Biotransformations using lipase enzymes in organic solvents

Sanvoisin, Jonathan

Biotransformations Using Lipase Enzymes in Organic Solvents

by

Jonathan Sanvoisin, M.Sc,

Graduate Society,

Department of Chemistry,

University of Durham.

This thesis was submitted for the degree of PhD at the University of Durham, August 1993.

Acknowledgements :

I would like to thank Dr David O'Hagan for his help and support during my three years research and for his patience during the editing and proof reading of this thesis. I would also like to thank Mr L. Lauchlan for his assistance with the gas chromatography, Dr R Matthews, Dr A Kenwright and Mrs J. Say for their technical assistance in interpreting NMR data, and Dr M Jones for mass spectra.

I would also like to recognise the efforts of Russell, Marion, Vicki, Jenny, Jeff, David, Raju, Sarah, Nikki, Andy and Patrick for maintaining my sanity.

Financial support from the SERC and the University of Durham is gratefully acknowledged.

Biotransformations Using Lipase Enzymes in Organic Solvents.

by

Jonathan Sanvoisin, M.Sc.

Submitted for the degree of PhD at the University of Durham,
August 1993.

Abstract :

Enzymatic transformations in organic solvents have recieved increasing attention over the past 10 years and lipases have become by far the most popular enzymes in this area.

The initial aim of the research was to assess the effect of small modifications to the acyl donor in the transesterification reactions mediated by the *Candida cylindracea* lipase. 2.2.2-Trichloroethyl butyrate (TCEB) was used as a standard for the rate studies. In the event the acyl donor, trichloroethyl methoxyacetate (TCEMA), accelerated the transesterification reaction with hexan-1-ol by an order of magnitude over that with TCEB. This observation, and the absence of an acceleration with trichloroethyl methoxypropionate (and ethyl 2-fluorobutyrate over ethyl butyrate) suggested that the effect is due to the β-oxygen.

A solvent activity profile indicated that the most hydrophobic solvents supported faster initial rates. This was attributed to the ability of the hydophilic solvents to strip the hydrated water from the enzyme surface thus deactivating it.

The switch to organic solvents allowed a wider temperature range to be studied. For the reactions between heptan-2-ol and TCEMA the reaction could be conducted in the temperature range -23^{o}C to 80^{o}C.

It was of interest to assess how the alcohol moiety effected the transesterification reaction. A series of alcohols were presented to the enzyme and a pattern emerged with substrates containing an acetylene functionality being processed faster than those with a vinyl group, which were faster than those containing a methyl group (all other groups being the same).

A series of heterocyclic alcohols were presented to the enzyme and it was observed that the order of reaction was thiophene > furan > pyridyl. The secondary alcohols in this series, 2-thiopheneethan-1-ol and o-pyridylethan-1-ol, were resolved at various temperatures from -15^{o}C to 50^{o}C with no variation in enantioselectivity. These are the first resolutions to be accomplished at temperatures below zero degrees.

Contents.

Page

Chapter 1. Introduction.

1.1. Proteins as Enzymes. 5
1.2. Enzyme Catalysis. 6
1.3. Enzyme Classification. 10
1.4. Enzymes in Organic Synthesis. 16

Part 1. Biotransformations in Aqueous Media.

1.5. Whole Cell Systems. 17
1.5.1. Reductions. 17
a) Regioselectivity. 19
b) Resolution. 19
1.5.2. Hydrolyses. 20
1.6. Isolated Enzyme Systems. 22
a) Co-factor Regeneration. 22
1.6.1. Reductions. 24
a) Prochiral Selectivity. 25
b) Prelog's Rule. 26
c) Hydrogenation of Double Bonds. 26
1.6.2. Oxidations. 27
a) Alcohols. 27
b) Prochiral Selectivity. 28
c) Other Compounds. 29
1.6.3. Other Enzymes of the EC1 Group. 29
a) Oxidations and Reductions. 29
1.6.4. Hydrolyses. 31
a) Resolutions. 31
b) Prochiral Selectivity. 34
c) Prochiral Cyclic Systems. 38
d) Racemisation (as a method of increasing yield). 39
i. Chemical Racemisation. 39
ii. Enzymatic Racemisation. 40
e) Acylations and Transacylations. 41

1.6.5. Other Biotransformations. 43
a) Transferases (EC2). 43
i. Co-factor Synthesis. 43
ii. Chemical Synthesis. 45
b) Lyases (EC4). 46
c) Isomerases (EC5). 50
d) Ligases (EC6). 50

Part 2 Biotransformations in Organic Solvents.
1.7. Introduction. 53
1.8. Co-Solvents. 53
1.9. Emulsions. 56
1.10. Reverse Micelles. 57
1.11. Pure Organic Solvents. 59
a) Synthesis. 59
b) Reaction Conditions. 60
1.11.1.
a) Resolutions. 61
b) Transesterifications. 63
c) Effect of Acyl Donor. 66
d) Bifuncional Compounds. 67
1.10.2. Other Biotransformations
Using Hydrolases. 68
1.10.3. Biotransformations Using
Other Enzymes. 70
a) EC1 Oxidoreductases. 70
b) EC4 Lyases. 71

Chapter 2. Effect of Modifying Acyl Donor.
Introduction. 72
Objective. 80
Results. 82
Discussion. 85
Conclusion. 96

Chapter 3. Variation of Solvent and Temperature.

A.1. Effect of Solvent on Rate Kinetics.

Introduction. 97

Objective. 99

Results and Discussion. 100

2. Effect of Solvent on Enantioselectivity.

Introduction. 102

Objective. 104

Results and Discussion. 104

Conclusion. 108

B. Effect of Temperature on Reaction Kinetics and Enantioselectivity.

Introduction. 109

1. Elevated Temperatures.

Results and Discussion. 110

2. Low Temperatures.

Introduction. 113

Objective. 114

Results and Discussion. 115

Conclusion. 117

Chapter 4. Structure Activity Relationships with The *Candida cylindracea* Lipase. Part 1.

