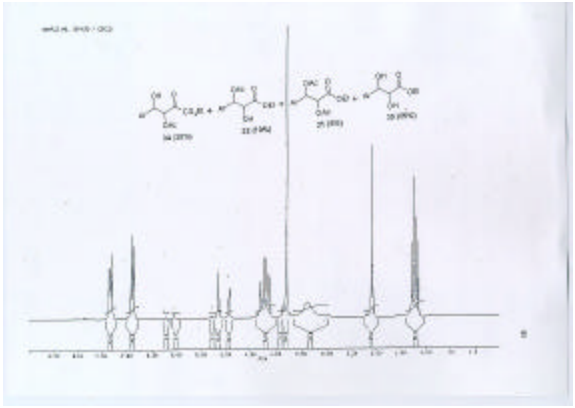


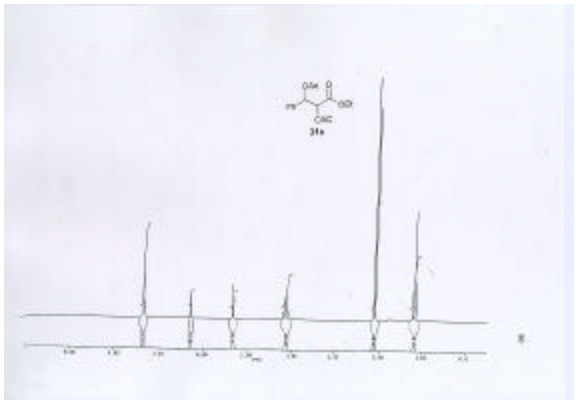


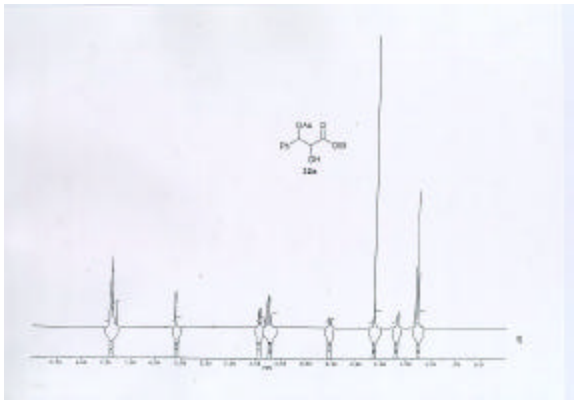





3.12
3.00
2.5







CHAPTER 3

MECHANISTIC STUDIES IN LIPASE CATALYZED HYDROLYSIS OF VICINAL DIACETATES: CORRELATING THE ACTUAL AND OBSERVED REGIOSELECTIVITY / ENANTIOSELECTIVITY

3.1 INTRODUCTION

A large number of *meso* and unsymmetrical vicinal diacetates with a wide range of structural diversities have been enzymatically hydrolyzed to obtain chiral molecules.[1] The enzymatic conversions of these diacetates have been carried out a t acidic, neutral or basic pH depending upon the enzyme used. Since intramolecular acyl migrations under various reaction conditions are well documented,[2,3] a major unresolved problem is the unambiguous determination of the actual and observed regioselectivities in enzymatic hydrolyses. For example, the literature reports on enzymatic hydrolysis of di- and triesters of glycerol reveal varying observations listed below.

(A) *The enzyme directly recognizes the primary acetate*.[4] Different esters of glycerol e.g. 2-oleoyl dipalmitin, 2-oleoyl distearin, 2-palmitoyl diolein and 1-oleoyl dipalmitin, were studied for the *in vitro* specificity of pancreatic lipase mediated hydrolysis. The course of the reaction, which was seen to be triglyceride to 1,2-diglyceride, to 2 -monoglyceride, does not depend on the type of fatty acid, the degree of unsaturation or the chain length. The specificity is towards the hydrolysis or transesterification at the primary esters or alcoholic function.

(B) *The enzyme directly recognize s the secondary acetate*.[5] Studies on the hydrolysis of secondary esters by panceratic lipases were carried out on glycerol derivatives,*viz*:1,3-*O*-benzylidiene-2-oleate glycerol and 1,3-dialkylether-2-oleate. These upon treatment with pancreatic lipase showed hydrolysis of secondary ester and in the absence of non-specific lipases, the hydrolysis was relatively slow. In a third substrate, a trioleate of glycerol, wherein only the secondary oleate was labeled, some amount of the label was found to be hydrolyze d and the probability of label migration on the primary alcohol followed by hydrolysis was not discussed.

In another study, the objective was to test various aspects of specificities of lipolyses, with different derivatives of triglycerides such as 2-oleodipalmitin, 1-oleo dipalmitin and 1-palmitodiolein. The results indicated that

i. both primary esters are hydrolyzed more rapidly than the secondary ester, with the order of products formed

Triglyceride ⟶ 1,2-diglyceride ⟶ 2-monoglyceride

ii. formation of some (10-30%) of 1-monoglyceride with its original acid chain indicating that no isomerization (secondary ester to primary) occurs and the 1-monoglyceride is a result of direct hydrolysis of secondary ester.

(C) *The enzyme first recognizes primary acetate followed by in-situ intramolecular acyl migration of the secondary ester to yield the secondary alcohol derivative.*[4a] When 2-oleyl dipalmitin was used for the hydrolysis, the diglyceride formed was 2-oleoyl palmitin and further hydrolysis gave 1-monoolein. Thus, the course of hydrolysis of triglyceride is a series of directed stepwise reactions from triglyceride to 1,2-diglyceride to 2-monoglyceride, which could be followed by isomersation of 2-monoglyceride to 1-monoglyceride. The key results obtained in another study of triglyceride hydrolysis are summarized here:

i) The hydrolysis of esters of oleic acid by pancreatic lipase depends on the nature of the alcohol. The two factors that influence the rate of the reaction are (a) inductive effect and (b) steric hindrance.

ii) Lipolysis promoted by electrophilic substituent as might be expected from a reaction involving nucleophilic attack on the carbonyl e.g. slow hydrolysis of oleoyl oleate as compared to 2-fluoroethyl oleate or *p*-nitrobenzyl oleate. The operation of such an inductive effect explains the following sequence in rate of hydrolysis triglyceride> 1,2-diglyceride> 1,3-diglyceride> 1-monoglyceride> 2-monoglycride.

iii) The bulkiness of the carbinol group perhaps inhibits lipolysis, as vinyl oleate is hydrolyzed but isopropyl or phenyl oleate does not. This steric effect explains lipase specificity for α-chains of triglycerides. Electron-withdrawing substituents can counteract this hindrance since 1,3-difluoroisopropyl oleate and *p*-nitrophenyl oleate are slowly hydrolysed.

(D) *Formation of a multiple point attachment of the substrate with enzyme.*[6] There could exist a possibility wherein the enzyme cavity may have more than one binding sites for the acetates, with some amino acid residues of the enzyme helping in simultaneous hydrolysis and shuffling of the hydrolyzed/unhydrolyzed acetyl moiety to aid the acyl migration.

E) *The recognition of the primary acetate in antibody mediated hydrolysis*[7] *of p-nitro substituted derivative of* ***10****.* An antibody-mediated hydrolysis of 2,3-diacetoxy-1-(*p*-nitrobenyloxy)glycerol in DMSO/aq. buffer at pH 8.0 was carried out. The hydrolysis was completely regioselective giving primary hydrolyzed product and also stereoselective (80% ee) at 36% conversion.

(F) *Formation of 1:4 ratio of primary:secondary hydrolysed products.*[8]

In lipase catalyzed hydrolysis[9] of aliphatic diacetates having one primary acetate and other secondary acetate, only the secondary acetate was found to be hydrolyzed. This was proved to arise from the direct hydrolysis of secondary acetate and not as a result of post hydrolytic acetyl migration through a mixed ester intermediate. Another report[10] has shown that the lipase catalyzed hydrolysis of *threo*-2,3-diacetoxybutanoic ester leads to both regio isomers of monoacetate, as a result of post hydrolytic acetyl migration on silica gel column.

3.2 DEFINITION OF THE PROBLEM

From the above observations it is obvious that there is no basis for clearly delineating the specificities in lipase hydrolysis of vicinal diacetate substrates (for convenience named as 1st-acyl and 2nd-acyl, see Figure **1**). If a lipase is shown to be regiospecific towards the hydrolysis of 1st-acyl, is this specificity a result of direct hydrolysis of the 1st-acyl ***(ACTUAL SELECTIVITY)*** or the lipase hydrolyses 2nd-acyl followed by the *in-situ* migration of 1st-acyl to give the *apparent* 1st-acyl hydrolysed product ***(OBSERVED SELECTIVITY)***. A literature search revealed lack of any or suitable direct and unambiguous methods to correlate the actual and observed specificity in such enzymatic hydrolysis of vicinal diacetates. In order to delineate the mechanisms of hydrolysis and migrations in enzymatic reactions, a simple unambiguous protocol using labeling technique is presented here to validate the actual and observed selectivities, along with a measure of enzyme selectivity efficiency.

3.2.1 Strategy

Our present strategy consists of an enzymatic hydrolysis of unsymmetrical diacetate, followed by labeling with CD_3COOD/DCC to give exclusive monolabeled

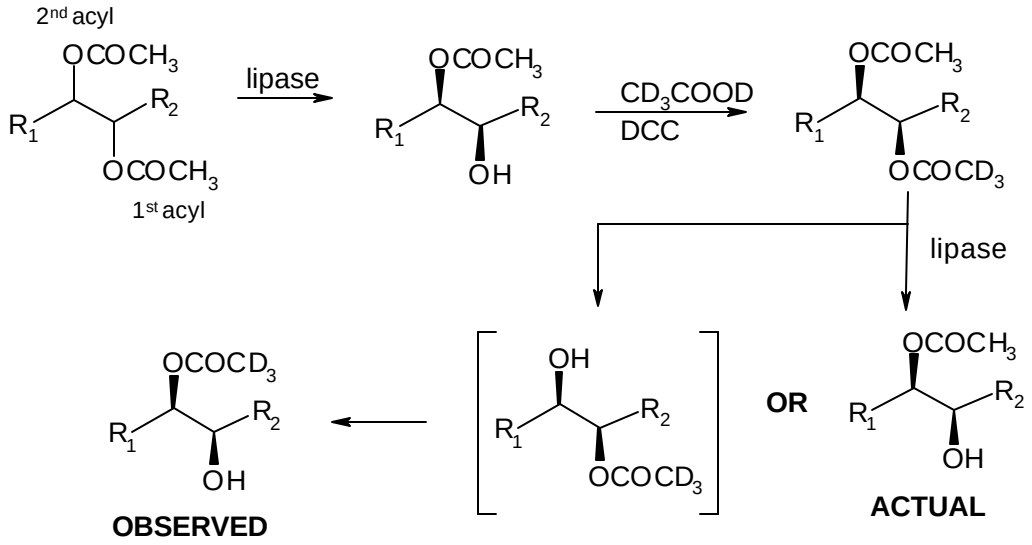


Figure 1

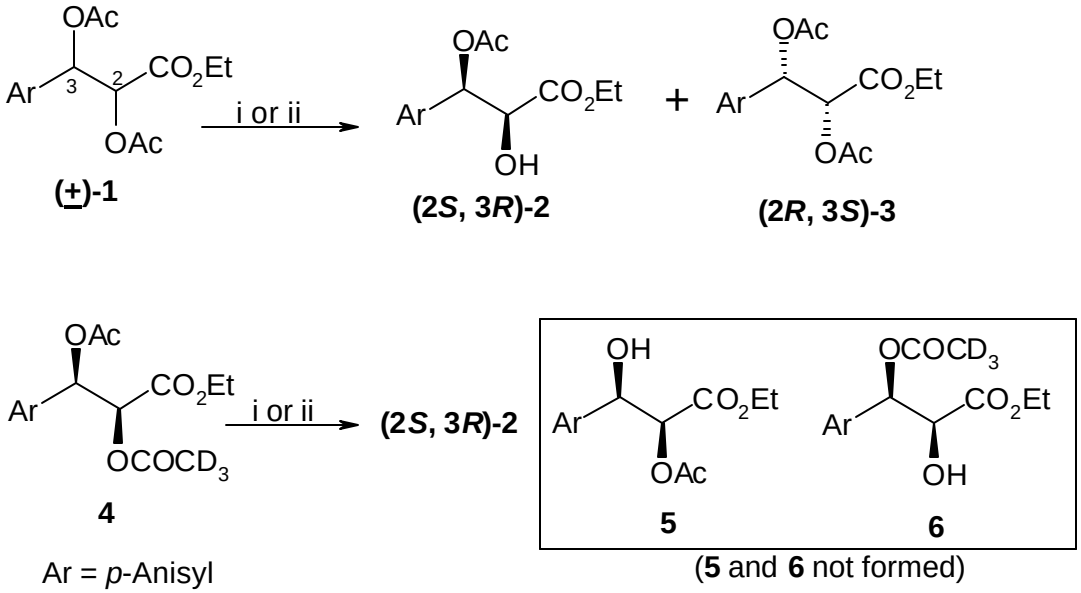
diacetate. Enzymatic rehydrolysis of the monolabeled diacetate under the identical set of reaction conditions aids in estimating the amount of label retention by ^{1}H NMR spectroscopy. The complete loss of label would then indicate the actual and observed regioselectivity to be the same. The retention of the label would be a consequence of *in-situ* intramolecular acyl migration and in such a condition, the actual and observed regioselectivity would be different. The amount of label lost will directly indicate the proportional extent of regioselective action of the enzyme.

3.3 RESULTS ON ACTUAL AND OBSERVED REGIOSELECTIVITY

3.3.1 Actual and Observed Regioselectivity in AmanoPS Reactions

As reported in Chapter 2, a remarkable chemo-, regio-, and enantioselective hydrolysis of the (±)-diacetate **1** was observed[11] using AmanoPS and pig liver acetone

Scheme 1

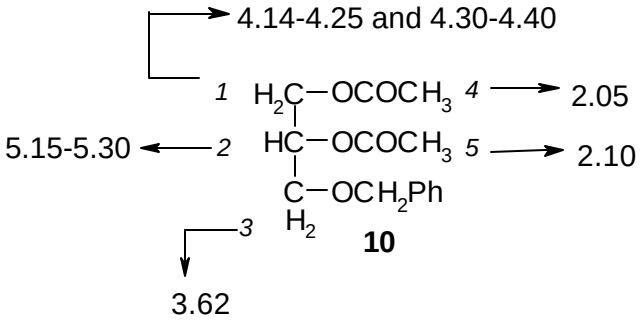


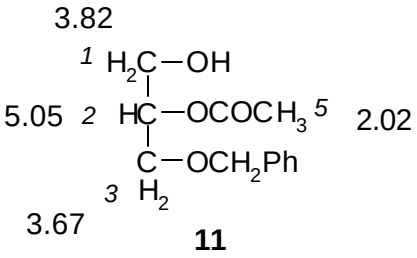
i) AmanoPS, C_6H_6:Petroleum ether (2:1), 50 mM sodium phosphate buffer pH 7.0 25° C; ii) PLAP, C_6H_6:Petroleum ether (2:1), 50 mM sodium phosphate buffer pH 8.0 37° C

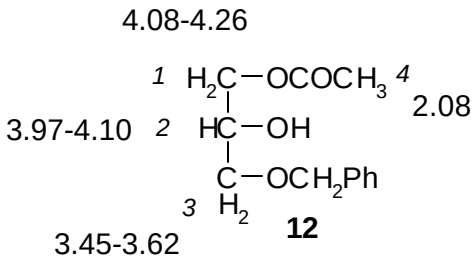
powder (PLAP) to obtain chiral precursors of clinically used (+)-diltiazem in very good yields and optical purities. As depicted in **Scheme 1**, the (±)-diacetate **1** was enzymatically hydrolyzed using AmanoPS at its optimum pH (7.0), to obtain a mixture of (2*S*, 3*R*)-hydroxyacetate **2** and the unreacted (2*R*, 3*S*)-diacetate **3**. The structural analysis of hydroxyacetate **2** using ^{1}H NMR data revealed that the enzymes AmanoPS and PLAP are nearly 100% chemoselective as the carbethoxy function was not hydrolyzed and about 98-99% enantioselective in their action with substrate (±)-diacetate **1** to give (2*S*, 3*R*)-**2.** This was further supported by optical rotation and confirmed by spectral analysis of the complex with chiral shift reagent and Mosher's ester of **2**. The enzymes were also seen to be ~100% regioselective in their action since only the C2-*O*-acetate of (±)-**1** is hydrolyzed, keeping the C3-*O*-acetate intact. The column chromatographically purified (2*S*, 3*R*)-hydroxyacetate **2** (98% ee) was reacylated using CD_3COOD and DCC as a coupling reagent to obtain 2*S*-deuterolabeled (2*S*, 3*R*) diacetate-**4**. The labeled diacetate **4** was resubjected to enzymatic hydrolysis using AmanoPS under the previous set of reaction conditions. The exclusive product formed in this experiment as identified by ^{1}H NMR and the corresponding MTPA derivative was **2** in 75% chemical yield and nearly 100% ee. The rate of enzymatic hydrolysis for this optically pure enantiomer (2*S*, 3*R*)-**4** was relatively faster (24 h) than for the racemic (±)-**1** (156-180 h).

In a second example to demonstrate the positive validity of this method, the (±)-glyceroldiacetate **10**,[12,13a] was chosen and was subjected to biphasic enzymatic hydrolysis using AmanoPS at pH 7.0 (**Scheme 2**). This reaction furnished a product mixture as revealed by ^{1}H NMR, (C1-hydroxy,C2-acetoxy) **11** and (C1-acetoxy,C2-hydroxy) **12**[13] in 9:1 ratio with a chemical yield of 55% in 2 h. In the control experiments without the enzyme AmanoPS at pH 7.0, the (±)-diacetate **10** remained unreacted, indicating the absence of any accompanying chemical hydrolysis.

The chemical shifts in ^{1}H NMR of all the three compounds are listed below and show different shifts. The characteristic shift in each is of C2 proton that differentiates each one of them, particularly the two hydroxy-acetates. The spectral details are given later in the experimental section along with the respective spectra. The mixture of hydroxyacetates (C1-hydroxy,C2-acetoxy) **11** and (C1-acetoxy,C2-hydroxy) **12**

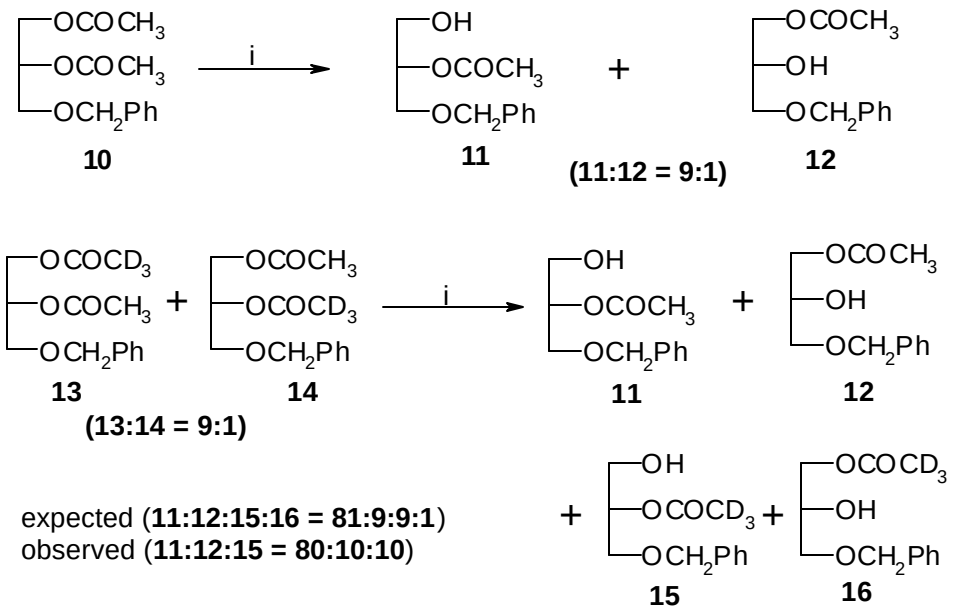






thus obtained was mono-labeled with CD_3COOD/DCC to yield a mixture of primary labelled diacetate-**13** and secondary labeled diacetate-**14** in 9:1 product ratio (page 135). This mixture of mono-labeled diacetates in AmanoPS catalyzed hydrolysis yielded a product mixture of **11**, **12** and **15** in 80:10:10 proportion (page 137). The ^{1}H NMR spectral data of the crude reaction mixture and the column purified products revealed that there was no intramolecular acyl migration during silica gel column chromatography.