A. Reaction Kinetics.

Introduction. 119

Results and Discussion. 122

1. Effect of Alkyl Chain. Length on Reactivity. 122

2. Reactivity of Various Primary Heterocyclic Alcohols. 125

3. Reactivity of a Range of Secondary Heterocyclic Alcohol. 133

B. Studies in Enantioselectivity.
1. Resolution of 2-Pyridylethan-1-ol at Room Temperature (25°C) 138
2. The Effect of Temperature on Enantioselectivity. 139
Conclusion. 148

Chapter 5. Structure Activity Relationships with the *Candida cylindracea* lipase. Part 2.
A. Structure Activity Relationships for a Range of Secondary Alcohols.
Introduction. 144
Results and Discussion. 146
B. Structure Activity Relationships for a Range of Tertiary Alcohols.
Introduction. 152
Results and Discussion. 153
Conclusion. 156

Chapter 6. Experimental. 157

References. 179

Appendix 1. Publications 1989-1993. 200

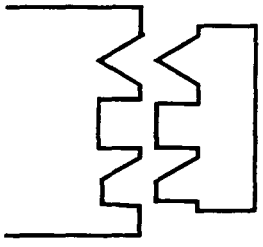
Appendix 2. Seminars and Conferences. 201

Chapter 1

Introduction

1.1 Proteins as Enzymes.

An enzyme is a protein that acts as a catalyst in biological systems. The enzyme is constructed in such a way as to create a 3 dimensional cleft or cavity, termed the active site, that will accept a substrate. In all enzymes of known structure substrate molecules are bound to the active site,"cleft", from which water is excluded unless it is a reactant[1]. The cleft contains polar residues that are essential for binding and catalysis. In 1890 Emil Fischer proposed the lock and key metaphor for substrate binding.

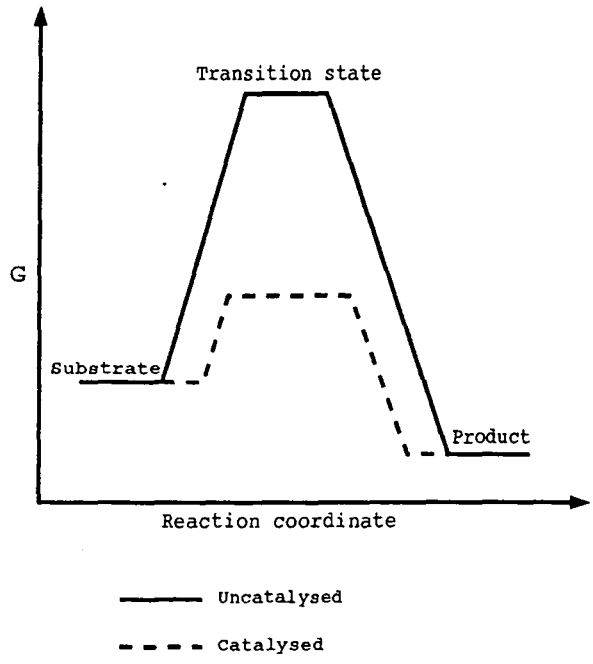


Enzyme Substrate

This model has proved useful, as a working hypothesis to rationalise substrate specificity and stereoselectivity, however, it has been shown more generally that active sites are not rigid and in certain cases a configurational change occurs with binding. This has led to a refinement of the Fisher model and is generally termed, "induced fit".

1.2 Enzyme Catalysis.

Current understanding of enzyme catalysis is dominated by the hypothesis of transition state binding introduced by Linus Pauling in 1948[2]. The quarternary folding of the protein generates a binding site that is tailored to the transition state of the reaction to be catalysed. Thus the enzyme stabilises the transition state and lowers the energy barrier for the enzyme reaction.

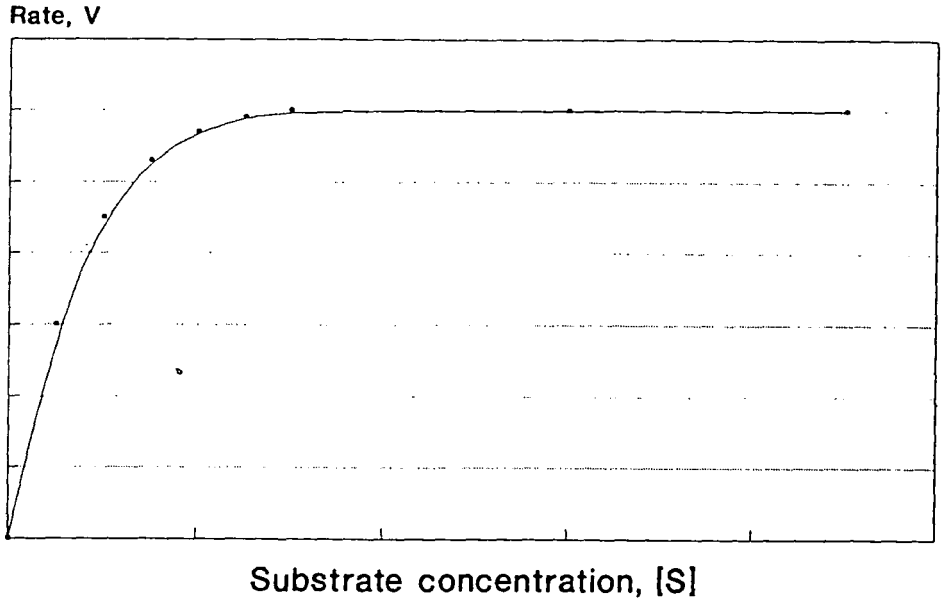


$$\Delta G^{\ddagger} = G_{transition\ state} - G_{substrate}$$

The reaction rate is proportional to the free energy difference between the substrate and the transition state, the free energy of activation ($\Delta G^{\ddagger}$). A decrease in $\Delta G^{\ddagger}$ results in a rate increase.