Scheme 2



i) AmanoPS, C_6H_6:Petroleum ether (2:1), 50 mM sodium phosphate buffer pH 7.0 25°C

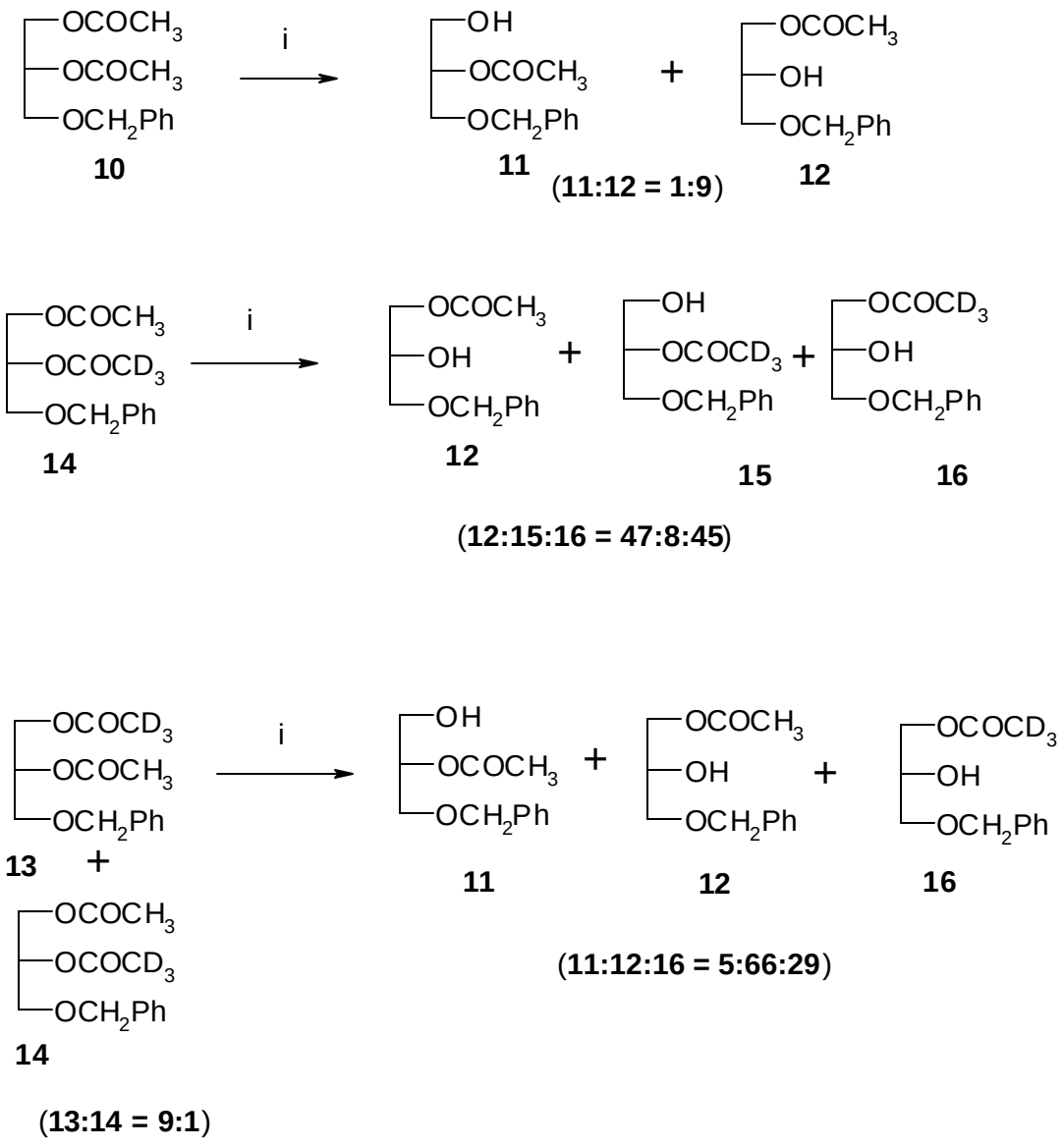
3.3.2 Actual and Observed Regioselectivity in PLAP Reactions

To examine whether these observations are general for other lipase hydrolysis, the enzymatic hydrolysis of (±)**-1** and (±)**-10** were studied with PLAP at its optimum pH (8.0). The optically pure mono-labeled diacetate **4** obtained *via* the PLAP catalyzed hydrolysis of (±)**-1** (**Scheme 1**), after rehydrolysis with PLAP also revealed a complete loss of label as previously observed in case of AmanoPS catalyzed hydrolysis.

In a second set of experiments, the (±)-diacetate **10** was subjected to biphasic enzymatic hydrolysis using PLAP[14] at pH 8.0 and the reaction upon 25% conversion (2 h)[15] gave a mixture of (C-1 hydroxy,C-2 acetoxy) **11** and (C1-acetoxy,C2-hydroxy) **12,** but in 1:9 proportion (reversal of AmanoPS expt) (**Scheme 3**). In control experiments without the enzyme PLAP at pH 8.0, the (±)-diacetate **10** remained unreacted. The column chromatographically purified compound **12** on coupling with CD_3COOD/DCC

gave the secondary labeled diacetate **14** (page 139), which was resubjected to enzymatic hydrolysis using PLAP at pH 8.0. The reaction upon 25% completion (2 h) yielded a mixture of **12, 15** and **16** in 47:8:45 ratio (page 141). The ^{1}H NMR spectral

Scheme 3



i) PLAP, C_6H_6:Petroleum ether (2:1), 50 mM sodium phosphate buffer pH 8.0 37^o C

data of the crude reaction mixture and column purified products revealed that there was no intramolecular acyl migration during silica gel column chromatography.

Similarly, the primary and secondary labeled mixture of diacetates **13+14** (9:1) on PLAP catalyzed hydrolysis at pH 8.0 furnished a mixture of **11, 12** and **16** in 5:66:29 proportion.

3.4 DISCUSSION ON ACTUAL AND OBSERVED REGIOSELECTIVITY

3.4.1 Actual and Observed Regioselectivity in AmanoPS Reactions

It was thought appropriate to examine the nearly 100% regioselective action of AmanoPS on (±)-**1** to give the hydroxyacetate **2** (**Scheme 1**), wherein, of the two acetates only C2 *O*-acetyl is hydrolyzed. In principle, the hydroxyacetate **2** can arise either from the direct hydrolysis of C2 *O*-acetate or *via* the hydroxyacetate-**5**, resulting from the hydrolysis of C3 *O*-acetate followed by *in-situ* intramolecular acyl migration (see **Scheme 1**). It is necessary to establish the real basis for the observed high regiospecificity. During the enzymatic hydrolysis of 2*S*-labeled diacetate-**4,** the exclusive product formed was **2** in high yield and optical purity as seen by PMR, optical rotation and mass spectral analysis. The rate of enzymatic hydrolysis for the optically pure enantiomer (2S, 3*R*)-**4** was relatively faster than the diacetate (±)-**1**. It is possible that the opposite isomer might be acting as a competitive inhibitor. The fact that the labeled compound **6** was not obtained proves that the compound **2** is formed from diacetate **4** by direct hydrolysis. This implies that **1** $\rightarrow$ **2** conversion is also by direct hydrolysis and *not via* acyl migration in the initially formed intermediate **5** (**Scheme 1**). Thus the true and observed regioselectivity of the hydrolysis with AmanoPS at pH 7.0 with the (±)-diacetate **1** is same and the enzyme is nearly 100% regioselective in its activity. In control experiments without enzyme AmanoPS at pH 7.0, the (±)-diacetate **1** and diacetate **4** (**Scheme 1**) remained unreacted, proving the absence of any accompanying chemical hydrolysis.

During the AmanoPS catalyzed hydrolysis of (±)-diacetate **10,** the products formed were (C1-hydroxy,C2-acetoxy) **11** (90%) and the (C1-acetoxy,C2-hydroxy) **12** (10%). In control experiments without the enzyme AmanoPS at pH 7.0, the (±)-diacetate **10** (**Scheme 2**) also remained unreacted, proving the absence of any accompanying chemical hydrolysis. The formation of 10% of (C1-acetoxy,C2-hydroxy) **12** can be explained due to two possibilities – (i) the enzyme is non-selective and hydrolyses both the 1^{o} and 2^{o} acetates such that along with the (C1-hydroxy,C2-acetoxy) **11,** (C1-acetoxy,C2-hydroxy) **12** (10%) is also formed and (ii) the enzyme regioselectively hydrolyses 1^{o} acetate to give exclusively (C1-hydroxy,C2-acetoxy) **11** followed by partial *in-situ* intamolecular acyl migration to give the (C1-acetoxy,C2-hydroxy) **12** in 10%. If the (C1-acetoxy,C2-hydroxy) **12** (10%) is formed from (C1-hydroxy,C2-acetoxy) **11** (90%) *via in-situ* intramolecular acyl migration, then one can follow the labeling technique wherein the retention of label would prove the presence of acyl migration. If we assume **12** in 10% arises from intramolecular acyl migration *via* **11**, then it should be possible to deduce the ratio of products arising from the labeled diacetate mixture **13**+**14** (90:10). The calculated ratios of the so formed products would then be **11+12** (90:10) arising from primary labeled diacetate-**13** and **15+16** (90:10) arising from **14**. Further, calculating the overall ratios from combined mixture of labeled diacetates **13+14** would then give **11+12+15+16** in *81:9:9:1* and the observed results are *80:10:10* (**Scheme 2**) (the product **16** was not observed in the limits of NMR resolution). The observed results convincingly indicate that in the enzymatic hydrolysis of both **10** and **13+14**, the enzyme AmanoPS recognizes the primary acetate in the substrate in nearly 100% regioselective (*sn*-1 specific) manner and **12** (10%) is formed by intramolecular acyl migration.

3.4.2 Actual and Observed Regioselectivity in PLAP Reactions

Similarly in PLAP catalyzed hydrolysis of the optically pure mono-labeled diacetate **4** obtained *via* the hydroxyacetate **2** (which in turn was obtained *via* PLAP catalyzed hydrolysis of (±)-**1**, rehydrolysis with PLAP at pH 8.0) also revealed a complete loss of label (**Scheme 1**), confirming that the actual and observed selectivity are same. In control experiments without the enzyme PLAP at pH 8.0, the diacetate (±)-**1** and **4** remained unreacted, proving the absence of any accompanying chemical hydrolysis. The ^{1}H NMR spectral data of the crude reaction products and the column purified products revealed that there was no intramolecular acyl migration during silica gel column chromatography.

The PLAP catalyzed hydrolysis of (±) glycerol diacetate **10** at pH 8.0 gave (C1-hydroxy,C2-acetoxy) **11** (10%) and (C1-acetoxy,C2-hydroxy) **12** (90%) (**Scheme 3**). This is interesting since the ratio of the products is exactly the opposite of that obtained in the AmanoPS hydrolysis of (±)-glycerol diacetate **10**. In this case also, it is possible that either PLAP hydrolyses both 1^{o} and 2^{o} acetates with different rates or the enzyme might hydrolyze 1^{o} acetate exclusively followed by *in-situ* intramolecular acyl migration of 2^{o} acetate to the 1^{o} hydroxy to give 90% of (C1-acetoxy,C2-hydroxy) **12**. Further, upon labeling the (C1-acetoxy,C2-hydroxy) **12** with CD_3COOD gave the monolabeled diacetate-**14** which on rehydrolysis under same conditions gave the mixture **12**+**15**+**16** in 47:8:45 ratio. As observed above almost equal amounts of compounds with secondary hydroxyls **12** and **16** were formed and in **16** the label was retained as a result of acyl migration from the 2^{o} position of **14**. It is interesting to ponder on the surprising formation of unlabeled **12**. As yet *sn*-2 specific lipases are unknown[5c] in the literature and hence it is unlikely that this arises from a direct hydrolysis of secondary acetate in **14**. Alternatively, it could arise as a result of retaining back of the primary

unlabeled acyl group from a transitory intermediate set up during the migration of the secondary acyl group. Similarly, the mixture **11**+**12** (obtained from AmanoPS hydrolysis of (±) glycerol diacetate **10**) upon labeling gave the monolabeled diacetates **13**+**14** (9:1), which on rehydrolysis under similar set of conditions (PLAP, pH 8.0) gave a mixture of **11**+**12**+**16** in 5:66:29 ratio. These results clearly suggest an intramolecular acyl migration in PLAP induced hydrolysis of both **14** and **13**+**14**. In the absence of PLAP, the mixture of hydroxyacetates **11+12** (9:1) at pH 8.0 showed very slow intramolecular acyl migration to yield **12** (24 h, 60%). Hence in PLAP catalyzed hydrolysis of (±)-**10**, the observed intramolecular a cyl migration seems to be a synergic effect of enzyme and pH. The observed difference in the amount of label retained in mixtures **12+15+16** and **11+12+16** in the above mentioned two experiments is interesting. It is possible that during the formation and cleavage of an unsymmetrical intermediate of substrate with the enzyme PLAP, the amount of label retained may depend on its position (1^o or 2^o) in the substrate. In PLAP catalyzed hydrolysis of (±)-**10** at pH 8.0, the observed regioselectivity is very high and the actual selectivity differs markedly depending upon the intramolecular acyl migration or multiple point attachment[6] to the enzyme and hydrolysis. Recent results also suggest that *sn*2-regioselectivity stems from acyl migration and an actual *sn*2-specific lipase may not exist.[5c]

3.5 APPLICATION TO *meso* DIACETATES

3.5.1 Background

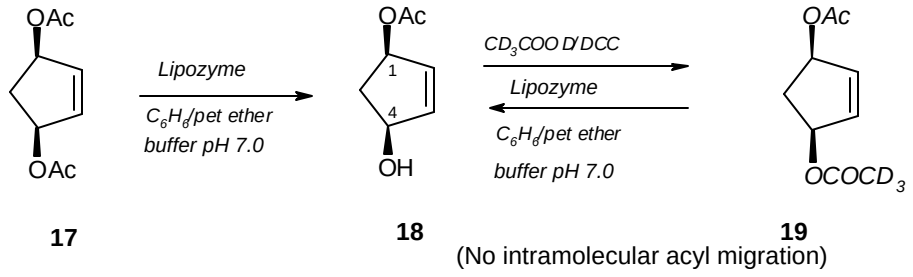
The *meso* diacetates also are potential candidates for enzymatic desymmetrization, to provide optically pure hydroxyacetates and a similar problem is faced for determining the actual and observed enantioselectivities in enzymatic hydrolysis. For e.g. the enantioselective hydrolysis of a *meso* diacetate, *cis* 1,4-

diacetoxycyclopent-2-ene (**17**) is reported[16] with Lipozyme lipase. In another example, lipase mediated hydrolysis of 1,2-diacetoxy cyclopentane (**21**) revealed varying observations *viz*: (a) formation of enantiopure[17] (*1R*,*2S*)-1-acetoxy,2-hydroxycyclopentane and (b) formation of optically inactive[18] (racemic) 1-acetoxy-2-hydroxycyclopentane. We opted to extend our labeling technique to correlate the actual and observed enantioselectivities in lipase mediated hydrolysis of above mentioned both the *meso* diacetates.

3.5.2 Results on Actual and Observed Enantioselectivity in *meso* Diacetates

1,4-Diacetoxycyclopent-2-ene (**17**) when hydrolyzed with Lipozyme at pH 7.0 in a biphasic system gave the (1*R*, 4*S*)-hydroxyacetate **18** with >92% ee (**Scheme 4a**).

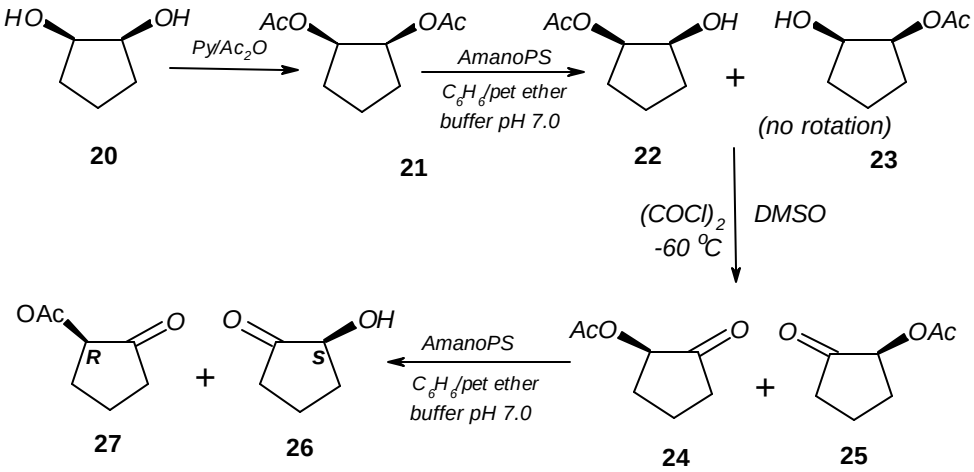
Scheme 4a



17 **18** **19**

(No intramolecular acyl migration)

Scheme 4b



20 **21** **22** **23**

27 **26** **24** **25**

These results are consistent with that observed in literature.[16] Among the enzymes screened were Lipozyme, PLAP and AmanoPS, of which Lipozyme showed good results. The labeling technique was extended to find the actual versus observed enantioselectivity of the enzyme on the diacetate **17**. It was observed that on labeling the 4*S*-hydroxy function by CD_3COOD/DCC coupling led to the exclusive monolabeled diacetate **19,** which underwent Lipozyme mediated biphasic hydrolysis to give back the (1*R*, 4*S*)-hydroxycetate **18**.

The diacetate **21** was subjected to Amano PS catalyzed biphasic hydrolysis at pH 7.0. At 60% conversion, a product mixture of hydroxyacetate **22** plus **23** and ureacted diacetate **21** in 12 h (**Scheme 4b**) was obtained. The product analysis by ^1H NMR indicated the product to be hydroxy acetate, but product (mixture) was optically inactive. This was then subjected to oxidation by oxalyl chloride and DMSO in dry CH_2Cl_2 to give (±)-2-acetoxy cyclopentanone (**24+25**) in 80% yield. The (±)-ketoacetate **24+25** on biphasic Amano PS catalyzed hydrolysis under similar set of conditions was stopped at 45% conversion (by PMR, page 150), to obtain the starting material keto-acetate **27** (55%) and 2-hydroxycyclopentanone (**26**) (45%). Further column chromatography gave pure 2-acetoxycyclopentanone (**27**) and was found to be optically active. The measured optical rotation was $+33.4^{o}$ (c 1, $CHCl_3$) and by comparison to the literature reports,[19] this isomer was assigned *S*-configuration. The 2-hydroxycyclopenatnone (**26**) could not be isolated and further experiments are needed. The present method could be a potential technique to obtain both the enantiomers of 2-hydroxycyclopentanones (**26**) and 2-acetoxycyclopentanones (**27**) in high optical purities and these are useful starting materials for syntheses of many naturally occurring bioactive products.

3.5.3 Discussion on Actual and Observed Enantioselectivity in *meso* Diacetates

It was reasoned that either the observed enantiomer of hydroxy acetate (1*R*, 4*S*)-**18** is a result of direct hydrolysis of '*pro*-*4S*-acetate' of the *meso* diacetate **17** or the enzyme recognizes the '*pro*-1*R*-acetate' followed by complete or nearly complete *in-situ* acyl migration. The fact that during the enzymatic rehydrolysis of monolabeled diacetate-**19**, the label was totally lost points to direct recognition of the *pro*-4*S*-acetoxy function by the enzyme. Hence the observed and actual enantioselectivities of Lipozyme are same with this *meso* diacetate-**17**.

Upon AmanoPS biphasic hydrolysis of diacetate-**21**, the hydroxy-acetates formed **22** plus **23** (identified by ^{1}H NMR) were found to be optically inactive. The racemic hydroxyacetate **22** plus **23** could be a result of non-selective random hydrolysis of both acetates. Alternatively the enzyme if believed to be enantioselective in its hydrolysis, the product may form by instantaneous *in-situ* acyl migration in a vicinal *cis*-cyclic system. The acyl migration could be kinetically favored such that both the enantiomers so formed would be in equal proportions giving the racemic hydroxyaetate **22** plus **23**. In this case, our labeling technique would prove to be ineffective, since the label would be on both the enantiomers of the resultant diacetate and hydrolysis would result in only shuffling of the label. The labeled hydroxyacetate would then be in 1:1 ratio, not enabling us to draw any definite inference. Hence it was planned to convert the hydroxy to keto by Swern oxidation to obtain (±)-2-acetoxycyclopentanone (**24+25**). If the enzyme were to be non-selective then one would get optically inactive 2-hydroxy cyclopentanone. If the enzyme is selective towards hydrolysis of one of the enantiomer and if the reactions are stopped at < 50% conversions, products would consist of optically pure 2-hydroxycyclopentanone (**26**) and the unreacted enantiopure 2-acetoxycyclo pentanone (**27**). The (±)-2-

acetoxycyclopentanone (**24+25**) upon AmanoPS hydrolysis offered the starting material keto-acetate **27** (55%) and 2-hydroxy cyclopentanone **26** (45%) as analyzed by ^{1}H NMR. Column chromatographic purification gave pure 2-acetoxycyclopentanone (**27**) in 49% overall yield and was found to be optically active. The rotation was measured to be +33.4^o (c 1, $CHCl_3$) which by comparison with the literature[19] was assigned *S*-configuration. This clearly indicates that the AmanoPS is enantioselective towards the ketoacetate-**24+25**, which is derived from the *meso* diacetate **21**. It is tempting to extend the enantiospecificity of the AmanoPS from the substrate keto-acetate **24+25** to the *meso* diacetate **21**, and the enzyme perhaps enantioselectively hydrolyzes one of the acetates in meso diacetate **21** followed by rapid intramolecular acyl migration to give the optically inactive hydroxyacetates **22+23**. Although the substrate structure of (±)-2-acetoxycyclopentanone (**24+25**) has been changed with respect to the parent diacetate **21**, the results may lead to some idea about the enantioselectivities of the enzyme towards the *meso* diacetates.

3.6 SUMMARY

In summary, with two unsymmetrical diacetates as examples, we have demonstrated a first simple method to correlate the actual and observed regioselectivity in enzymatic hydrolysis of unsymmetrical diacetates and to measure the enzyme selectivity efficiency.[20] The simple method consists of enzymatic hydrolysis of unsymmetrical diacetate followed by labeling of the formed hydroxyacetate with CD_3COOD/DCC, and enzymatic rehydrolysis of labeled compound under the identical reaction conditions to estimate the amount of label retained by ^{1}H NMR spectroscopy. The isotopic effect on rate and selectivity of enzymatic hydrolysis has not been taken into account in this method. The labeled compounds were characterized by using ^{1}H NMR and mass spectral data, while the quantitative estimation of the label was performed using ^{1}H NMR data (relative integrations of methyl group from -OAc). The

amount of label lost directly indicates the extent of regioselective action of the enzyme. By suitable manipulations in reaction conditions (enzyme type, solvent system, pH range and temperature) it may be possible to get both the isomers with high regioselectivity by complete prevention or forcing the acyl migration. The same strategy has been also extended to *meso* diacetate **17** to show the actual and observed enantioselectivities by exclusively labeling the pro-*R* or pro-*S* acetate. In yet another strategy on *meso* diacetates, it is plausible to correlate the actual and observed enantioselectivities and more work in this direction is necessary. The strategy used is, enzymatic hydrolysis of a *meso* diacetate to give the racemic hydroxyacetate, followed by converting the hydroxy function to its keto derivative (in this case) and carrying out the enzymatic rehydrolysis under same conditions. The formation of optically pure hydroxyketone/ unreacted keto-acetate leads to the inference that the observed and actual enantioselectivities are different due to the acyl migration. The labeling strategy may also be useful for assessing the actual and observed regioselectivities in enzymatic hydrolysis of polyacylated systems including sugars.[2k]

3.7 EXPERIMENTAL SECTION

DCC and CD_3COOD (99.5%) were obtained from Aldrich Chemical Co. The substrate diacetates (±)-**1**,[11] (±)-**10**[12,13a] and (2*S*, 3*R*)-hydroxyacetate **2**[11] were prepared as reported before. The biphasic enzymatic hydrolysis of (±)-**1** and (-)-**4** using AmanoPS (800 U) and PLAP (20 U) were carried out using known[11] procedures. The activity of lipase powder has been expressed in terms of units, with 1 unit corresponding to micromoles of butyric acid (estimation by GC) liberated from glyceryl tributyrate per minute per milligram of enzyme powder. The term usual work-up refers to extraction with ethyl acetate, washing the organic layer with water, and brine, drying of organic layer over Na_2SO_4, and concentration under vacuo.

Preparation of 1,3-dioxolone-4-methanol-2,2-dimethyl-glycerol (7)[21]: To a 500 mL two-necked RB flask with dean-stark apparatus acetone (80 mL, 1.020 mol) and glycerol (25 g, 275 mmol) was charged and to it low boiling (40-60 fraction, 80 mL) of petroleum ether was added along with cat. amount of *p*-TSA (750 mg) and the reaction mixture was stirred under reflux, the formed water was removed azeotropically and the heating was stopped after 24 h. The reaction mixture on cooling was stirred with NaOAc (750 mg) for 45 min and filtered, concentrated and distilled under reduced pressure (100 ^{o}C at 12 mm of Hg) to give the product as colorless oil in 85% yield.

Preparation of 1,3-dioxolone-4-benzylmethyl-2,2-dimethyl-glycerol (8)[12]: In a three necked-flask NaH (60% suspension in paraffin oil, 2 g, 48 mmol) was washed with petroleum ether (20 mL X 3) and dried under vacuum, a slurry was made by adding anhyd. THF (30 mL). To this suspension a solution of acetonide **7** (5.4 g, 36 mmol) in anhyd. THF (15 mL) was slowly added at 0 ^{o}C and stirred for 1 h. BnBr (5.5 mL, 45 mmol) was added at rt and stirred overnight. The reaction was quenched by adding methanol, then reaction mixture was filtered over celite bed, concentrated, to this ethyl acetate was added and water wash, followed by brine, drying over Na_2SO_4 and concentrating under vacuo gave yellowish oil in 82% yield.

1-Benzylglycerol 9[12]: The solution of acetonide **8** (2.22 g, 10 mmol) in 15% acetic acid (100 mL) in distilled water was stirred for 4-5 h at 90^{o}C. The reaction mixture was cooled and extracted with ethyl acetate (30 mL X 3) and this was then washed with water, 10% bicarbonate solution, water and then with brine. The organic layer was dried over Na_2SO_4 and concentrated under vacuo to give colorless oil in quantitative yield.

1,2-Diacetyl-3-benzylglycerol (10): To a stirred solution of diol (1.82 g, 10 mmol) in pyridine (20 mL) was added acetic anhydride (15 mL) and the reaction mixture was kept in dark for 24 h at rt. On usual aqueous work-up, ether extraction followed by

concentration and silica-gel column chromatography purification gave pure diacetate **10**, 2.40 g (90.2% yield).

AmanoPS Catalyzed Biphasic Hydrolysis of 10: A solution of diacetate **10** (1 mmol) in petroleum ether:benzene (2:1) mixture (20 mL) was added to a suspension of AmanoPS (125 mg) in 50 mM sodium phosphate buffer (10 mL) pH 7.0 at 25^{0}C. After 2 h, the reaction mixture was filtered through celite and on usual work-up followed by silica gel column chromatographic removal of the unreacted diacetate (elution with 20% ethyl acetate : pet ether), furnished **11+12** (9:1) as a thick oil in 55% yield. In the spectral data only signals due to the major component **11** have been listed below. Similarly **13+14** on AmanoPS catalyzed hydrolysis furnished a mixture of **11+12+15** as a thick oil in 80:10:10 proportion.

PLAP Catalyzed Biphasic Hydrolysis of 10: A solution of diacetate **10** (5 mmol) in petroleum ether:benzene (2:1) mixture (150 mL) was added to a suspension of PLAP (500 mg) in 50 mM sodium phosphate buffer (75 mL) pH 8.0 at 25 ^{0}C. The pH was maintained at 8.0 using auto-stat with 0.1 M NaOH solution. At the end of 2 h, the reaction mixture was filtered through celite and on usual work-up followed by silica gel column chromatographic purification (elution with 20% ethyl acetate:pet ether), first gave pure **12** as a thick oil (20% yield) and then a mixture of **11** and **12** in 5% yield.

Similarly **13** on PLAP catalyzed furnished a mixture of **12+15+16** as thick oil in 47:8:45 proportion. While **13+14** (9:1) on PLAP catalyzed hydrolysis furnished a mixture of **11+12+16** as thick oil in 5:66:29 proportion.

Labeling of hydroxyacetate-2 with CD_3COOD/DCC: To a stirred solution of hydroxyacetate **2** (1 mmol), CD_3COOD (72 mg, 1.2 mmol), and cat. amount of DMAP in EDC (5 mL) was added a solution of DCC (247 mg, 1.2 mmol) in EDC (2 mL) in a dropwise fashion at rt. The reaction mixture was further stirred at rt for 45 min and then filtered through celite, the residue was washed with EDC and the organic layer was

concentrated in vacuo. The residue on usual work-up followed by silica gel column chromatographic purification (elution with 10% ethyl acetate:pet ether) gave the corresponding mono-labeled compound **4** in 90-95% yields as a thick oil.

By following a similar procedure, **11+12** (9:1) gave the mixture of **13+14** (9:1) as thick oil, **11** gave **14** as thick oil and **18** gave **19** as a thick oil, all in 90-95%yield

1,4-Diacetoxycyclopent-2-ene 17: To a stirred solution of diol (1.0 g, 10 mmol) in pyridine (20 mL) was added acetic anhydride (15 mL) and the reaction mixture was kept in dark for 24 h at rt. On usual aqueous work-up, ether extraction followed by concentration and column purification gave pure diacetate, 1.67 g (91.0% yield).

1-Acetoxy-4-hydroxycyclopent-2-ene (18): A solution of (±)-diacetate **17** (184 mg, 1 mmol) in petroleum ether: benzene (2:1) mixture (10 mL) was added to a suspension of Lipozyme lipase (200 mg) in 5 mM aq. sodium phosphate (5 mL) at pH 7.0. The reaction mixture was stirred at 25 ^{o}C for 36 h after which it was filtered through celite and extracted with ethyl acetate (25 mL x 3). The combined organic layer after usual work-up and concentration in vacuo gave an oily residue, which was subjected to column chromatography. Elution with 12% ethyl acetate:pet ether gave diacetate (**17**), 64.5 mg (35% yield) and with 18% ethyl acetate:pet ether gave hydroxy-aceate (**18**) 118 mg (60.0% yield)): [α]$^{25}_{D}$ = +66.4^{o} (c 1.0, $CHCl_3$) [lit. +66.3^{o} c 1.0, $CHCl_3$ >99 %ee][16]

1,2-Dihydroxyc yclopenetane (20): To a stirred solution of cyclopentene (1.15 g, 17 mmol) in *t*-butanol:water (125 mL) was added K_3FeCN_6 (16.8 g, 51 mmol) and K_2CO_3 (7.04 g, 51 mmol) the reaction mixture was cooled to 0^{o}C. To this mixture, was added dropwise a solution of OsO_4 (120 mg, 0.03 equiv.) in *t*-butanol. The reaction mixture was stirred at rt. for 2 days, and reaction was quenched with an aqueous solution of sodium sulphite (50 mL, 20%). The mixture was stirred at rt. for 1 h, filtered through

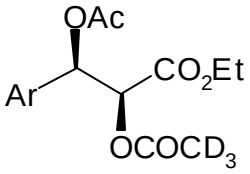
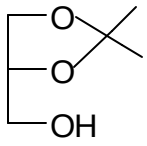
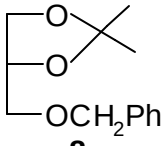
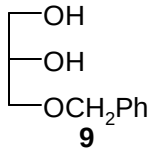
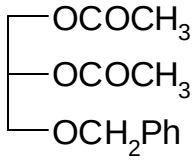
celite, concentrated in vacuo and extracted with ethyl acetate (50 mL x 3). The organic layer upon usual work-up gave a residue which was chromatographed on silica gel to obtain pure diol **20**, 1.13 g (65.0% yield).

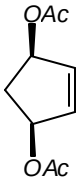
1,2-Diacetoxycyclopentane (21): To a stirred solution of diol **20** (1.02 g, 10 mmol) in pyridine (20 mL) was added acetic anhydride (15 mL) and the reaction mixture was kept in dark for 24 h at rt. On usual aqueous work-up, ether extraction followed by concentration and silica-gel column chromatographic purification gave pure diacetate **21**, 1.57 g (84.5% yield).

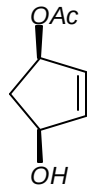
1-Acetoxy-2-hydroxycyclopentane (22 and **23):** A solution of (±)-diacetate **21** (186 mg, 1 mmol) in petroleum ether: benzene (2:1) mixture (10 mL) was added to a suspension of Amano PS lipase (100 mg) in 5 mM aq. sodium phosphate (5 mL) at pH 7.0. The reaction mixture was stirred at 25 °C for 12 h after which it was filtered through celite and extracted with ethyl acetate (15 mL x 3). The combined organic layer after usual work-up and concentration in vacuo gave an oily residue which was subjected to silica-gel column chromatography. Elution with 10% ethyl acetate:pet ether gave diacetoxy **21**, 65.1 mg (35% yield) and with 15% ethyl acetate:pet ether gave hydroxy-acetate **22** and **23** 86.5 mg (60.0% yield)):$[\alpha]^{25}_{D} = 0^{o}$ (c 1.0, $CHCl_3$).

1-Oxo-2-acetoxycyclopentane (24 and **25):** A solution of oxalyl chloride (0.2 mL, 2.2 mmol) in dry CH_2Cl_2 (5 mL) was placed in a two necked RB flask kept under argon at –60 °C, to it a solution of DMSO (0.35 mL) in dry CH_2Cl_2 (2 mL) was added dropwise over a period of 5 min. hydroxyacetate solution (288 mg, 2 mmol) in dry CH_2Cl_2 (5 mL) was charged dropwise over period of 5 min and stirred at –60 °C for a period of 15 min, TEA (1.5 mL, 10 mmol) was added and stirred at rt for 5 min. Water (5mL) was added to it and extracted with CH_2Cl_2 (7 mL x 3), and the organic layer was washed with brine, dried over Na_2SO_4 and concentrated under vacuo and silica-gel column chromatographed to give colorless oil in 229 mg, 80% yield.

AmanoPS Catalyzed Biphasic Hydrolysis of 1-oxo-2-acetoxycyclopentane (24+25): A solution of (±)-ketoacetate **24+25** (215 mg, 1.5 mmol) in petroleum ether: benzene (2:1) mixture (20 mL) was added to a suspension of Amano PS lipase (100 mg) in 5 mM aq. sodium phosphate (5 mL) at pH 7.0. The reaction mixture was stirred at 25 ^{o}C for 10 h after which it was filtered through celite and extracted with ethyl acetate (15 mL x 3). The combined organic layer after usual work-up and concentration in vacuo gave an oily residue, which was subjected to column chromatography. The spectral analysis showed 45% conversion (45% of hydroxyketone **26**) and 55% of unreacted acetoxyketone **27**. Elution with 10% ethyl acetate:pet ether gave acetoxyketone **27** 105.5 mg (49% yield):[$\alpha]^{25}_{D}$ = +33.4^{o} (c 1.0, $CHCl_3$).[19] We were unable to isolate pure hydroxyketone **26** and further work is in progress.

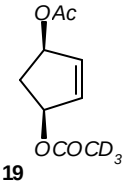
Structure (No.)	IR (cm^{-1}) PMR (d) CMR (d) and Mass spectral data
 **4**	**4**: IR (neat) ν_{max} 1740, 1730, 1720 cm^{-1}. ^1H NMR ($CDCl_3$, 200 MHz) δ 1.18 (t, *J* = 7.0 Hz, 3H), 2.10 (s, 3H), 3.80 (s, 3H), 4.15 (q, *J* = 7.0 Hz, 2H), 5.30 (d, *J* = 4.4 Hz, 1H), 6.22 (d, *J* = 4.4 Hz, 1H), 6.88(d, *J* = 8.8 Hz, 2H), 7.32 (d, *J* = 8.8 Hz, 2H). MS (m/e) 327, 264, 222, 179, 151, 137, 121.
 **7**	**7**: ^1H NMR ($CDCl_3$, 200 MHz) δ 1.36 (s, 3H), 1.43 (s, 3H), 2.25 (bs, 1H), 3.58 (dd, *J* = 3.5 & 3.2 Hz, 1H), 3.72 (dd, *J* = 3.4 & 3.5 Hz, 1H), 3.78 (dd, *J* = 5.4 & 5.6 Hz, 1H), 4.03 (dd, *J* = 5.4 & 5.6 Hz, 1H) 4.15-4.30 (dd, *J* = 6 Hz, 1H).
 **8**	**8**: ^1H NMR ($CDCl_3$, 200 MHz) δ 1.38 (s, 3H), 1.43 (s, 3H), 3.48 (dd, *J* = 4.8 & 4.8 Hz, 1H), 3.58 (dd, *J* = 4.9 & 4.9 Hz, 1H), 3.73 (dd, *J* = 5.8 & 6.1 Hz, 1H), 4.07 (dd, *J* = 5.8 & 5.8 Hz, 1H), 4.32 (quin, *J* = 6 Hz, 1H), 4.58 (d, *J* = 1.5 Hz, 2H), 7.20-7.40 (m, 5H).
 **9**	**9**: ^1H NMR ($CDCl_3$, 200 MHz) δ 3.50-3.60 (m, 2H), 3.60-3.80 (m, 2H), 3.85-4.00 (m, 1H), 4.55 (s, 2H), 5.00-5.50 (bs, 2H), 7.35 (bs, 5H).
 **10**	**10**: ^1H NMR ($CDCl_3$, 200 MHz) δ 2.05 (s, 3H), 2.10 (s, 3H), 3.62 (d, *J* = 5 Hz, 2H), 4.14-4.25 (dd, *J* = 12 & 7 Hz, 1H), 4.30-4.40 (dd, *J* =12 & 5 Hz, 1H), 4.55 (d, *J* = 2 Hz, 2H), 5.15-5.30 (m, 1H), 7.25-7.40 (m, 5H), ^{13}C NMR ($CDCl_3$, 50 MHz): 20.4, 20.7, 62.6, 68.0, 70.2, 73.1, 127.5, 127.6, 128.3, 137.7, 169.9, 170.2.