Generally the rate, V, varies with the substrate concentration, [S], in a manner shown in Graph 1.

Graph 1.



At low [S], V is proportional to [S] for a fixed enzyme concentration. At high [S] however, V becomes independent of [S].

In 1913[4], Leonor Michaelis and Maud Menten proposed a simple model to account for the observed kinetic characteristics of enzyme processes. A key feature of their model involves a specific enzyme substrate complex (ES);

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + P \qquad 1$$

The enzyme, E, combines with the substrate, S, to form the complex, ES, with a rate constant, k_1. ES complex has two possible fates; it can, either dissociate to E and S with rate constant, k_2, or it can proceed to product, P with rate constant, k_3. It is assumed that no product reverts to the initial substrate, a condition that holds for the initial stage of the reaction before the concentration of product is appreciable.

To derive the Michaelis-Menten equation an expression for the rate of catalysis is required;

$$V = k_3 [ES] \qquad 2$$

We need to express [ES] in known quantities, eg

$$\text{Rate formation ES} = k_1[E][S]$$

$$\text{Rate of breakdown ES} = (k_2 + k_3)[ES]$$

In a steady state situation the rate of formation and breakdown of ES complex is the same. Therefore -

$$[ES] = \frac{[E][S]}{(k_2 + k_3)/k_1} \qquad 3$$

Equation 3 can be simplified by defining a new constant, K_M, termed the Michaelis constant;

$$K_M = \frac{k_2 + k_3}{k_1} \qquad 4$$

This simplifies 3 to -

$$[ES] = \frac{[E][S]}{K_M} \qquad 5$$

The concentration of uncombined substrate is equal to the total substrate if the enzyme concentration is much lower. The concentration of enzyme is that of the uncombined , E_T, minus the [ES];

$$[E] = [E_T] - [ES] \qquad 6$$

substituting equation 6 into 5 and solve for [ES] gives -

$$[ES] = [E_T] \frac{[S]}{[S] + K_M} \qquad 7$$

Substituting 7 into equation 2 gives -

$$V = k_3 [E_T] \frac{[S]}{[S] + K_M} \qquad 8$$

V_{max} is obtained when all the enzyme sites are saturated with substrate ie, when [S] is much greater than K_M, so that $[S]/([S]+K_M)$ approaches 1 thus;

$$V_{max} = k_3[E_T] \qquad 9$$

Combining equations 8 and 9 gives the Michaelis-Menten equation :

$$V = V_{max} \frac{[S]}{[S] + K_M} \qquad 10$$

This equation accounts for the kinetic data in Graph 1. When $[S] << K_M$ then $V = [S]V_{max}/K_M$. Consequently the rate is proportional to the substrate concentration. When $[S] >> K_M$

then $V = V_{max}$. ie the rate is maximal.
It is convenient to express the Michaelis-Menten equation in a form that gives a straight line plot. This can be done by taking the reciprocal of both sides giving :

$$\frac{1}{V} = \frac{1}{V_{max}} + \frac{K_M}{V_{max}} \cdot \frac{1}{[S]} \qquad 11$$

The value of K_M for a particular enzyme depends on substrate and reaction conditions and is related to the binding strength of the enzyme substrate complex.

eg :

Enzyme	Substrate	K_M
Chymotrypsin	Ac-L-tryptophanamide	5×10^{-3}M
Lysozyme	Hexa-N-Acglucosamine	6×10^{-6}M
Pyruvate carboxylase	Pyruvate	4×10^{-4}M
"	HCO_3^-	1×10^{-3}M
"	ATP	6×10^{-5}M

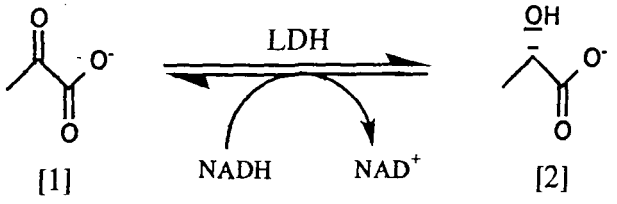
Clearly a smaller value of K_M suggests efficient binding. For a measure of how good a particular substrate is K_m/V_{max} is usually used, as this is a more accurate expression of turnover, rather than binding.

1.3 Enzyme Classification.

In 1979 the International Union of Biochemistry set out a six group classification system to categorise all enzymes[5]. Each enzyme was given an EC number consisting of 4 digits. The first digit is the enzyme group; EC 1 the oxidoreductases, EC 2 the transferases, EC 3 the hydrolases, EC 4 the lyases, EC 5 the isomerases and EC 6 the ligases. Examples of each group are given,

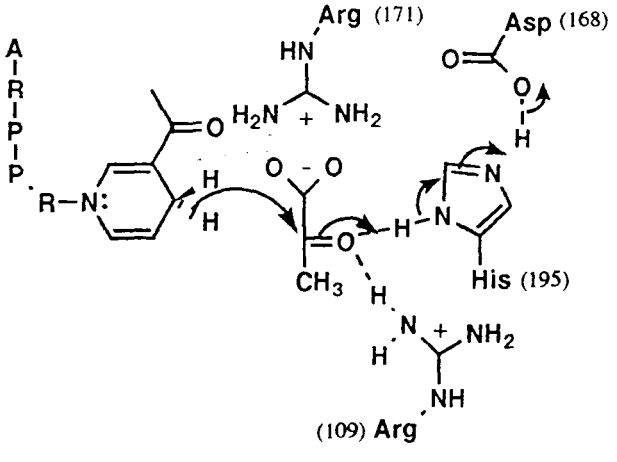
EC1.