OH OCOCH$_3$ OCH$_2$Ph **11**	**11**: IR (neat) ν_{max} 3440, 1730 cm^{-1}. ^{1}H NMR (CDCl$_3$, 200 MHz) δ 2.12 (s, 3H), 3.67 (d, *J* = 5.5 Hz, 2H), 3.82 (d, *J* = 5.5 Hz, 2H), 4.56 (d, *J* = 2.5 Hz, 2H), 5.05 (quin, *J* = 5.5 Hz, 1H), 7.20-7.45 (m, 5H). ^{13}C NMR (CDCl$_3$, 50 MHz): 20.8, 62.0, 68.7, 73.2 (2C), 127.4, 127.6, 128.2, 137.6, 170.6. MS (m/e) 225, 207, 154, 137, 117, 105, 91.
OCOCH$_3$ OH OCH$_2$Ph **12**	**12**: IR (neat) ν_{max} 3440, 1720 cm^{-1}. ^{1}H NMR (CDCl$_3$, 200 MHz) δ 2.08 (s, 3H), 2.60 (bs, 1H), 3.45-3.62 (m, 2H), 3.97-4.10 (m, 1H), 4.08-4.26 (m, 2H), 4.58 (s, 2H), 7.20-7.48 (m, 5H). ^{13}C NMR (CDCl$_3$, 50 MHz) 21.0, 65.8, 68.9, 71.2, 73.6, 128.0 (2C), 128.7, 138.0, 178.3.
OCOCD$_3$ OCOCH$_3$ OCH$_2$Ph **13**	**13:** ^{1}H NMR (CDCl$_3$, 200 MHz) δ 2.10 (s, 3H), 3.60 (d, *J* = 5Hz, 2H), 4.12-4.25 (dd, *J* = 12 & 7 Hz, 1H), 4.30-4.42 (dd, *J* =12 & 5 Hz, 1H), 4.54 (d, *J* = 2 Hz, 2H), 5.23 (q, *J* = 6 Hz 1H), 7.20-7.45 (m, 5H); MS (m/e) 270, 210, 181, 162, 137, 117, 91, 43.
OCOCH$_3$ OCOCD$_3$ OCH$_2$Ph **14**	**14:** ^{1}H NMR (CDCl$_3$, 200 MHz) δ 2.06 (s, 3H), 3.60 (d, *J* = 5Hz, 2H), 4.12-4.26 (dd, *J* = 12 & 7 Hz, 1H), 4.28-4.40 (dd, *J* =12 & 5 Hz, 1H), 4.56 (d, *J* = 2 Hz, 2H), 5.16-5.30 (m, 1H), 7.25-7.45 (m, 5H); MS (m/e) 270, 207, 181, 162, 120, 91, 46.
 **17**	**17**: ^{1}H NMR (CDCl$_3$, 200 MHz) δ 1.75 (td, *J* = 15 & 5 Hz, 1H), 2.07 (s, 6H), 2.88 (td, *J* = 15 & 7.5 Hz, 1H), 5.50-5.60 (m, 2H), 6.10 (s, 2H).



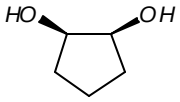
18

18: ^{1}H NMR ($CDCl_3$, 200 MHz) δ 1.65 (td, *J* = 15 & 5Hz, 1H), 1.88 (bs, 1H), 2.05 (s, 3H), 2.80 (td, *J* = 15 & 7.5 Hz, 1H), 4.67-4.78 (m, 1H), 5.45-5.55 (m, 1H), 5.98 (d, *J* = 8 Hz, 1H), 6.12 (d, *J* = 8 Hz, 1H). MS (m/e) 142, 125, 111, 98, 81, 70, 60, 54. IR (neat) ν_{max} 3400, 1720 cm^{-1}.



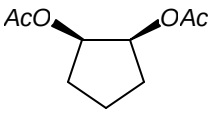
19

19: ^{1}H NMR ($CDCl_3$, 200 MHz) δ δ 1.75 (td, *J* = 15 & 5Hz, 1H), 2.07 (s, 3H), 2.80 (td, *J* = 15 & 7.5Hz, 1H), 5.50-5.60 (m, 2H), 6.10 (s, 2H).



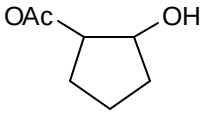
20

20: ^{1}H NMR ($CDCl_3$, 200 MHz) δ 1.35-2.00 (complex m, 6H), 3.75-4.15 (m, 2H). IR (neat) ν_{max} 3400 cm^{-1}.



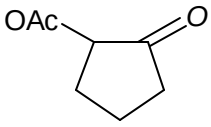
21

21: ^{1}H NMR ($CDCl_3$, 200 MHz) δ 1.50-2.10 (m, 6H), 2.04 (s, 6H), 5.00-5.25 (m, 2H). IR (neat) ν_{max} 3380, 1690 cm^{-1}.



22+23

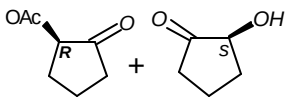
22+23: 1.40-2.05 (m, 6H), 2.08 (s, 3H), 2.25 (bs, 1H), 4.15 (q, *J* = 4.8 Hz, 1H), 4.87-5.03 (m, 1H). IR (neat) ν_{max} = 3320, 1695 cm^{-1}



24+25

24+25: ^{1}H NMR ($CDCl_3$, 200 MHz) δ1.70-2.00 (m, 2H), 2.13 (s, 3H), 2.20-2.50 (m, 4H), 5.07 (t, *J* = 8.8 Hz, 1H); ^{13}C NMR ($CDCl_3$, 50 MHz): 17.0, 20.5, 28.2, 34.6, 75.5, 169.9, 212.2.

MS (m/e) 143, 142, 99, 86, 71. IR (neat) ν_{max} 1749 cm^{-1}



27 **26**

Please see page 150.

3.8 REFERENCES AND NOTES

1 (a) Schmid, R. D.; Verger, R. *Angew. Chem. Int. Ed. Engl.* **1998**, *37*, 1608. (b) Kazlauskas, R. J.; Bornscheuer, U. T. *Biotechnology-Series, Vol. 8a*, VCH-Wiley, Wienheim 1998, p-37. (c) Theil, F. *Chem. Rev.* **1995**, *95*, 2203. (d) Mori, K. *Synlett* **1995**, 1097. (e) Wong, C. -H.; Whitesides, G. M. *Enzymes in Synthetic Organic Chemistry, Pergamon*, 1994 and references cited therein 1 a – e.

2 (a) Sugihara, J. M. *Adv. Carbohydr. Chem. I* **1953**, *8*, 1. (b) Angyal, S. J.; Melrose, G. J. H. *J. Chem. Soc.* **1965**, 6494 and 6501. (c) Welsh, L. H. *J. Org. Chem.* **1967**, *32*, 119. (d) Albert, R.; Dax, K.; Stuetz, A. E.; Weidman, H. *J. Carbohydr. Chem.* **1983**, *2*, 289. (e) Breitgoff, D.; Laumen, K.; Schneider, M. P. *J. Chem. Soc., Chem. Commun.* **1986**, 1523. (f) Belluci, G.; Bianchini, R.; Vechiani, S. *J. Org. Chem.* **1987**, *52*, 3355. (g) Liu, K. K. C.; Nozaki, K.; Wong, C. -H. *Biocatalysis* **1990**, *3*, 169. (h) Heisler, A.; Rabiller, C.; Hublin, L. *Biotechnol. Lett.* **1991**, *13*, 327. (i) Millqvist- Fureby, A.; Virto, C.; Adlercreutz, P.; Mattiason, B. *Biocatal. Biotransorm.* **1996**, *14,* 89. (j) D' Arrigo, P.; Servi, S. *Trends biotechnol.* **1997**, *15*, 90. (k) Horrobin, T. H.; Tran, C. H.; Crout, D. *J. Chem. Soc., Perkin Trans.1* **1998**, 1069.

3 For the recent example on intermolecular acyl migration in solid state see, Praveen, T.; Samanta, U.; Das, T.; Shahidhar, M. S.; Chakrabarti, P. *J. Am. Chem. Soc.* **1998**, *120*, 3842.

4 (a) Mattson, F. H.; Beck, L. W. *J. Biol. Chem.* **1955,** *214*, 115. (b) Mattson, F. H.; Beck, L. W. *J. Biol. Chem.* **1956,** *219*, 735.

5 (a) Mattson, F.; Volpenhein, R. *J. Lipid Res.* **1968**, *9*, 79. (b) Desnuelle, P.; Savary, P. *Biochim. Biophys. Acta*. **1956**, *21*, 349. (c) Briand, D.; Duberucq, E.; Galzy, P. *Eur. J. Biochem.* **1995**, *228*, 169.

6 Brockerhoff, H. *Biochim. Biophys. Acta.* **1968**, *159*, 296.

7 Ikeda, K.; Achiwa, K. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 225. The ^{1}H NMR data reported by the authors for the *p*-nitro derivative of **11** fits in well with the alternate

structure, ie *p*-nitro derivative of **12** and our method will be directly useful to confirm actual and observed selectivities in this example.

8 Iriuchijima, S.; Natsuko, K. *Agric. Biol. Chem.* **1982**, *46*, 1153.

9 Parmar, V. S.; Sinha, R.; Bisht, K. S.; Gupta, S.; Prasad, A. K.; Taneja, P. *Tetrahedron*, **1993**, *49*, 4107.

10 Kirschning, A.; Kreimeyer, M.; Blanke, H.-P. *Tetrahedron Asymm.* **1993**, *4*, 2347.

11 Desai, S. B.; Argade, N. P.; Ganesh, K. N. *J. Org. Chem.* **1996**, *61*, 6730.

12 Menger, F. M; Chen, X. Y.; Brocchini, S.; Hopkins, H. P.; Hamilton, D. *J. Am. Chem. Soc.* **1993**, *115*, 6600.

13 (a) Herradon, B.; Cueto, S.; Morcuende, A.; Valverde, S. *Tetrahedron Assym.* **1993**, *4*, 845. (b) Argazzoni, F.; Maconi, E.; Potenza, D.; Scolastico, C. *Synthesis* **1989**, 225.

14 The AmanoPS and PLAP catalyzed hydrolysis of (±)-**10** was non-stereoselective, see Taterie, N. H.; Bailey, R. A.; Kates, M. *Arch. Biochem. Biophys.* **1958**, *78*, 319.

15 PLAP catalyzed hydrolysis of (±)-**10** was arrested when the corresponding diol formation was detected on tlc (~25% conversion, 2 h).

16 Johnson, C. R.; Bis, S. J. *Tetrahedron Lett.* **1992**, *33*, 7287.

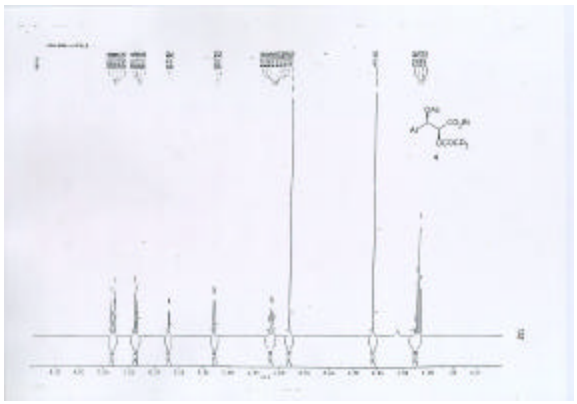
17 Xie, Z. -F.; Suemune, H.; Sakai, K. *J. C. S. Chem. Commun.* **1987**, *11*, 838.

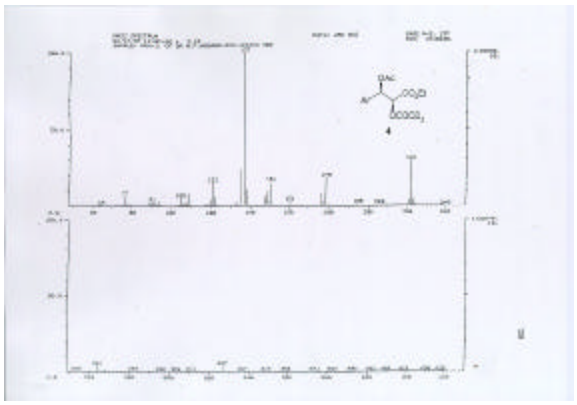
18 Crout, D. H. G.; Gaudet, V. S. B.; Laumen, K.; Schneider, M. P.; *J.C.S. Chem. Commun.* **1986**, 808.

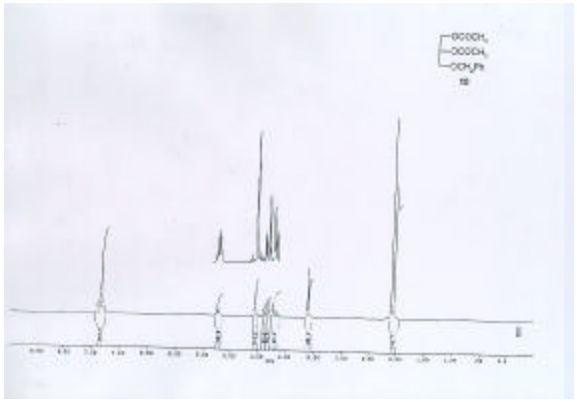
19 Tang, S.; Kennedy, R. M. *Tetrahedron Lett.* **1992**, *33*, 7823.

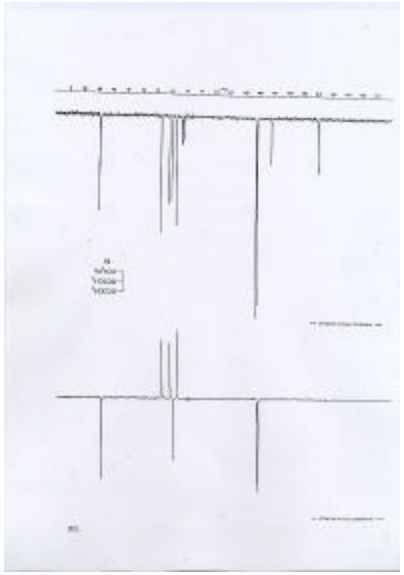
20 Desai, S. B.; Argade, N. P.; Ganesh, K. N. *J. Org. Chem.* **1999**, *64*, 8105.

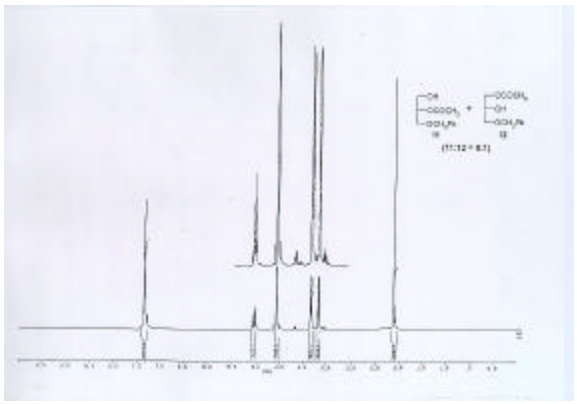
21 Renoll, M. Newman, M. S. Org. Syn. Coll. Vol. *3*, **1955**, 502.

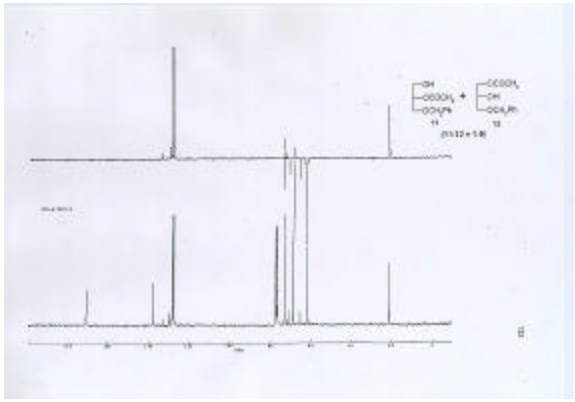


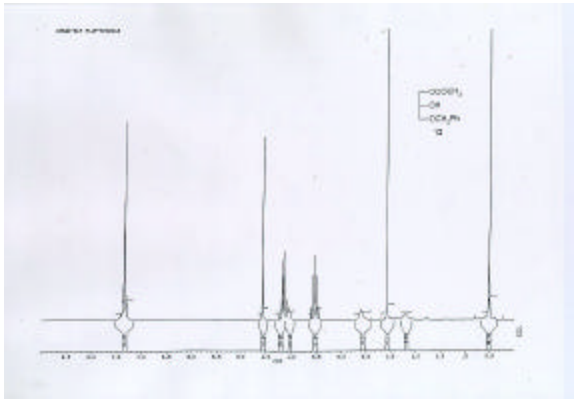


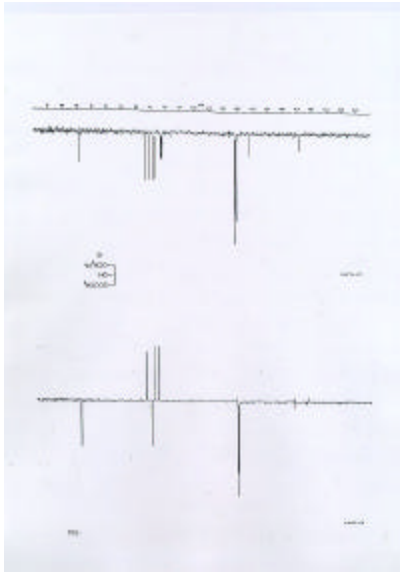


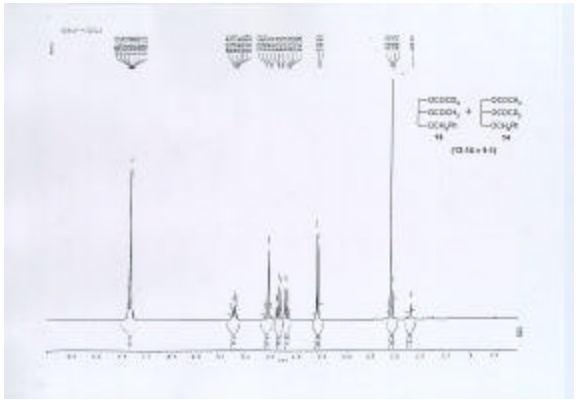


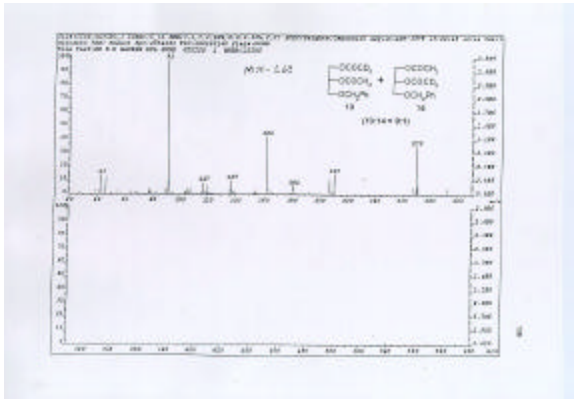


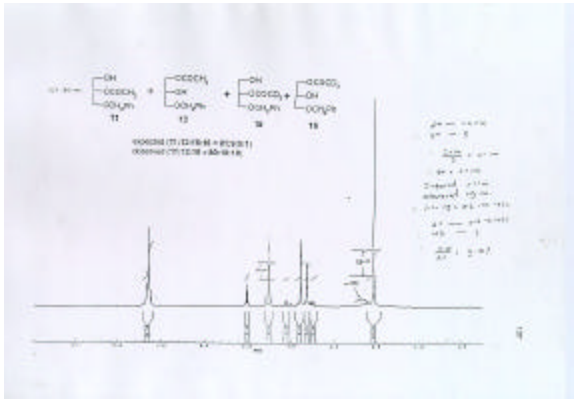


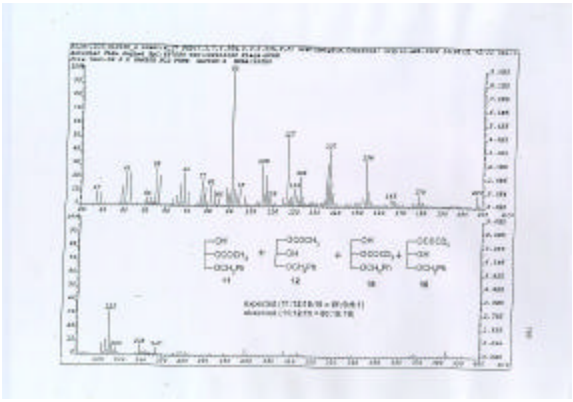


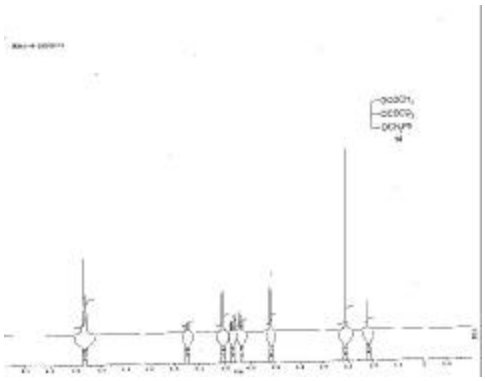


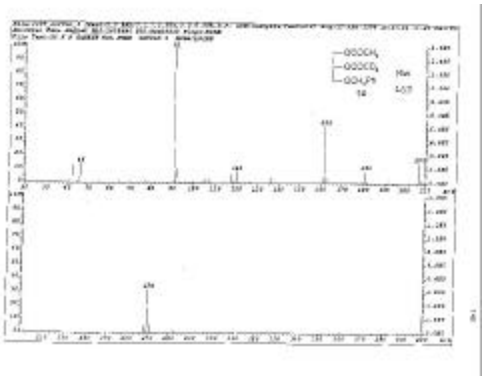


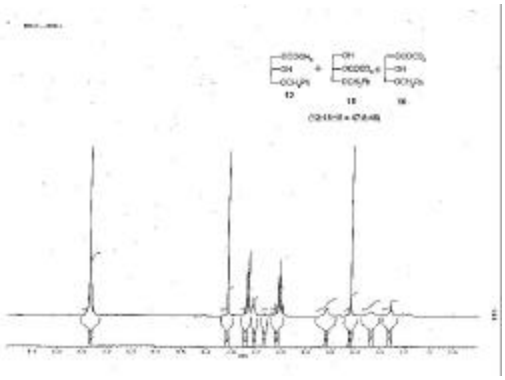


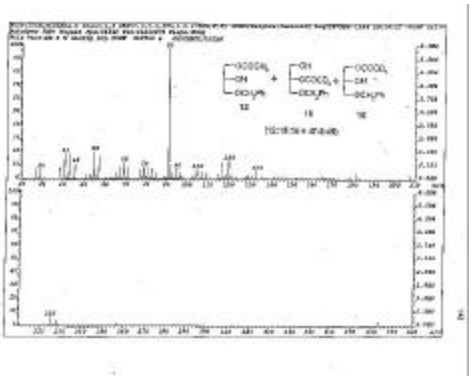


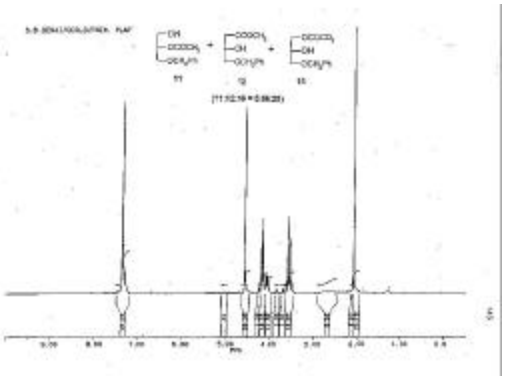


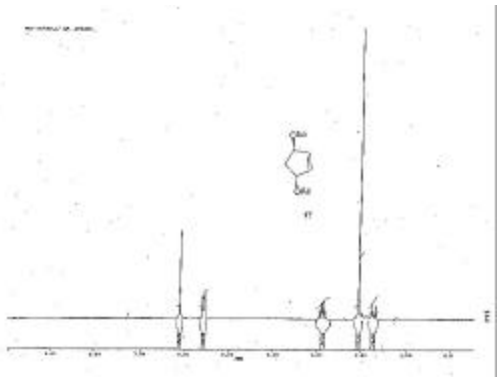


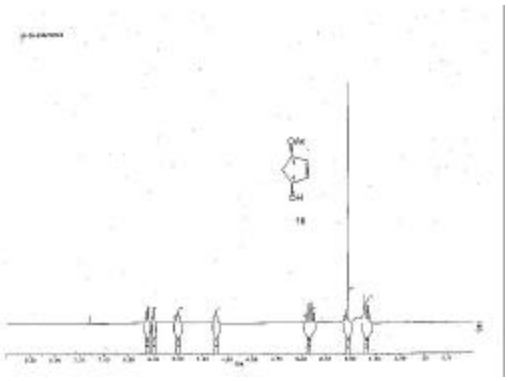


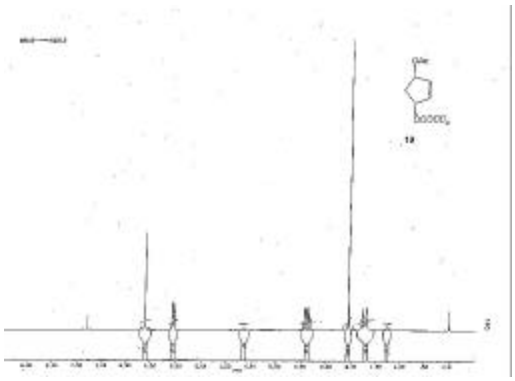


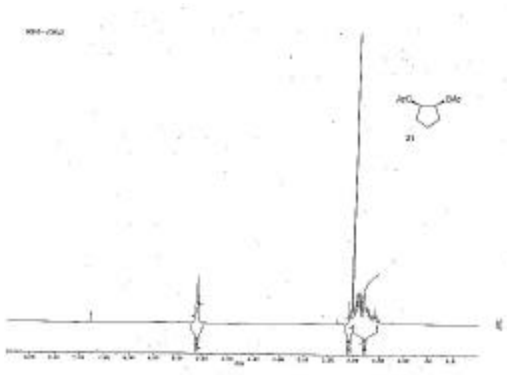


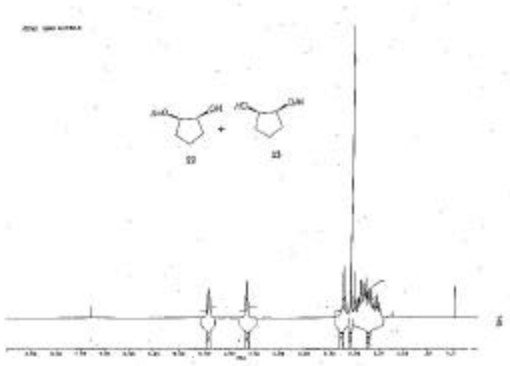


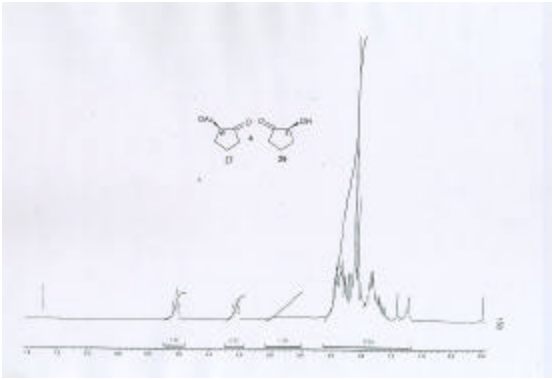












CHAPTER 4

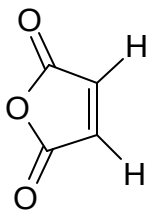
A FACILE SYNTHESIS OF RAS FARNESYL-PROTEIN TRANSFERASE INHIBITOR CHAETOMELLIC ACID A: SYNTHESIS AND AN ATTEMPTED ENZYMATIC RESOLUTION OF ITS CHIRAL ANALOGUES

SECTION A

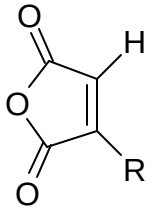
4.1 INTRODUCTION

4.1.1 Maleic Anhydride Derivatives and their Applications

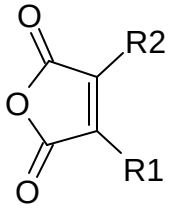
Maleic anhydride (2,5-furandione) **1** has been used by chemists since very early research times and it is prepared by vanadium pentoxide oxidation of butadiene or benzene. A vast array of nucleophilic reactions undergone by maleic anhydrides confer on them a high synthetic potential. In the past century, several symmetrically and unsymmetrically substituted maleic anhydrides have been prepared. The list of known mono and disubstituted maleic anhydrides is very large and some of them are represented below:



1



2



3

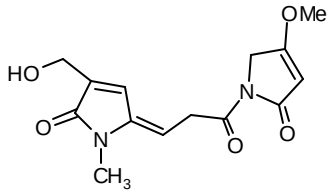
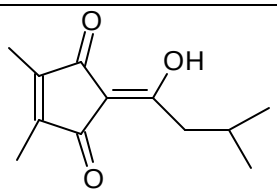
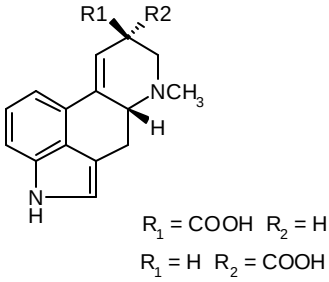
R/ R1/ R2 = Alkyl, OH, alkoxy, halo, phenyl, benzyl etc

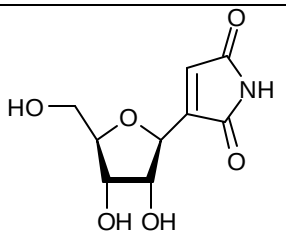
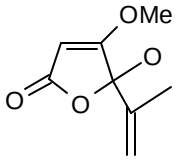
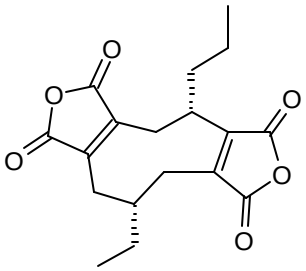
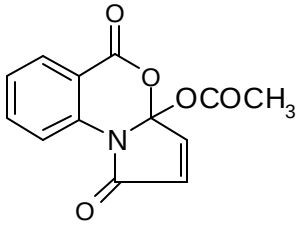
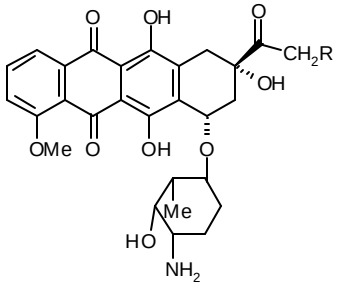
A large number of applications of maleic anhydrides have been reported and as such maleic anhydrides and their derivatives have been often used to model

- Compounds highlighting regiochemical dichotomy
- Heterocyclic skeletons
- Natural products and their precursors
- Bioactive molecules and
- Series of polymers with tailored material characteristics

A few representative important examples on above-mentioned applications are summarized in Table **1**. The synthesis of antibiotics showdomycin and *epi*showdomycin *via* maleimide-TPP adduct is interesting and practical, while the conversion of dimethylmaleic anhydride to adriamycin and daunorubicin are of commercial interest.

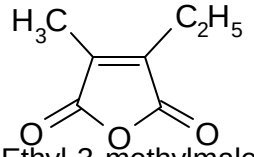
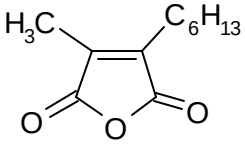
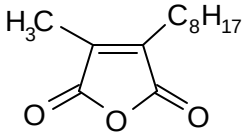
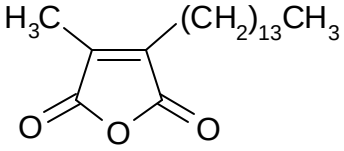
Table 1: Important Synthetic Applications of Symmetrically/Unsymmetrically Substituted Maleic Anhydrides

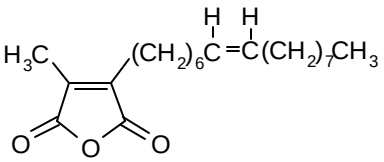
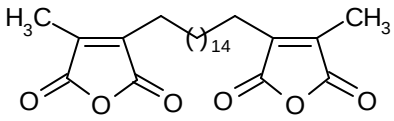
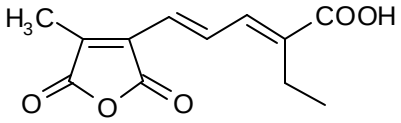
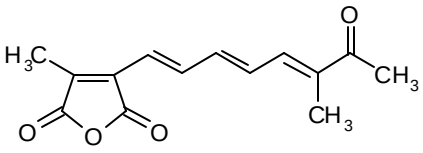
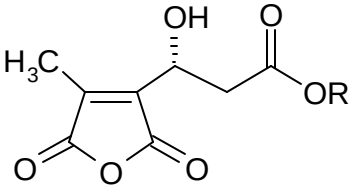
No.	Compound Synthesized	Source and Activity	Characterization	Ref.
1	 Pukelimide	*Lyngbya majuscula* (marine blue-green algae) irritant	IR, UV, PMR, Mass	1
2	 Calythrone	*Calythrix tetragona* (from oil)	UV, IR,PMR, mp of its Cu-salt	2
3	 Lysergic Acid	*Claviceps paspali* and *Claviceps purpurea* (ergot fungus) Hallucinogenic and anti- serotonin	IR, UV, PMR, Mass	3

4	 Showdomycin	*Streptomyces showdoensis* Antibiotic	IR, PMR, CMR, Mass.	4
5	 Penicillic Acid	*Streptomces spp* Antibiotic	IR, PMR, CMR, Mass	5
6	 Byssochlamic Acid	*Byssochlamys fulva*	IR, PMR, CMR, Mass	6
7	 Pyrrolobenzoxazinone	Synthetic	IR, PMR, Mass etc	7
8	 (R=OH/ H) Adriamycin/ Daunorubicin	*Streptomyces peucetius* Antibiotic, anti-cancer, immunomodulator and super oxide radical generation.	IR, PMR, CMR, Mass	8

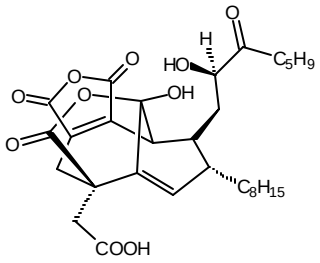
Recently several alkyl methyl subsituted maleic anhydrides have been isolated as biaoctive natural products and are listed in Table **2**. The structural features of these molecules reveal that half part of these molecules may constitute from pyruvic acid while the remaining half from the respective carboxylic acid derivative.

Table 2: Recently Isolated Naturally Occurring Alkyl Methyl Substituted Maleic Anhydrides

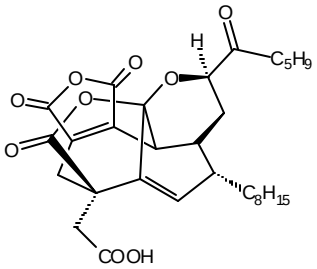
No	Compound Structure	Source/ bioactivity	Characterization	Ref
1	 2-Ethyl-3-methylmaleic anhydride	*Paederia foetida L.*. (from volatile oil) *Sambucus nigra L.* fruit	PMR, CMR, Mass, C,H-analysis	9
2	 2-Hexyl-3-methylmaleic anhydride	*Agropyrum repens* rhizome	PMR, CMR, Mass, C,H-analysis	10
3	 2-Octyl-3-methylmaleic anhydride	*Pseudomonas cepacia* A-1419	PMR, CMR, Mass C,H-analysis	11
4	 Chaetomellic anhydride A	*Chaetomella acutiseta* Ras farnesyl-protein transferase inhibitor	PMR, CMR, Mass, C,H-analysis	12

5	 Chaetomellic anhydride B	*Chaetomella acutiseta* Ras farnesyl-protein transferase inhibitor	PMR, CMR, Mass, C,H-analysis	12
6	 Tyromycin A	*Tyromyces lacteus* Aminopeptidase inhibitor, potential cytostatic activity	IR, PMR, CMR, Mass.	13, 14
7	 Telefairic Anhydride	*Xylaria telefairii* not known	PMR, CMR, Mass C,H-analysis	15
8	 Graphenone	Lichen mycobiont *Graphis scripta*	IR, PMR, CMR, Mass	16
9	 Maleic anhydride segment of Tautomycin	*Streptomyces spiroverticillactus* Antifungal activity (against *Sclerotina*) type1 and 2A protein phosphatase inhibitor	IR, PMR, CMR, Mass.	17

Recently two more interesting molecules having disubstituted maleic anhydride functionality, termed as the phomoidrides, (CP-263,114 and CP-225,917) have been



CP-263,114

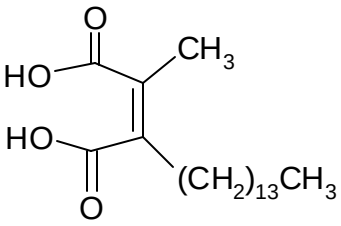


CP-225,917

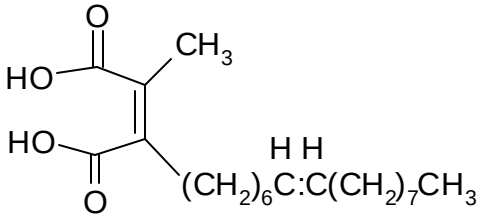
identified to have anticancer (farnesyl-protein transferase inhibitor) and anticardiovascular properties. Nicolaou *et al*[18] are the first group to achieve the total synthesis of these structurally interesting and biologically important molecules.

4.1.2 Alkylmethylmaleic Anhydrides: Chaetomellic Anhydride A

Chaetomellic acid A (**4**) and Chaetomellic acid B (**5**) have been isolated from *Chaetomella acutiseta*, by a group of scientists[19] at Merck in 1993, and the structure was elucidated by IR, PMR, CMR, and mass spectral data. The position and geometry of the

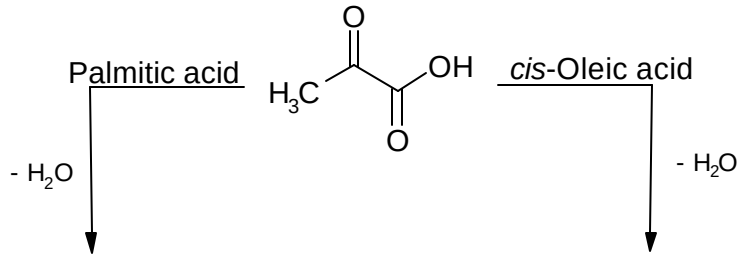


Chaetomellic Acid A (4)



Chaetomellic Acid B (5)

double bond in Chaetomellic acid B was established by MS analysis of the monoepoxide (prepared by reacting **5** with *meta*-chloroperbenzoic acid in CH_2Cl_2). It appears that both acids[19] may be produced in nature from the condensation of pyruvic acid with palmitic acid/ *cis*-oleic acid.



Chaetomellic acid A (4) **Chaetomellic acid B (5)**

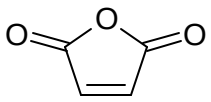
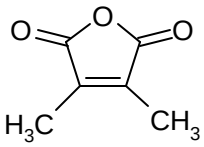
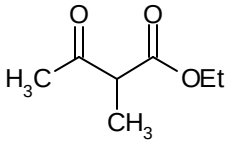
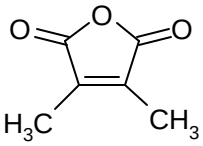
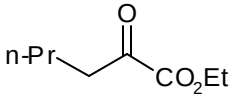
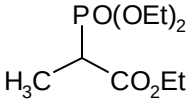
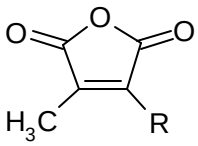
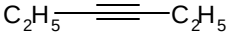
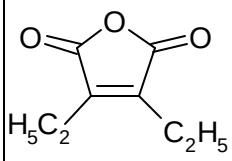
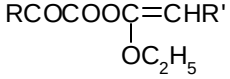
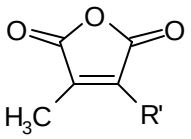
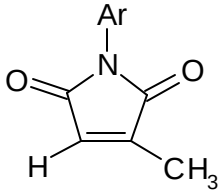
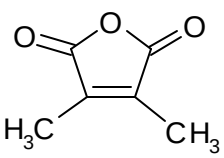
Both the diacids **4** and **5** are proved to be active inhibitors of Ras farnesyl-protein transferase enzyme, in their dianionic form. Mutated forms of *ras* oncogenes are associated with about 25% of human tumors.[19] The *ras* gene encodes a 21kD protein, called p21 or Ras, associated with the plasma membrane. The Ras protein functions by binding GTP to interact with effector molecules and this interaction is terminated by GTPase activity that converts Ras into complex with GDP. Inhibitors directed towards Ras function may have advantages over existing chemotherapeutic agents, the biochemical analysis of Ras function has revealed potential points of intervention.[20] One of these areas is the post-translation processing that is required to appropriately modify Ras prior to membrane association. The first and the obligatory step in Ras processing is Ras farnesylation by farnesyl-protein transferase (FPTase).[21] Genetic experiments have shown that farnesylation is required for Ras cell transforming activity. FPTase may, therefore, represent a target for chemotherapeutic intervention of human tumors mutated *ras* genes. FPTase utilizes farnesyl pyrophosphate (FPP) to modify the Cys residue at the C-terminus of Ras known as a CaaX box (C, Cys; a, an aliphatic amino acid; X, another amino acid). Analyses of the substrate requirements for FPTase have shown that enzyme binds with selective isoprenoid pyrophosphatase and CaaX tetrapeptides.[22] Potential peptide inhibitors [e.g. CVLS (IC_{50} = 2 μM), CVIM (0.1 μM), and CVFM (0.02 μM)][20] of FPTase can

be designed from the substrate. However, peptides are not the most desired compounds to be considered as therapeutic agents and there are interests to find non-peptide inhibitors of FPTase. For this purpose, microbial fermentation procedures were evaluated as sources for natural product inhibitors of FPTase. Among the compounds identified[19] were two novel dicarboxylic acids- Chaetomellic anhydride A and B, isolated from coelomycete, *Chaetomella acutiseta.* These inhibit FPTase (recombinant human enzyme purified to homogeneity) with IC_{50} values of 55 and 185 nM respectively.