The oxidoreductases catalyse reactions involving oxygenation, such as C-H to C-OH. Cytochrome P_{450} enzymes mediate such transformations also the overall removal or addition of hydrogen atom equivalents eg. CH(OH) reversibly to C=O (alcohol dehydrogenase) and CH-CH reversibly to C=C (dehydratase). These reactions require a co-factor as the hydride donor or acceptor. An example of this group of enzymes is lactate dehydrogenase (LDH) (EC1.1.1.27) which catalyses the interconversion of L-lactate [1] and pyruvate [2].



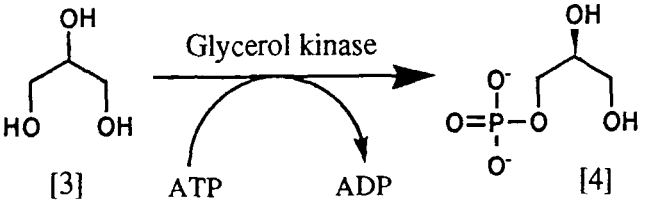
The co-factor in this reaction is NADH (nicotinamide adenine dinucleotide).

The quarternary structure of the dehydrogenase allows the lactate to bind in such a way to allow hydrogen bonding interactions of the carbonyl with arginine-171 and histidine-195. The 2 carbonyl carbon of pyruvate is optimally positioned for reduction with respect to the C-4 of the nicotinamide ring[6], as shown below



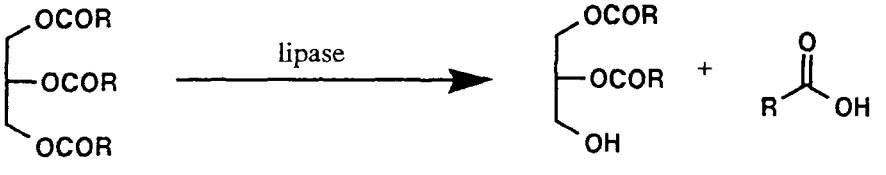
EC2.

Group 2 are the transferases which catalyse the transfer of acyl, sugar, phosphoryl, aldehyde or ketone moieties from one molecule to another. An example of this class is glycerol kinase (EC2.7.1.30) which catalyses the transfer of the γ-phosphoryl group of a nucleoside triphosphate to glycerol [3]. This activates the glycerol for subsequent transformations in the glycolytic pathway. Glycerol-3-phosphate [4] is also important in the regeneration of NAD^+ in the mitochondria.



EC3.

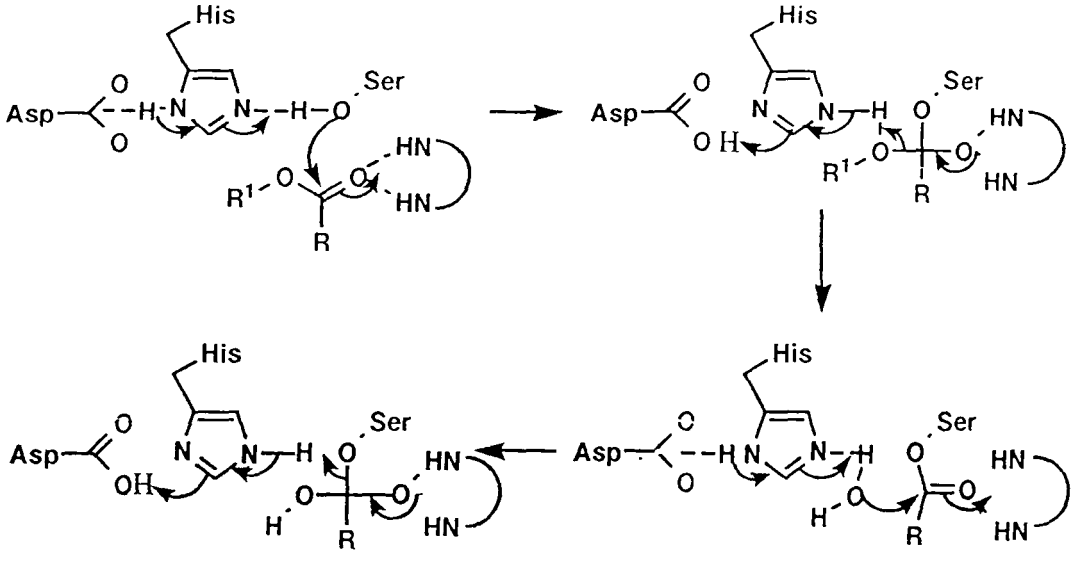
Group 3 are the hydrolases. These enzymes catalyse the formation and cleavage of ester, amide, glycoside and peptide bonds. An important group within the EC3 group are the lipases which catalyses the hydrolysis of triacyl glycerols [5] eg;



[4]