In the present work, it was proposed to synthesize Chaetomellic acid A anhydride (teradecylmethylmaleic anhydride A) and its chiral analogues to study their bioactivity against Ras FPTase. The literature search revealed several potential methods for the synthesis of alkylmethylmaleic anhydrides and these are summarized in Table **3**. The synthesis of dimethylmaleic anhydride (entry1, Table **3**) *via* the condensation of 2-amino pyridine with 2 equiv. of maleic anhydride through *in-situ* formation of nitrogen ylide is an elegant method, used several times earlier in this laboratory to obtain gram quantities dimethylmaleic anhydride in >90% yield. Recently,[29] another new method has been developed in this laboratory to obtain dimethylmaleic anhydride by Wittig reaction of methyl maleimide and triphenylphosphine adduct with paraformaldehyde in more than 75% yield.

The provision of facile synthetic approaches to the bioactive natural product chaetomellic acid A anhydride is a task of current interest[30-36] and within five years of its isolation eight syntheses have appeared in literature including three from this laboratory. The sixth approach will be discussed in **section B** as a part of this dissertation. The chemistry of the seven alternate syntheses of **6** is briefly discussed in following schemes.[30-36]

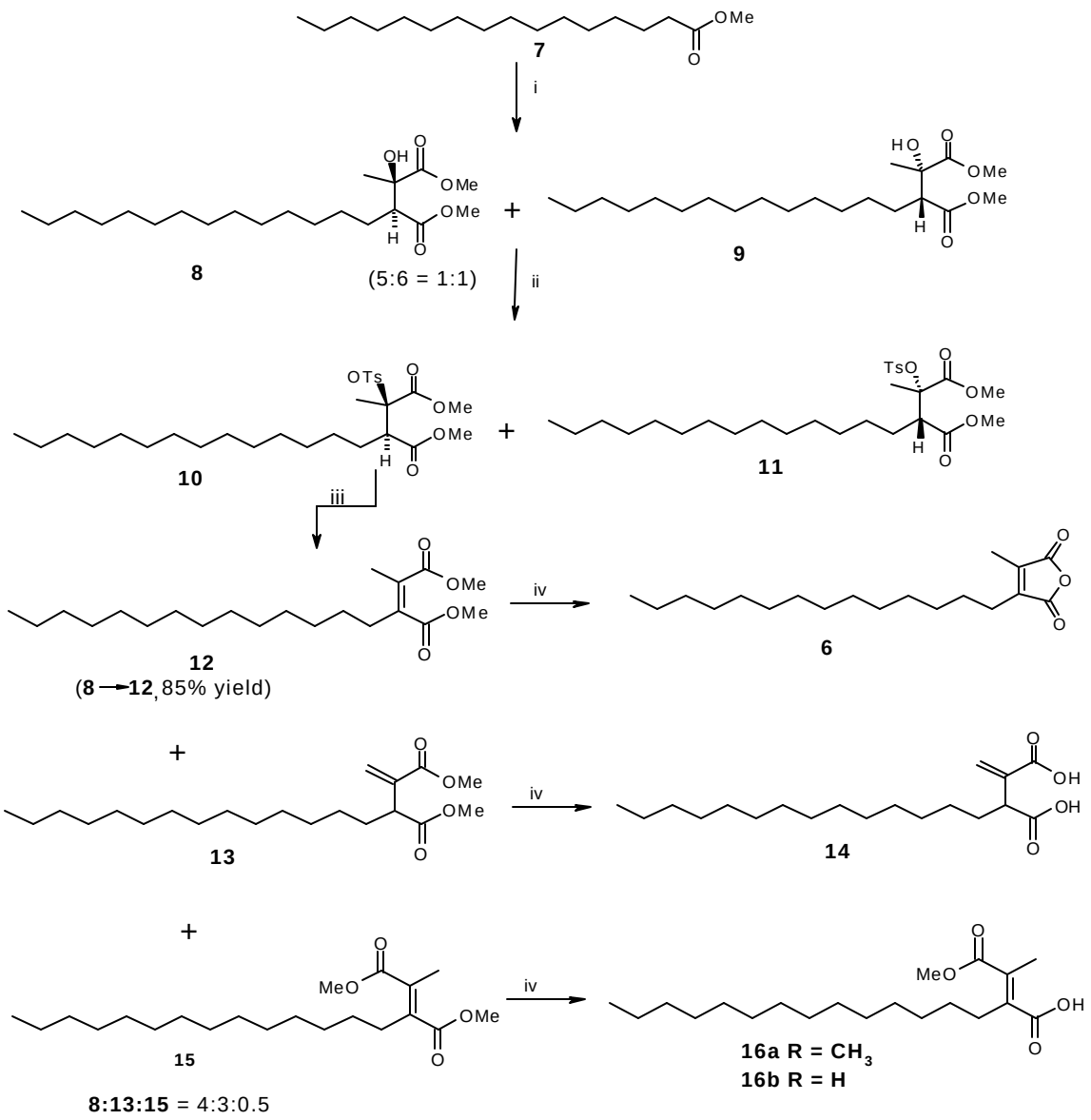
Table 3: Known Approaches for the Synthesis of Alkylmethylmaleic Anhydrides

No.	Starting material	Reagents/ Conditions	Product	Overall yield	Ref
1	Maleic Anhydride 	2-Aminopyridine, AcOH, reflux		75%	23
2	Ethyl 2-methyl acetoacetate 	i) NaCN/ H_2O ii)H_2SO_4		*	24
3	α-keto ester 	NaH/ 1,2-dimethoxy ethane 	 R= CH_3, C_4H_9	32% 56%	25, 26
4	Diethylacetylene 	$Ir(CO)_3Br$/THF/reflux/ under CO or inert/ 4N HNO_3		*	27
5	1-Ethoxy-1-alkenyl esters 	Pyrolysis of 1-Ethoxy-1-alkenyl ester (ketoacid/DCM 1-ethoxy-1-alkyne)	 R'=CH_3, C_4H_9	30% 44%	28
6	Methyl maleimide 	i)PPh_3, AcOH, $(CH_2O)_n$, reflux ii) Alkaline Hydrolysis		75%	29

* Not reported

The first biogenetic type of four-step synthesis of **6** with 18% overall yield involves[30] the base catalyzed nonstereospecific aldol condensation of methyl palmitate with methyl pyruvate followed by tosylation, elimination and acid hydrolysis as shown in **Scheme 1**.

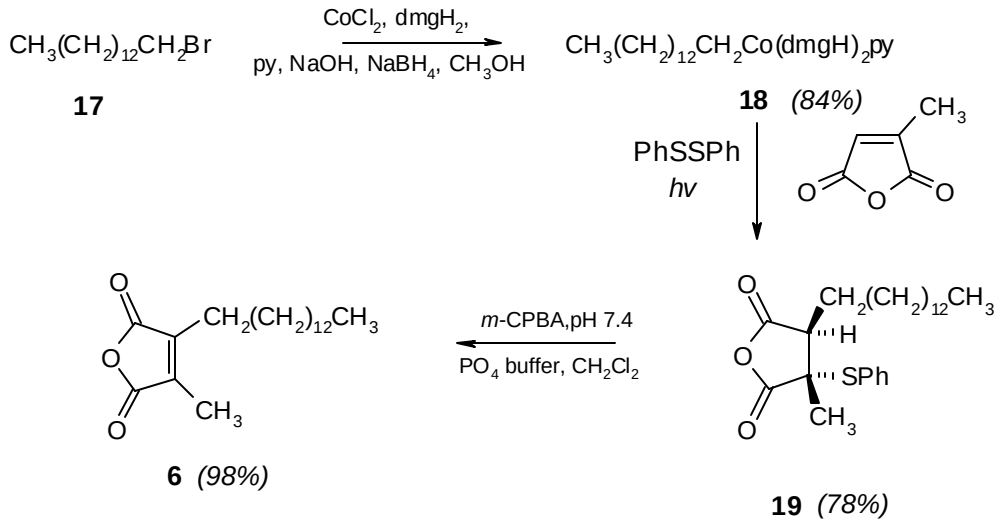
Scheme 1



Scheme 1: (i) LDA, THF, -78 to -10 °C, methyl pyruvate; (ii) CH_2Cl_2, pyridine, 40 °C, 2,6-Di-*tert*-butyl-4-methylpyridine, *p*-toluenesulfonic anhydride; (iii) Toluene, DBU, reflux (iv) a. 1N NaOH-CH_3OH-THF-H_2O, 80 °C; b. 4N HCl

The second three-step synthesis[31a] with 64% overall yield uses photochemical doubly chemoselective cross coupling of myristyl cobaloxime with citraconic anhydride and diphenyl disulfide. The oxidation of the sulfide in **19** to the sulfoxide with *meta*-chloroperbenzoic acid at 0 °C, with an *in-situ syn*-elimination under the reaction conditions provides the Chaetomellic Anhydride A (**6**) in 98% yield (**scheme 2**). A facile elimination in

Scheme 2

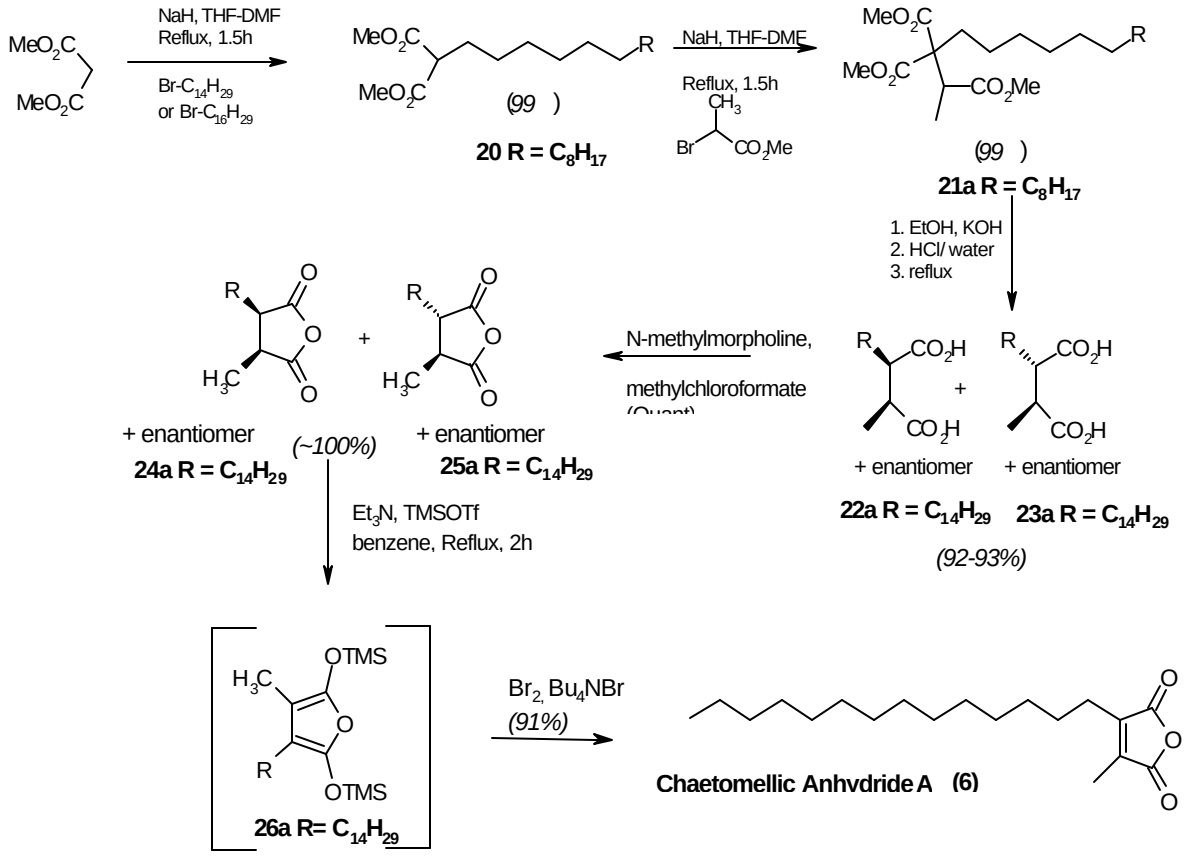


the intermediate sulfoxide establishes the *trans* stereochemical relationship of the thiophenyl and myristyl substituents in **19**, which is the predicted stereochemistry based on the steric effects.[31b]

The third five-step synthesis of **6** with 83% overall yield involves[32] a novel succinate to maleate oxidation (**scheme 3**). The succinate diastereomers (*threo* and *erythro*) were prepared using standard base catalyzed condensation (alkylation) of dimethyl malonate with 1-bromotetradecane, followed by condensation with methyl 2-bromopropionate offering the triester, which on hydrolysis followed by decarboxylation gave the corresponding 2-tetradecyl-3-methyl succinic acids **22** and **23**. Further the oxidative

sequence for the conversion of succinic-type anhydrides to maleic anhydrides was carried out by reaction of the succinic anhydrides with Et_3N and TMSOTf in benzene under reflux

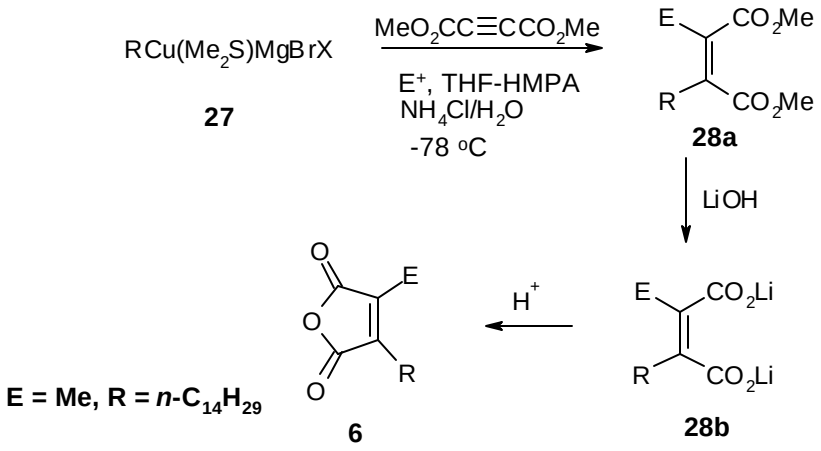
Scheme 3



to give the corresponding 3-tetradecyl-4-methyl-2,5-bis[(trimethylsilyl)oxy]furan **26**. Subsequent desilylation using pure tetra-*n*-butylammonium bromide in dry methylene chloride, followed by addition of pure bromine at 0 °C provides Chaetomellic Anhydride A. The authors have skillfully extended the same strategy to celebrate the first synthesis of Chaetomellic Anhydride B.

The fourth approach involves a two-step stereospecific synthesis by Vederas *et al*[33] using reaction of organocuprates and acetylenedicarboxylate with 78% overall yield. Thus, the Michael addition of the organocopper reagent **27** to dimethyl acetylenedicarboxylate in the presence of HMPA, followed by capture of the following enolate with methyl iodide and hydrolysis generates chaetomellic acid A (**6**) in 78% yield (**Scheme 4**). Similarly analogues wherein tetradecyl group was substituted with farnesyl/ gernylgeranyl moieties were

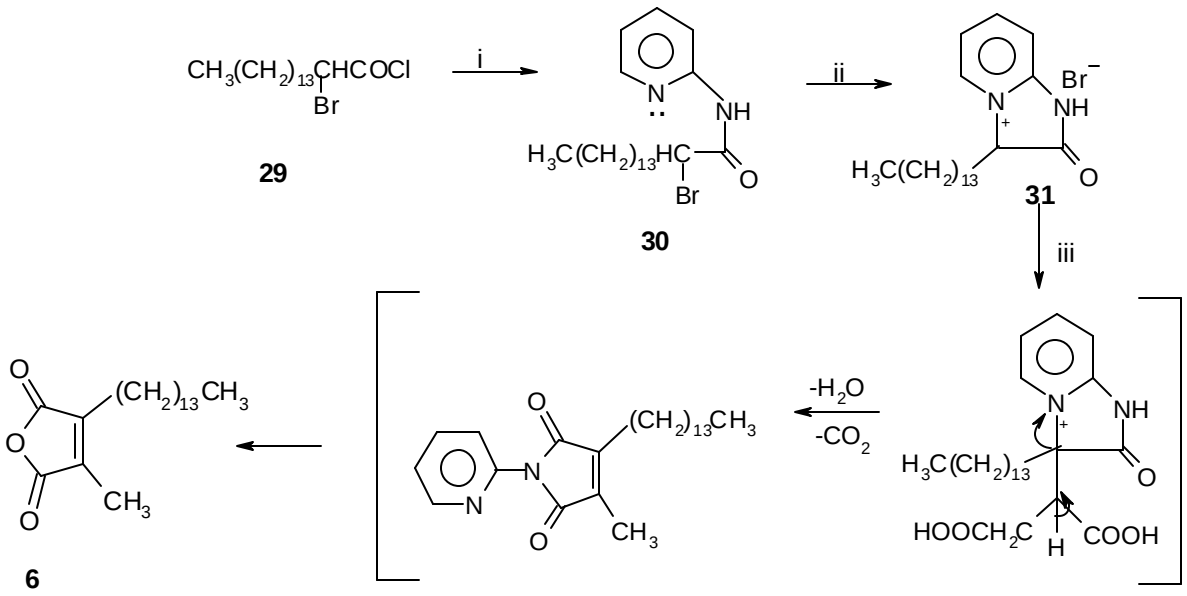
Scheme 4



prepared and the farnesyl substituted compound showed 7 times more potency than Chaetomellic Anhydride A.

A recent three-step approach employs[34] the condensation of imidazopyridinium bromide **31** obtained from the reaction of 2-bromopalmitoyl chloride and 2-aminopyridine with maleic anhydride in the presence of NaOAc/AcOH to form **6** in 62% overall yield.

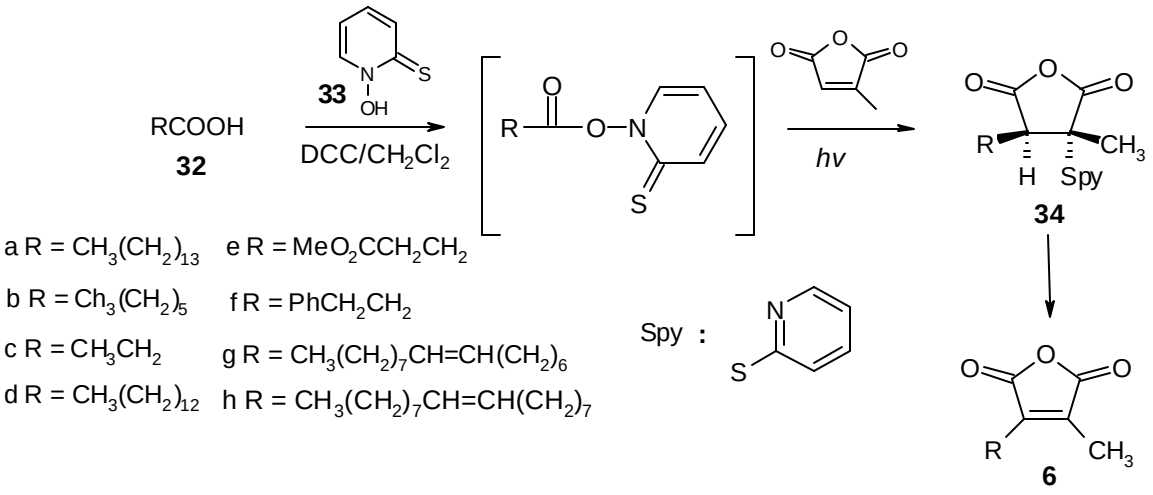
Scheme 5



(i) 2-Aminopyridine, TEA, Et_2O, rt; (ii) *t*-BuOH, reflux; (iii) maleic anhydride, NaOAc, AcOH, reflux

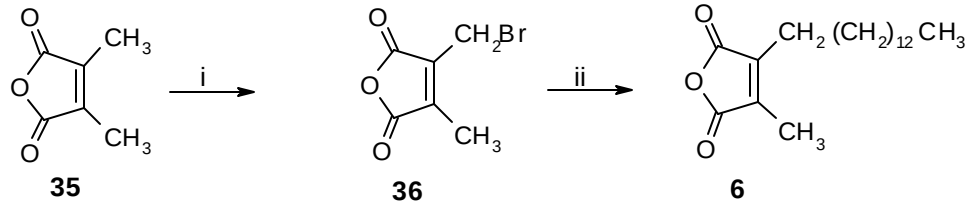
Another report by Samadi *et al*[35] uses one step synthesis by Barton radical decarboxylation; namely irradiation of thiohydroxamic esters derived from carboxylic acids, in the presence of citraconic anhydride to afford chaetomellic anhydride A and Chaetomellic anhydride B in overall yield of 70%. Later, the same group extended this approach to complete the first synthesis of Tyromycin A (Entry 6, Table **2**).