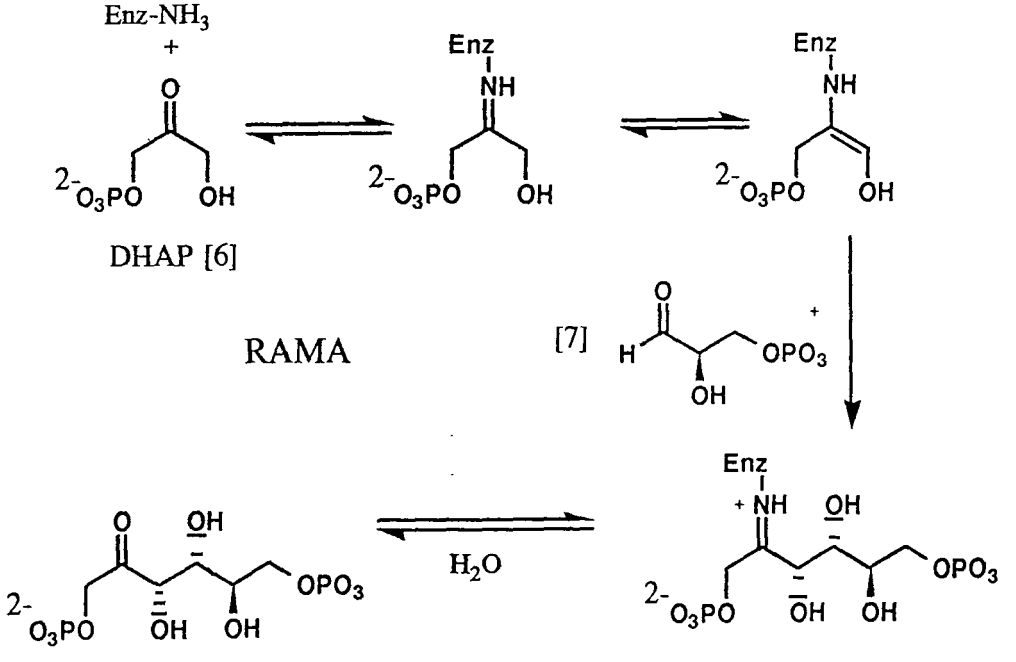
In general these enzymes have catalytically functional glutamate, histidine and serine residues. In the case of the lipase from *Candida cylindracea,* the quarternary folding of the enzyme brings these three residues into close proximity. This, "catalytic triad", is conserved over a wide range of serine proteases eg porcine pancreatic lipase, α-chymotrypsin (the glutamate is replaced by an aspartate) and

trypsin. The mechanism can be considered as follows[6];



EC4.

The lyases provide the fourth group in the classification system. These enzymes mediate the addition of HX to C=C, C=N and C=O groups. A classical example is rabbit muscle aldolase (RAMA). This enzyme catalyses the addition of dihydroxyacetone phosphate (DHAP)[6] to glyceraldehyde-3-phosphate [7]. A free lysine amino group is catalytically functional in this transformation as shown below.

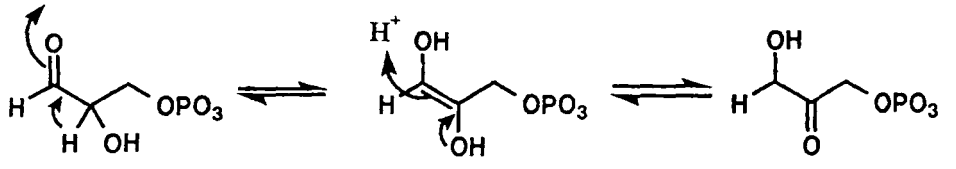


This reaction proceeds *via* an enzyme-imine intermediate. DHAP forms an imine with the lysine amine residue at the active site. The imine enolises and then a condensation occurs to form the new C-C bond. The iminium species then undergoes hydrolysis to release the product, fructose-1,6-diphosphate [8] and the free enzyme.

EC5.

The fifth group are the isomerases which catalyse cis/trans interconversions, C=C migrations and racemisations.

An important enzyme in the glycolysis pathway is triosephosphate isomerase which interconverts dihydroxyacetone phosphate (DHAP)[6] and glyceraldehyde-3-phosphate (G-3-P)[7] *via* an enediol, eg;



[7] [6]

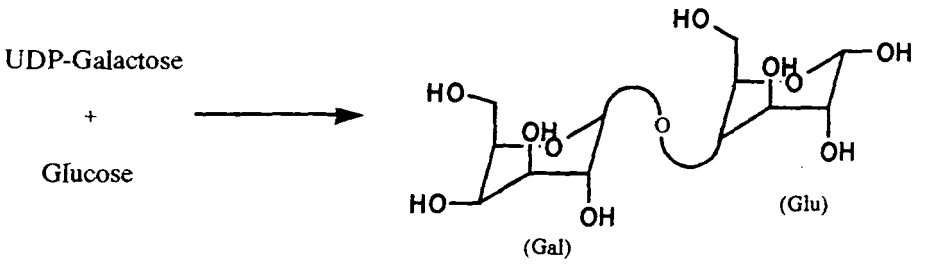
One molecule each of G-3-P and DHAP are produced from the breakdown of F-6-P on the glycolytic pathway. DHAP is then converted readily to G-3-P by this isomerase. At equilibrium 96% of the triose exists as DHAP.

EC6.

The last group of enzymes are the ligases, often termed the synthetases. These enzymes catalyse the formation of C-C, C-S, C-N and C-O bonds.

An important energy store in plants and animals are polysaccharides. An early step is the synthesis of disaccharides employs a specific synthetase, lactose synthetase for the synthesis of lactose [9] from galactose

and glucose subunits, eg;



α-Lactose [9]

Lactose synthetase consists of two subunits. The catalytic subunit, galatosyl transferase, normally transfers galactose from UDP-galactose to N-acetylglucosamine to form N-acetyllactosamine. The second subunit is a modifier, α-lactalbumin, which changes the specificity of the enzyme from N-acetylglucosamine to glucose, thus forming lactose.

1.4 Enzymes in Organic Synthesis.

Until recently biotransformations has been largely ignored by the chemical community, however there are a number of obvious advantages to using enzymatic systems over the more usual chemical methods eg -

a) The reactions occur under mild conditions eg 20-30°C and neutral pH.

b) Enzymes are generally regio- and stereo-selective

c) They are catalytic.

d) Due to their use in the pharmaceutical, food industries and in soap powders there are a range of enzymes that are commercially available in bulk quantities eg. porcine pancreatic lipase[7] (EC number 3.1.1.3) cost - 1 US dollar for 5 x 10^7units. In a recent publication there are listed 350 commercially available enzymes[8]. Of these 125 were hydrolases and a further 90 were oxidoreductases (plus 90 transferases, 35 lyases, 6 isomerases and 5 ligases). The quality, quantity and cost will continue to become more favourable due to improving methods of microbiology and molecular biology.

Clearly for the chemist, the most useful enzymes are those with a broad substrate specificity, but that retain good stereospecificity. These requirements seem antithetical but it is possible to find enzymes that satisfy both. It has been stated[7] that enzymes from mammalian sources in general satisfy this condition best, unfortunately, however, they are generally more expensive than the wider range from microbial systems.

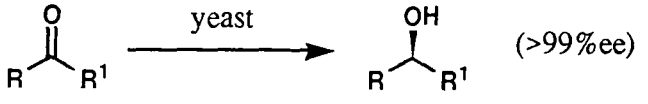
Part 1. Biotransformations in Aqueous Media.

1.5. Whole Cell Systems.

1.5.1. Reductions.

The earliest reports of biocatalysts was their use by the Greeks and Romans to brew wine and to make natural dyes over 2000 years ago. With the fragmentation of modern science living organisms and enzymes have been largely the domain of biologists. Reports of bakers' yeast reductions by organic chemists emerged in the literature in the 1950's and bakers' yeast has occupied a central role in biocatalysis ever since.

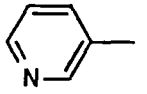
The technology of yeast reactions is very simple eg- sucrose in tap water with a ketone is incubated with bakers' yeast and after several days the product can be isolated by solvent extraction[10]. For large scale production it is possible to use actively fermenting bakers yeast as the reagent[11]. The use of microbial reduction of ketones stereospecifically is of great use in generating precursors in natural product synthesis. A wide range of structures can be accessed in this way[12-14], eg;



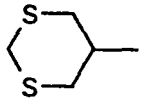
[10] [11]

a) R = $CH_2COOC_2H_5$, $R^1 = CH_3$

b) R = $(CH_2)_2CH{=}CH_2$, $R^1 = CH_2COOH$

c) R = 

$R^1 = CH_3$

d) R = 

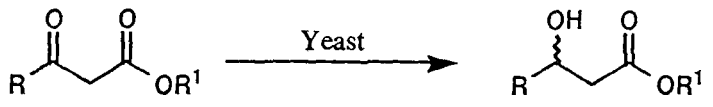
$R^1 = (CH_2)_3COOCH_3$

e) R = Aryl, $R^1 = CF_3$

For example the reduction of [10a], [10b] and [10c] has been carried out using bakers' yeast. The reduced products, [11a] and [11b], have been employed in the total synthesis

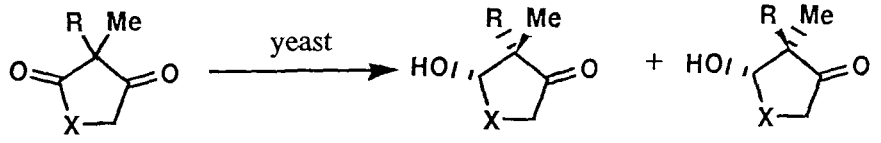
of various natural products. Using the yeast *Sporotrichum exile* compound [11c] has been prepared and used as a precursor in the synthesis of the pharmacologically significant alloheteroyohimbine alkaloids[15] and [11d] generated after *Kloeckera corticis* reduction of the ketone [10d] has been employed in the synthesis of arachidonic acid metabolites[16]. The aromatic trifluoromethyl alcohols [11e] are generated by reduction of the ketone [10e] using yeast and are useful as chiral solvents[17] and for inducing asymmetric reactions[18, 19].

A wide variety of β-ketoesters[20], β-ketoacids (potassium salt)[20a, 21] and prochiral cyclic ketones[22] are accepted as substrates in these biotransformations. eg. See Tables 1.1 and 1.2;



R^1	R	Configuration	%ee	%Yield
Me	Me	S	87	23
Me	$PhCH_2OCO(CH_2)_2$	S	2	24
C_8H_7	CH_2N_3	R	100	80
K	CH_3	S	>96	34

Table 1.1 : Enantioselectivity of the reduction of several β-keto esters using Bakers' yeast.

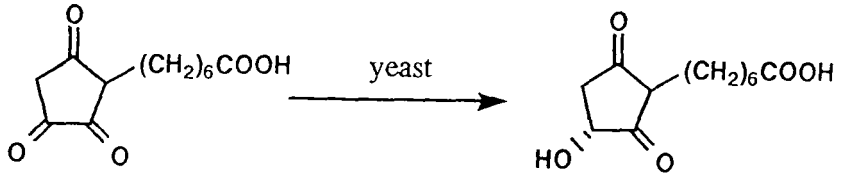


X	R	(2S,3S):(2R,3S)	(2S,3S) %ee	%yield
CH_2	$(CH_3)_2=CH_2$	100:0	98	69
CH_2	$HCCCH_2$	100:0	>90	65
$(CH_2)_4$	$H_2C=CHCH_2$	82:18	>98	5

Table 1.2 : Enantioselectivity of the reduction of a range of cyclic diesters with Bakers' yeast.

1.5.1.a. Regioselectivity.

In cases where there is more than one reducible functionality in the substrate, biocatalysis can be employed to achieve regiospecific reduction as well as prochiral selectivity[23,24], eg;

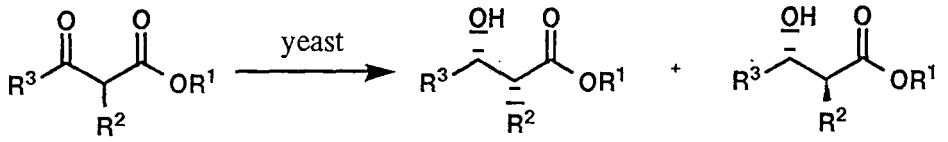


[12]

For example the trioxo-cyclopentane derivative [12] can be reduced selectively (98%ee) with bakers' yeast. It can also be reduced using a soluble rhodium catalyst with the asymmetry being induced using chiral phosphine ligands[24]. The selectivity of this method generates a product with only 68% ee. Both chemical and microbial based routes have been used in the total synthesis of prostaglandin E_1[24]

1.5.1.b. Resolution.