Scheme 6



The most recent report[36] from this laboratory demonstrates the chemoselective carbon-carbon coupling of (bromomethyl)methylmaleic anhydride and organocuprates (excess of *n*-tridecylmagnesiumbromide and cat. CuI) to give chaetomellic anhydride A in moderate yield (55%) (**Scheme 7**).

Scheme 7



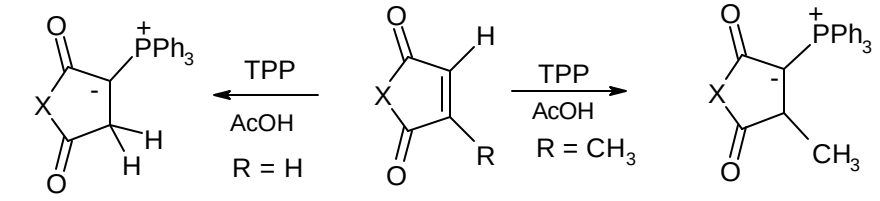
(i) NBS, Benzoyl peroxide, CCl_4, reflux 10h. (ii) $CH_3(CH_2)_{12}MgX$, Et_2O/THF, HMPA, CuI, -5 to 0 °C, 8h.

In the above described seven syntheses of **6**, interesting, novel chemistry has been reported with each synthesis having its own advantages. It appears that among these, the present 2-step approach to Chaetomellic acid A with 89% overall yield is the most efficient and practical. The sixth synthesis of Chaetomellic acid A and its derivatization to chiral analogues followed by an attempted enzymatic resolution are described in section B.

SECTION B

4.2 PRESENT WORK: RESULTS AND DISCUSSION

The formation of ylide adducts from the reaction of triphenylphosphine (TPP) with maleic anhydride, maleimide, citraconic anhydride, and citraconimide was established[37] three decades ago.



(Maleic anhydride/ imide-TPP adduct)

X = O, NAr

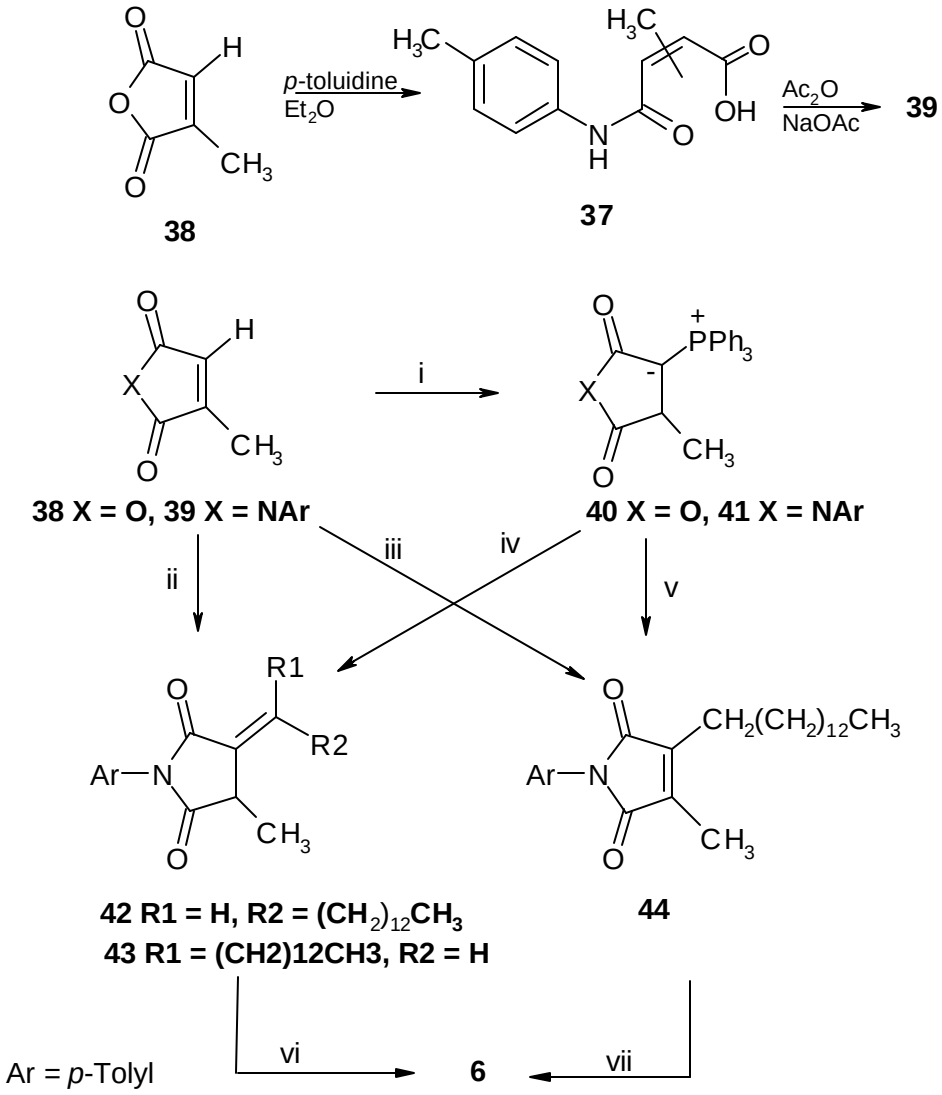
38 X = O
39 X = NAr

40 X = O
41 X = NAr

The phosphoranes generated from maleic anhydride and maleimide have been used for the synthesis of butenolides,[38] furans,[38] intermediates to lysergic acid,[3] showdomycin,[4] quinioline derivatives,[39] and bioactive molecules.[40] The ylides methyl(triphenylphosphoranylidene)succinic anhydride (**40**) and methyl-N-*p*-tolyl(triphenylphosphoranylidene)succinimide (**41**) respectively obtained from citraconic anhydride (**38**) and citraconimide **39** were found to be more stable, as the preliminary attempts to condense them with benzaldehyde were unsuccessful.[37] It was proposed to study the reactivity of the ylides **40** and **41** with aliphatic aldehydes to design a facile approach to chateomellic acid A anhydride (**6**) and other dialkyl-substituted maleic anhydrides.[9-11,41] All attempts to condense **40** with tetradecanal in various solvents (acetic acid, pyridine, methanol, acetone, chloroform, and benzene) under reflux conditions met

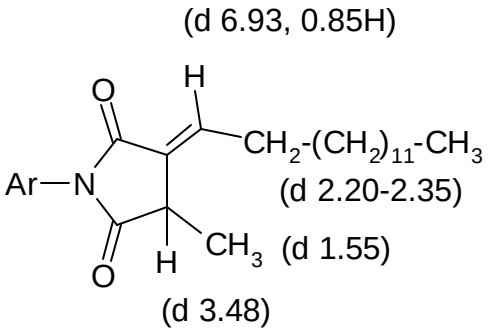
with failure. The relatively less stable citraconimide-TPP adduct **41** condensed very smoothly with tetradecanal in refluxing glacial acetic acid to yield a mixture of geometric isomers **42** plus **43** in 70% yield (**Scheme 8**). There was no reaction in any other solvents from the above

Scheme 8

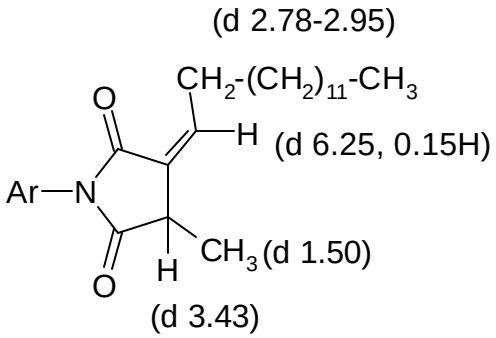


(i) PPh_3, AcOH, △, 2h. (ii) PPh_3, AcOH, $CH_3(CH2)_{12}CHO$, reflux, 18h
(iii) (a) condition ii, (b) △, 140-150 °C, 30 min.
(iv) AcOH, $CH_3(CH2)_{12}CHO$, reflux, 18h. (v) (a) condition iv,
(b) △, 140-150 °C, 30 min. (vi) (a) CH_3ONa/ CH_3OH, reflux, 2h,
(b) H^+ /HCl. (vii) $KOH/H_2O/CH_3OH$, reflux, 2h. (b) H+ /HCl.

list. Since the isolated yield of adduct **41** was only 45-50%, a direct reaction of imide **39** with tetradecanal was carried out in presence of TPP. A mixture of equivalent amounts of imide **39** and TPP and 1.5 equiv. of 80% pure tetradecanal was refluxed in glacial acetic acid for 18 h; when the glacial acetic acid was distilled off in vacuo at 50 ^{o}C bath temperature, the reaction furnished exclusively the mixture of **42** and **43** in 71% yield (*E:Z* = 85:15, by integration of vinylic protons in ^{1}H NMR, page 182) *via* **41**. The same relative ratio was also seen for other protons - allylic methylene (*E*, 2.20-2.35: *Z*,2.78-2.95), methyl (*E*,1.55 : *Z*,1.50), and methyne (*E* 3.48 : *Z* 3.43), the corresponding peaks of the minor *Z*-isomer are more upfield shifted with respect to the protons of the *E*-isomer, but interestingly the allylic methylene protons in case of the *Z*-isomer moves more downfield than the *E*-isomer because of peri interaction with imide carbonyl moiety.



E-isomer



Z-isomer

In the same reaction, when acetic acid was distilled off under normal atmospheric pressure at 140-150 ^{o}C and the oily residue was further heated at the same temperature for the next 30 min, the above reaction directly gave maleimide derivative **44**, via **41** and **42** plus **43** in 91% yield. Thus, both condensation and isomerization of double bond (exo to endo) are achieved in one pot. The mixture of **42** plus **43** on treatment with sodium methoxide in methanol under reflux conditions followed by acidification directly gave chaetomellic acid A anhydride (**6**) in 62% yield.

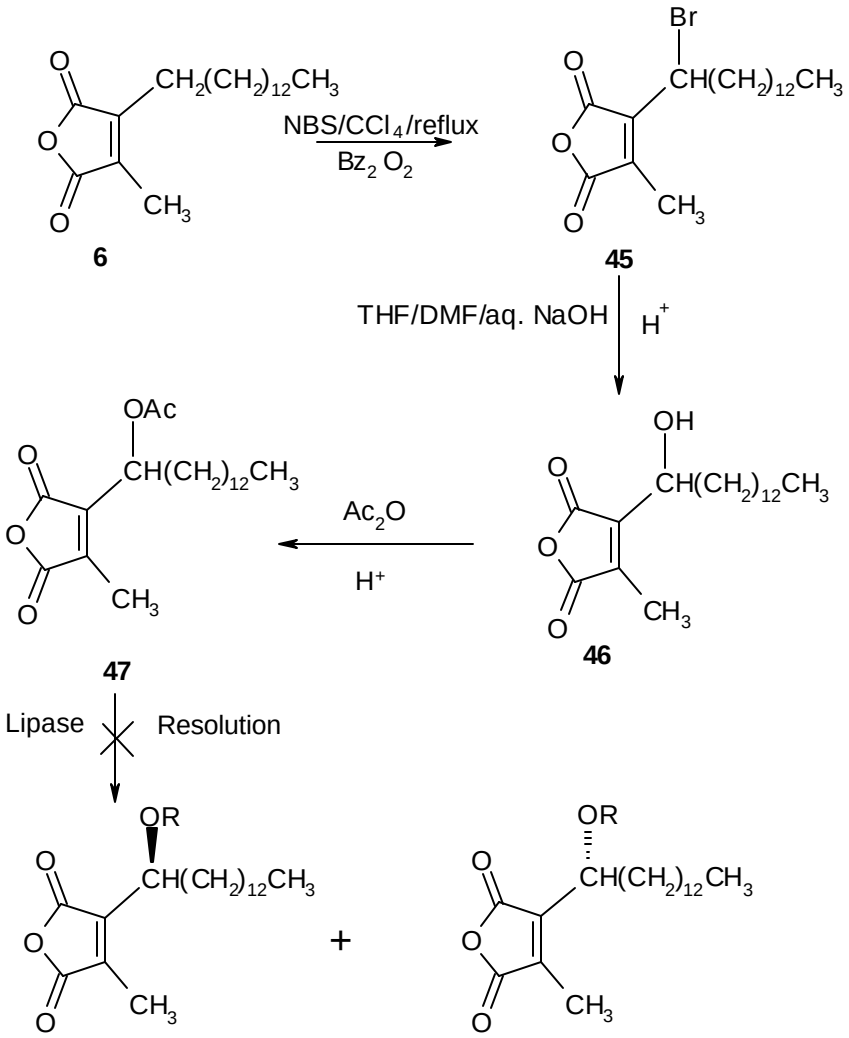
The trisubstituted exocyclic to tetrasubstituted endocyclic double bond isomerization is thermodynamically favored. It is possible that during the hydrolysis of **42**+**43** mixture, the first step is the double bond isomerization while the second step may involve hydrolysis of imide moiety to get exclusively the *cis* isomer. Various conditions for the alkaline hydrolysis of **44** were tried and the best results were obtained with THF-methanol-water (1:1:1) mixture as solvent system. The disubstituted maleimide **44** on alkaline hydrolysis under reflux conditions in THF-methanol-water as solvent system followed by acidification gave chaetomellic acid A anhydride (**6)** in two steps (**39** to **44** to **6**) with 89% overall yield. [42] It is known[19] that **6** in basic medium stays in the biologically active dianionic form.

4.2.1 Chiral Analogues of Chaetomellic Acid A

As discussed earlier, it was proposed to synthesize chiral analogues of Chaetomellic acid to study the structure-activity relationship. It was also planned to resolve these analogues by lipases. It is known that substitution of tetradecyl moiety of chaetomellic anhydride with farnesyl led to 7-fold increase[33] in the ras transferase protien inhibiton activity. One of the related compounds with better binding abilities than the parent Chaetomellic acid A is α-(hydroxyfarnesyl)phosphonate.[43] Apart from being a competitive inhibitor, it is also known to block ras processing *in vivo*. Considering the changes in the inhibition function related to the structural features of these molecules, the synthesis of hydroxy derivative of Chaetomellic acid A was executed to study its effect on Ras Farnesyl protein transferase inhibition. The overall strategy involved generation of a chiral center in racemic form at the allylic methylene moiety by NBS bromination and later carrying the subsequent substitution of the bromo derivative. The notion was, being a chiral molecule the enzyme might recognize only one of the enantiomer, effectively lowering the

concentration (IC_{50}) of the chiral molecule to be administered. The mixtures of enantiomers in the racemic compound were intended to be resolved by substituting the bromo with acetoxy via hydroxy derivative, consequently carrying out lipase mediated enantioselective hydrolysis of the acetoxy derivative to give pure enantiomers of the hydroxy and unreacted acetoxy dertivative (**scheme 9**). Apart from the enantomerically pure derivatives, the

Scheme 9



R = H or $COCH_3$

racemic forms of the hydroxy and the acetoxy derivative were also expected to show better binding abilities with FPTase. Hence using NBS/CCl_4, chaetomellic acid underwent

chemoselective bromination at allylic position to furnish the bromo derivative exclusively at the allylic methylene position and the primary allylic methyl was untouched. The secondary carbon function is then generated as a new chiral centre. The bromination of **6** carried under reflux with NBS/benzoyl peroxide in CCl_4 gave 3(α-bromotetradecyl)-4-methyl-2,5-furandione **(45)** (90%).

The bromo derivative **45** was then subjected to alkaline hydrolysis with THF-DMF-aq NaOH (1:1:1) to furnish 3(α-hydroxytetradecyl)4-methyl-2,5-furandione **(46)** in 80% yield. Attempts to oxidize the allyilc methyl group using SeO_2 in ethanol or acetic acid to obtain the other regio-isomer of hydroxy derivative was unsuccessful. The (±)-hydroxy-chaetomellic acid **46** when acetylated with Ac_2O and cat. H_2SO_4, to obtain the acetoxy derivative **47** (80% yield) which was subjected for enzymatic resolution with lipases, AmanoPS, PLAP so as to furnish the enantiopure isomers of alcohol **46**. All such attempts met with failure and the present substrates were not acted upon by the enzymes. Further changes in reaction conditions and scanning for different enzymes (tailor-made) for the resolution is warranted.

4.3 SUMMARY

In summary, we have demonstrated for the first time that the citraconimide-TPP adduct **41** condenses with aliphatic aldehyde (tetradecanal), providing a facile two-step synthesis of chaetomellic acid A anhydride (**6**) with 89% overall yield. This also has the potential for access to analogues of **6** and hence a new convenient and efficient method to model a variety of other dialkyl substituted maleic anhydride derivatives.

4.4 EXPERIMENTAL SECTION

***p*-Methyl methylmaleanilic acids (37)**: To a stirred solution of citraconic anhydride (3.82g, 3.5 mmol) in ether (25 mL) at room temperature was added a solution of *p*-toluidine (3.65 g, 3.5 mmol) in ether (25 mL) with constant stirring in a dropwise fashion over a period of 10 min. The reaction mixture was stirred at room temperature for 50 min and product precipitated was filtered, washed with ether (10 mL x 2) dried under vacuo to obtain a mixture of α-and β-isomer in a ratio of 9:1 (7.3 gm, 95% yield); mp 195-200 °C. The above mixture of acids on recrystallization from methanol gave exclusively the α-isomer (mp 201 °C).

N-*p*-Tolylcitraconimide (39). A mixture of Methylmaleanilic acids (9:1) (2.2g, 10 mmol) in Ac_2O (15 mL) and fused NaOAc (100 mg) was heated in water bath at 60-70 °C for 45 min. The reaction mixture was cooled to rt and poured in of ice cold water (200 mL). The formed precipitate was filtered, washed with excess of water and on recrystallization from ethanol gave **39** in 90% yield mp 115-116 °C.

Methyl(triphenylphosphoranyldiene)succinic Anhydride (40) and Methyl-*N*-*p*-tolyl(triphenylphosphoranyldiene)succinimide (41). These were prepared using literature procedures.[37] **40**: mp 180-181°C, **41**: mp 98-102 °C.

3-(*E:Z*)-Tetradecylidine-4-methyl-N-*p*-tolylsuccinimides (42/43). A mixture of citraconimide **39** (400 mg, 2 mmol), triphenylphosphine (524 mg, 2 mmol), and tetradecanal (630 mg, 3 mmol, 80%purity) in glacial acetic acid (10 mL) was refluxed with stirring for 18h. Acetic acid was distilled off in vacuo at 50 °C, and the residue was dissolved in ethyl acetate (25 mL). The organic layer was washed with water and brine and dried over Na_2SO_4. Concentration of the organic layer in vacuo followed by silica gel

column chromatographic purification of the residue using a mixture of petroleum ether and ethyl acetate (9:1) gave a mixture of **42** and **43** (**42**:**43** = 85:15 by ^{1}H NMR): 560 mg (71% yield); mp 53-57 ^{o}C.