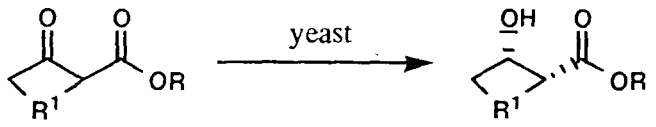
In the above examples reduction is of a prochiral ketone. An alternative strategy involves a racemic substrate such as 2-substituted β-ketoesters [13][25] and cyclic β-keto esters [14][26]. Reduction is stereospecific but both enantiomers are accepted, generating diastereomers. See tables 1.2 and 1.3



[13] Syn Anti

			Syn		Anti			
R^1	R^2	R^3	Config	%ee	Config	%ee	Ratio	Yield
Et	Me	Me	(2R,3S)	100	(2S,3S)	100	80:20	75%
nOct	Me	Me	(2R,3S)	100	(2S,3S)	High	95:5	82%
Me	Me	CH_2OCH_2Ph	(2R,3R)	68	(2S,3R)	Low	10:90	50%
Me	SMe	Me	(2R,3S)	>96	(2S,3S)	>96	72:28	72%

Table 1.3 : Resolution of β-keto esters with B. Yeast.

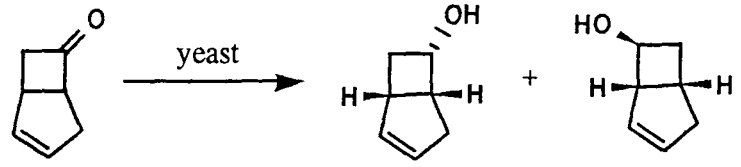


[14]

		(2R,3S)	
R^1	R	%ee	Yield
$(CH_2)_2$	Et	99	80%
SCH_2	Me	85	low
CH_2SCH_2	Me	70	71%
$N(Et)CH_2$	Et	73	65

Table 1.4 : Resolution of cyclic β-keto esters with Baker's yeast.

Similarly the stereospecific reduction of both enantiomers of bicyclo[3.2.0]heptanone [15] afforded [16a] in (90%ee, 56% yield)[27]. Again both of the diastereomers have proved useful for the preparation of precursors to prostaglandins and related compounds.

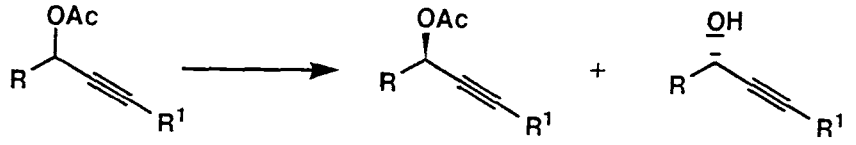


[15] [16a] [16b]

1.5.2. Hydrolyses.

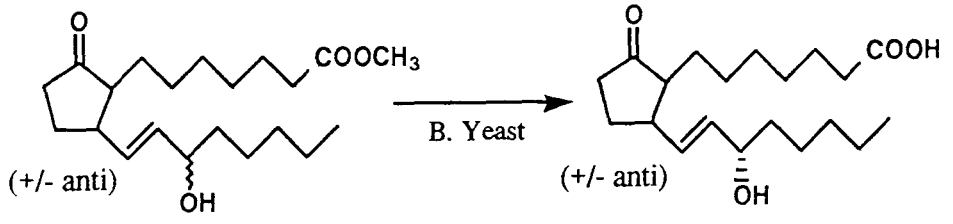
Classically chiral alcohols have been resolved into their component enantiomers by a method that entails preparing the half phthalate carboxylic acid and then preparing salts with brucine, dehydoabiethylamine or α-methylbenzylamine[28a]. These systems are, however, not effective for alkyl and alkynyl carbinols [17]. Methods involving actively growing micro-organisms are attractive here due to the availability of micro-organisms and the mildness of the reaction conditions, eg - 2g of the acetates of racemic alkyl and alkynyl

carbinols have been hydrolysed in 3 days at 30°C with *Bacillus substilis* grown on 2% nutrient broth, to provide the corresponding alcohol [18] in >98%ee[28].



[17] [18]

The hydrolytic resolution of the methyl esters of prostaglandin intermediates have also been achieved using yeasts[28] eg;



4 diastereomers 2 diastereomers

1.6. Isolated Enzyme Systems.

More recently isolated enzyme preparations have become available and are becoming increasingly attractive to organic chemists as catalytic systems. Their availability is due to their use as catalysts is the food and pharmacological industries as well as the developments in molecular biology that have lead to the over production of many enzymes in microbial culture. Cell free preparations are attractive to the chemist for a number of reasons. The most important is that the chosen enzyme will catalyse a single reaction and unwanted side reactions are rarely observed. In addition the temperature and pH can be varied for optimal product yield. Other manipulations become possible such as immobilisation and the addition of organic co-solvents. The work up of enzyme reactions is rarely a problem and is generally much easier than for whole cell biotransformations.

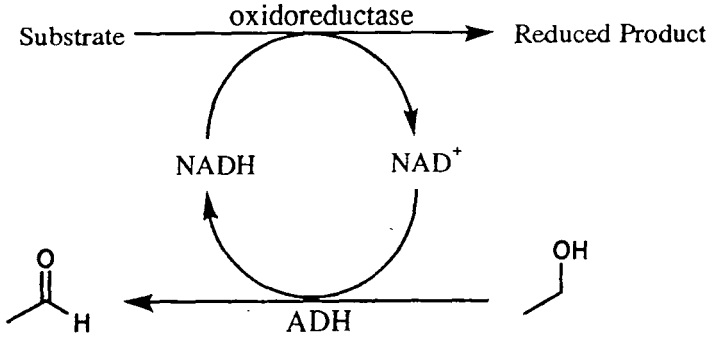
1.6.a. Co-factor Regeneration.