Similarly the adduct **41** with tetradecanal under the same set of conditions gave a mixture of **42** and **43** in 70 –75% yield.

***N-p*-Tolylchaetomellic acid A imide (44).** The imide **44** was prepared using the same procedure as described for the preparation of **42** and **43**, except that the acetic acid was distilled off slowly over a period of 15 min at 140 –150 ^{o}C bath temperature and the oily residue was further heated with stirring for 30 min at same bath temperature to obtain **44**: 720 mg (91% yield); mp 74 –76 ^{o}C.

Similarly the adduct **41** with tertradacanal under the same set of conditions gave imide **44**, in 88 – 90% yield.

Chaetomellic Acid A anhydride (6). (**a**) A mixture of **43** plus **44** (100 mg) in a solution sodium methoxide (100 mg) in methanol (5 mL) was refluxed for 2 h with stirring. The methanol was removed in vacuo. The residue was acidified with dilute HCl and ether extracted (10 mL x 2), and the organic layer was washed with water, brine and dried over Na_2SO_4. Concentration in vacuo followed by silica gel column chromatographic purification of the residue furnished pure **6** (thick oil): 48 mg (62% yield). (**b**) To the solution of imide **44** (100 mg) in THF-methanol mixture (6 mL, THF:MeOH = 1:2) was added a solution of KOH (300 mg) in water (1 mL), and the reaction mixture was refluxed for 2 h with stirring. The solvent mixture was removed in vacuo, and the residue was acidified with dilute HCl. Repetition of the above workup procedure followed by silica gel column chromatographic purification furnished pure **6**: 76 mg (98% yield).

3(α-Bromotetradecyl)-4-methyl-2,5-furandione (45): To a solution of Chaetomellic anhydride A (**6**, 616 mg, 2 mmol) in CCl_4 (10 mL), N-bromosuccinimide (1.78 g, 10 mmol) and cat. amount of benzoyl peroxide was added, the solution was then refluxed with stirring for 9 h. The reaction mixture was allowed to cool to room temperature and then filtered, the residue was washed with CCl_4 (5 mL X 3); the combined organic layer was then washed with water, brine and dried over Na_2SO_4. Concentration of the organic layer in vacuo followed by silica-gel column chromatographic purification gave white fluffy solid **45**: 700 mg (90% yield); mp 42-44 ^oC.

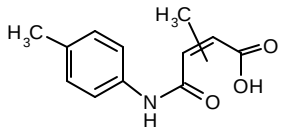
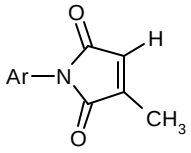
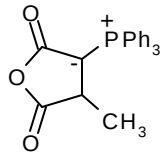
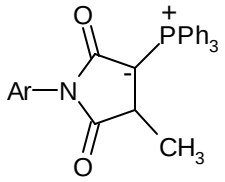
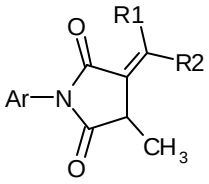
3-(α-Hydroxytetradecyl)-4-methyl-2,5-furandione (46): A solution of **45** (774 mg, 2 mmol), in THF:DMF:aq. NaOH (1:1:1, 10 mL) was stirred overnight at rt and to it 0.1N HCl was added till pH 2. The reaction mixture was then extracted with ethyl acetate (10 mL X 3), the combined organic layer was then washed with water, brine, dried over Na_2SO_4 concentrated in vacuo and column purified to give thick oily residue **46**: 520 mg (~80% yield).

3-(α-Acetoxytetradecyl)-4-methyl-2,5-furandione (47):

To a solution of α-hydroxy derivative of chaetomellic anhydride (**46**, 646mg, 2 mmol) in acetic anhydride (5 mL), a drop of conc. H_2SO_4 was added and stirred overnight under nitrogen at rt. The acetic anhydride was removed in vacuo and the resulting oily residue was purified by silica gel column chromatography (elution with petroleum ether/ethyl acetate 9:1) to offer **47**: 540mg (80% yield).

Attempted Resolution of 3-(α-acetoxytetradecyl)-4-methyl-2,5-furandione (47): A solution of (±)-acetate **47** (365 mg, 1 mmol) in petroleum ether : benzene (2:1) mixture (20 mL) was added to a suspension of Amano PS lipase (275 mg) in 5 mM aq. sodium

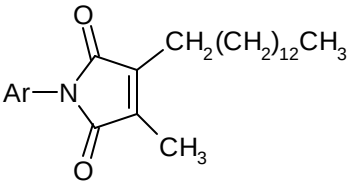
phosphate (10 mL) at pH 7.0. The reaction mixture was stirred at 25°C upto 120 h, monitored at intervals with tlc, after which it was filtered through celite and extracted with ethyl acetate (25 mL x 3). The combined organic layer after usual work-up and concentration in vacuo gave an oily residue, which was subjected to column chromatography. The starting material was recovered back with no hydrolyzed product, the lipase source was changed using the above procedure *viz*: PLAP, Amano PS and Amano "AY 30". The absence of any reaction taking place on the (±)-acetate **47** was also confirmed by PMR of the reaction mixture, evident from the recovery of the starting material spectrum.

Structure (No.)	IR (cm^{-1}) PMR (d) CMR (d) and Mass spectral data
 **37**	IR (nujol) ν_{max} 3400, 1715, 1660, 1620 cm^{-1}.
 **39**	^{1}H NMR ($CDCl_3$, 200 MHz) δ 2.18 (d, *J* = 2 Hz, 3H), 2.38 (s, 3H), 6.47 (q, *J* = 2 Hz, 1H), 7.20 (d, *J* = 10 Hz, 2H), 7.27 (d, *J* = 10 Hz, 2H); IR (nujol) ν_{max} 1710, 1685 cm^{-1}.
 **40**	^{1}H NMR ($CDCl_3$, 200 MHz) δ 0.82 (d, *J* = 7 Hz, 3H), 3.36 (q, *J* = 8 Hz, 1H), 7.30-7.80 (m, 15H).
 **41**	^{1}H NMR ($CDCl_3$+ DMSO-d_6, 200 MHz) δ 1.15 (d, *J* = 7Hz, 3H), 3.10 (s, 3H), 3.85-4.20 (m, 1H), 7.30-7.80 (m, 19H).
 **42 R1 = H, R2 = $(CH_2)_{12}CH_3$** **43 R1 = $(CH_2)_{12}CH_3$, R2 = H**	^{1}H NMR ($CDCl_3$, 200 MHz) δ 0.9 (t, *J* = 8 Hz, 3H), 1.30 (bs, 20H), 1.45 -1.60 (m, 2H), 1.53 (d, *J* = 10 Hz, 3H), 2.20 - 2.35 (m, 2H), 2.40 (s, 3H), 3.30 – 3.55 (m, 1H), 6.25 (dt, *J* = 10 and 4 Hz, 0.15H), 6.93 (dt, *J* = 10 and 4 Hz, 0.85H), 7.20 (d, *J* = 13 Hz, 2H), 7.30 (d, *J* = 13 Hz, 2H);

^{13}C NMR ($CDCl_3$, 50 MHz) δ 14.1, 16.4, 21.6, 22.7, 28.4- 29.7 (10 X CH_2), 32.0, 37.5, 126.2 (2-carbons, see protons coupled ^{13}C NMR data), 129.6, 130.8, 138.2, 140.3, 168.7, 177.2; the signals for C–4 methyl carbon, C-4 carbon and the proton bearing the olefinic carbon from the corresponding (*Z*)-isomer appeared at δ 16.2, 39.5, and 144.1, respectively. Proton-coupled ^{13}C NMR ($CDCl_3$, 50 MHz) δ 13.8- 38.0 (complex pattern, proton bearing saturated carbons), 126.3 (d), 126.4 (s), 129.8 (d), 130.8 (s), 138.4 (s), 140.6 (d), 168.9 (s), 168.9 (s), 177.4 (s);

MS (m/e) 397, 242, 229, 216, 203, 133, 118, 107, 95, 91, 81, 67, 55;

IR ($CHCl_3$) ν_{max} 1705, 1655 cm^{-1}.



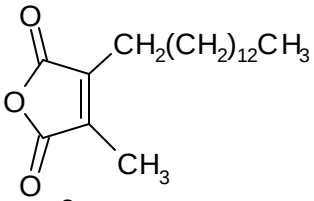
44

^{1}H NMR ($CDCl_3$, 200 MHz) δ 0.90 (t, *J* = 7 Hz, 3H), 1.30 (bs, 22H), 1.60 (m, 2H), 2.06 (s, 3H), 2.38 (s, 3H), 2.47 (t, *J* = 7Hz, 2H), 7.24 (bs, 4H);

^{13}C NMR ($CDCl_3$, 50 MHz) δ 9.0, 14.3, 21.3, 22.9, 24.0, 28.4, 29.5 – 29.9, (9 X CH_2), 32.1, 125.9 (2-carbons), 129.8, 137.3, 137.4, 141.5, 171.0, 171.3;

MS (m/e) 397, 382, 294, 228, 215, 203, 183, 149, 107, 91, 81, 67, 57;

IR (Nujol) ν_{max} 1710, 1690, 1650 cm^{-1}.



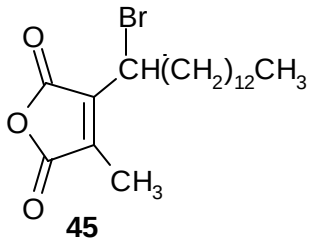
6

^{1}H NMR ($CDCl_3$, 200 MHz) δ 0.88 (t, *J* = 7 Hz, 3H), 1.15-1.45 (bs, 22H), 1.56 (m, 2H), 2.07 (s, 3H), 2.45 (t, *J* = 7 Hz, 2H);

^{13}C NMR ($CDCl_3$, 50 MHz) δ 9.6, 14.3, 22.9, 24.6, 27.7, 29.0 - 31.0 (9 x CH_2), 32.1, 140.6, 144.9, 166.0,166.4;

MS (m/e) 308, 290, 206, 191, 168, 150, 136, 126, 115, 105, 95, 91, 81, 69;

IR (neat) ν_{max} 2960, 2940, 2925, 2860, 1770, 1680, cm^{-1}.



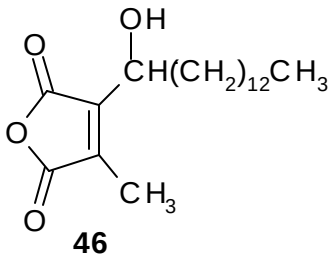
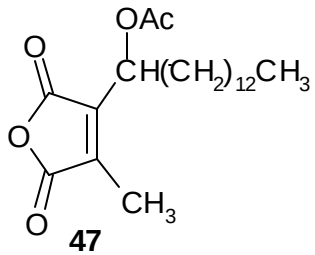
45

^{1}H NMR ($CDCl_3$, 200 MHz), δ 0.89 (t, *J* = 8 Hz, 3H), 1.18-1.45 (bs, 22H), 1.45-1.75 (m, 2H), 2.18 (s, 3H), 4.79 (t, *J* = 8 Hz, 1H);

^{13}C NMR ($CDCl_3$, 75 MHz) δ 9.9, 13.9, 22.5, 27.9, 28.6-29.5 (8 X CH_2), 31.8, 35.9, 39.4, 141.6, 141.8, 163.2, 165.0;

MS (m/e) 388, 387, 386, 385, 289, 261, 233, 191, 177, 163, 151, 139, 126, 111, 95, 81, 69, 57;

IR ($CHCl_3$) ν_{max} 2925, 2352, 1770, 1765 cm^{-1}.

 **46**	^{1}H NMR ($CDCl_3$, 200 MHz), δ 0.89 (t, *J* = 8 Hz, 3H), 1.26 (bs, 22H), 1.65-1.90 (m, 2H), 2.19 (s, 3H), 2.50-2.80 (bs, 1H), 4.71 (t, *J* = 8 Hz, 1H); ^{13}C NMR ($CDCl_3$, 50 MHz) δ 9.7, 13.7, 22.8, 25.5, 28-30 (8 X CH_2), 31.0, 35.6, 66.7, 141.5, 144.4, 164.8, 165.6; IR (neat) ν_{max} 3444, 2924, 1766 cm^{-1}.
 **47**	^{1}H NMR ($CDCl_3$, 200 MHz), δ 0.86 (t, *J* = 6 Hz, 3H), 1.24 (bs, 22H), 1.75-2.00 (m, 2H), 2.10 (s, 3H), 2.16 (s, 3H), 5.50-5.60 (m, 1H); MS (m/e) 324, 306, 261, 233, 193, 181, 151, 142, 126, 111, 95, 83, 69, 57; IR (neat) ν_{max} 1772, 1749 cm^{-1}

4.5 REFERENCES:

1. James, G. D.; Patenden, G.; Mills, S. D. *Tetrahedron Lett.* **1985**, *26*, 3617.

2. Gedge, D. R.; Pattenden, G. *J. C. S., Chem. Commun*. **1978**, 880.

3. Armstrong, V. W.; Coulton, S.; Ramage, R. *Tetrahedron Lett*. **1976**, 4311.

4. (a) Barett, A. G. M.; Broughton, H. B.; Attwood, S. V.; Gunatilaka, A. A. L. *J. Org. Chem.* **1986**, *51*, 495. (b) Barett, A. G. M.; Broughton, H. B. *J. Org. Chem.* **1984**, *49*, 3673.

5. Yeh, C. L.; Colwell, W. T.; DeGraw, J. I. *Tetrahedron Lett.* **1978**, *42*, 3987.

6. (a) White, J. D.; Dillon, M. P.; Bultin, R. J. *J. Am. Chem. Soc.* **1992**, *114*, 9673. (b) Stork, G.; Tabak, J. M. *J. Am. Chem. Soc.* **1972**, *94*, 4735. (c) Schaumann, E.; Ketcham, R. *Angew. Chem. Int. Ed. Engl.* **1982**, *21*, 225.

7. Balasubramaniyan, V.; Argade, N. P. *Tetrahedron* **1989**, *45*, 835.

8. Baurain, R. *Ger. Offen.* **1978**, *2*, 604,756.

9. Wong, K. C.; Tan, G. L. *Flavour Fragrance J.* **1994**, *9*, 25.

10. Boesel, R.; Schilcher, H. *Planta Med.* **1989**, *55*, 399.

11. Poll, L.; Lewis, M. J. *Lebensm. –Wiss. U. –Technol.* **1986**, *19*, 258.

12. See refs. 30-36 for synthesis of Chaetomellic acid A and see refs. 32 and 33 for Chaetomellic acid B.

13. Poigny, S.; Guyot, M.; Samadi, M. *J. Org. Chem.* **1998**, *63*, 1342.

14. Weber, W.; Semar, M.; Anke, T.; Bross, M.; Steglich, W. *Planta. Med.* **1992**, *58*, 56.

15. Adeboya, M. O.; Edwards, R. L.; Laessee, T.; Maitland, D. J.; Whalley, A. J. S. *Liebigs Ann. Chem.* **1996**, 1437.

16. Miyagawa, H.; Hamada, N.; Sato, M.; Uneo, T. *Phytochemistry* **1994**, *36*, 1319.

17. (a) Naganawa, A.; Ichikawa, Y.; Isobe, M. *Tetrahedron* **1994,** *30*, 8969 and refs. Cited therein. (b) Cheng, X. -C.; Kihara, T.; Kusakabe, H.; Magae, J.; Kobayashi, Y.; Fang, R. -P.; Ni, Z. -F.; Shen, Y. -C.; Ko, K.; Yamaguchi, I.; Isono, K. *J. Antibiot.* **1987**, *40*, 907.

18. Nicolaou, K. C.; Baran, P. S.; Zhong, Y. Li.; Choi, H. S.; Yoon, W. H.; He, Y.; Fong, K. C. *Angew. Chem. Int. Ed. Engl.* **1999**, *38*, 1669 and 1676.

19. Singh, S. B.; Zink, D. L.; Liesch, J. M.; Goetz, M. A.; Jenkins, R. G.; Nallin-Omstead, M.; Silverman, K. C.; Bills, G. F.; Mosley, R. T.; Gibbs, J. B.; Albers,-Schonberg, G.; Lingham, R. B. *Tetrahedon* **1993**, *49*, 5917.

20. Gibbs, J. B. *Semin. Can. Biol.* **1992**, *3,* 383.

21. (a) Gibbs, J. B. *Cell* **1991**, *65*, 1 and ref. cited therein (b) Der. C. J.; Cox, A. D. *Cancer Cells* **1991**, *3*, 331.

22. (a) Reiss, Y.; Goldstein, J. L.; Seabra, M. C.; Casey, P. J.; Brown, M. S. *Cell* **1990**, *62*, 81. (b) Schaber, M. D.; O' Hara, M. B.; Garsky, V. M.; Mosser, S. D.; Bergstrom, J. D.; Moores, S. L.; Marshall, M. S.; Friedman, P. A.; Dixon, R. A. F.; Gibbs, J. B. *J. Biol. Chem.* **1990**, *265*, 14701.

23. (a) Baumann, M. E.; Bosshard, H.; Breitenstein, W.; Rihs, G. *Helv. Chem. Acta* **1986**, *69*, 396. (b) Bauman, M. E.; Bosshard, H.; Breitenstein, W.; Rihs, G.; Winkler, T. *Helv. Chem. Acta* **1984**, *67*, 1897. (c) Bauman, M. E.; Bosshard, H. *Helv. Chem. Acta* **1978**, *61*, 2751.

24. Janitchke, L.; Kreiser, W. *Synthesis* **1976**, 314.

25. Huff, R.K.; Moppett, C. E.; Sutherland, J. K. *J. Chem. Soc. Sec.(C)* **1968**, 2725.

26. Jondiko, I. J. O.; Pattenden, G. *J. Chem Soc., Perkin Trans. 1* **1983**, 467.

27. Canziani, F.; Malatesta, M. C.; Longoni, G. J. *J. Chem. Soc., Chem. Commun.* **1975**, 267.

28. Newman, M. S.; Stalick, M. S. *J. Org. Chem.* **1973**, *38*, 3386.

29. Mangaleshwaran, S.unpublished results.

30. Singh, S. B. *Tetrahedron Lett.* **1994**, *35*, 6521.

31. (a)Branchaud, B. P.; Slade, R. M. *Tetrahedron Lett.* **1994**, *35*, 4071. (b) Branchaud, B. P.; Slade, R. M.; Janisse, S. K. *Tetrahedron Lett.* **1993**, *34*, 7885.

32. Kates, M. J.; Schauble, J. H.; *J. Org. Chem.* **1996**, *61*, 4164.

33. Ratemi, E. S.; Dolence, J. M.; Poulter, C. D.; Vederas, J. C. *J. Org. Chem.* **1996**, *61*, 6296.

34. Argade, N.P.; Naik, R. H. *Bioorg. Med. Chem.* **1996**, *4*, 881.

35. Poigny, S.; Guyot, M.; Samadi, M. *J. Chem. Soc., Pekin Trans. 1* **1997**, 2175.

36. Deshpande, A. M.; Natu, A. A.; Argade, N. P. *J. Org. Chem.* **1998**, *63*, 9557.

37. Hedaya, E.; Theodoropopulos, S. *Tetrahedron* **1968**, *28*, 2241 and ref. cited therein.

38. McMurry, J. E.; Donovan, S. F. *Tetrahedron Lett.* **1977**, 2869.

39. Argade, N. P.; Babar, S. M.; Balasubramaniyan, V. *Org. Prep. Proc. Int.* **1994**, *26*, 362.

40. Doulut, S.; Dubuc, I.; Rodriguez, M.; Vecchini, F.; Fulcrand, H.; Barelli, H.; Checler, F.; Bourdel, E.; Aumelas, A. *J. Med. Chem.* **1993**, *36*, 1369.

41. Itoh, S.; Esaki, N.; Masaki, K.; Blank, W.; Soda, K.; *Ferment. Bioeng.* **1994**, *77*, 513.

42. Desai, S. B.; Argade, N. P. *J. Org. Chem.* **1997**, *62*, 4862.

43. Gibbs, J. B.; Pompliano, D. L.; Mosser, S. D.; Rands, E.; Lingham, R. B.; Singh, S. B.; Scolnick, E. M.; Kohl, N. E.; Oliff, A. *Biochemistry* **1993**, *266*, 7617.