In whole cell systems (eg bakers' yeast) the problem of co-factor regeneration does not arise as the cells naturally recycle the co-factor using linked enzyme systems.

However, when the chemist uses an oxidoreductase enzyme the co-factor needs to be supplied. To supply such an expensive reagent in stoichiometric amounts is prohibitive and efficient methods of recycling are required. Several attractive methods for regenerating NAD(P)H from NAD(P) *in situ* have been developed.

eg - For small scale reactions the addition of ethanol with

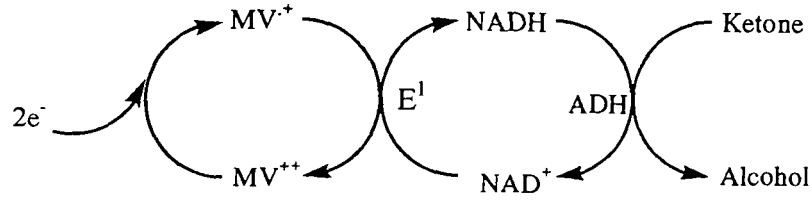
an alcohol dehydrogenase can efficiently recycle the NAD(P)H by oxidation of the ethanol to acetaldehyde[29];



An alternative method employs dithionite. This has been used to regenerate NAD(P)H in small scale reactions and its consumption can be monitored spectrophotometrically[30].

In larger scale processes the most popular method employs a formate dehydrogenase coupled system. Formate is oxidized to carbon dioxide and water with concomitant reduction of the co-factor. The clear advantage here is the lack of contaminating by-products making workup easier

A third method has been developed which involves electrochemical reduction of the co-factor *via* an intermediate methyl viologen carrier. The enoate reductase, E_1, can then be used directly to reduce the double bond of α,β-unsaturated carboxylic acids[31], eg;

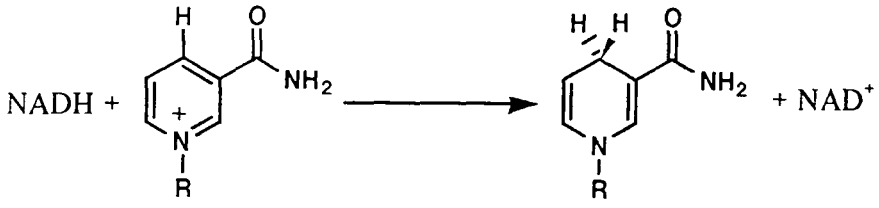


MV^{++} =1,1-dimethyl-4,4'-bipyridinum dication

This carrier system will also accept electrons directly from hydrogen using a hydrogenase enzyme. In some micro-organisms gaseous hydrogen can be used as the electron donor instead of sugar (in resting cells). The advantage here is again the lack of side reactions, as hydrogen donates electrons directly to the $NAD(P)^+$ without the need for the

intervening glycolysis reactions that conspire to slow the sugar based reactions.

When oxido-reductases are used as oxidising agents there is a need to regenerate NAD(P)$^+$ from NAD(P)H. The addition of pyridinium salts or flavin-mononucleotide (FMN) to the reaction has been used to achieve this[32], eg;



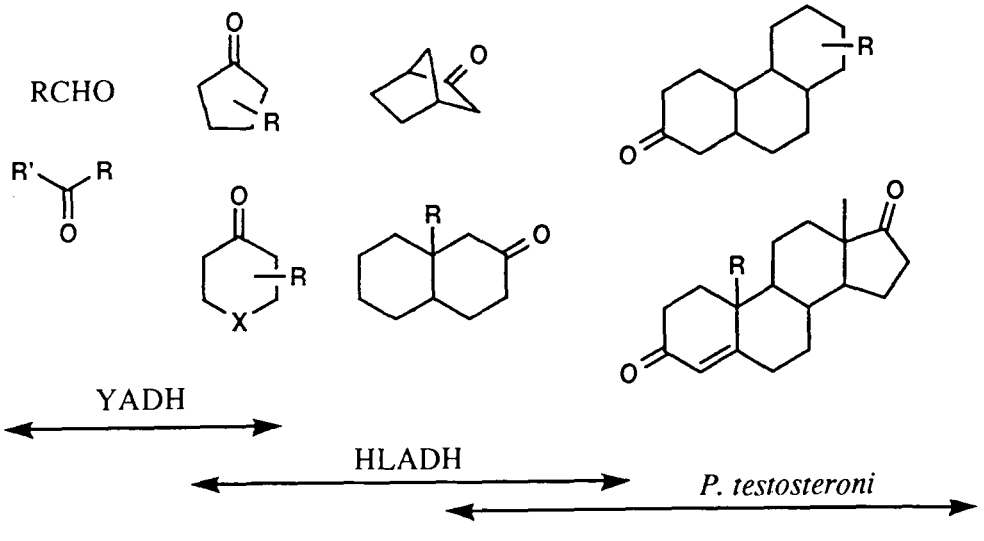
R = β-1(2',3',4',6'-tetra-o-acetyl-β-D-glucopyranosidyl)

X = Ac

FMN is relatively cheap, commercially availible and so it can be used in stoichiometric amounts in small preparative scale reactions.

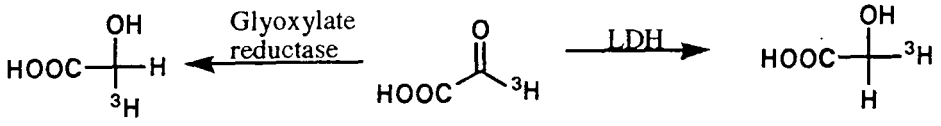
1.6.1. Reductions.

With the problem of co-factor regeneration effectively solved for dehydrogenases, the use of isolated enzymes for biotransformations becomes practical. By judicial choice of enzyme source a wide range of homochiral compounds can be accessed[9], eg;



1.6.1.a. Prochiral Selectivity.

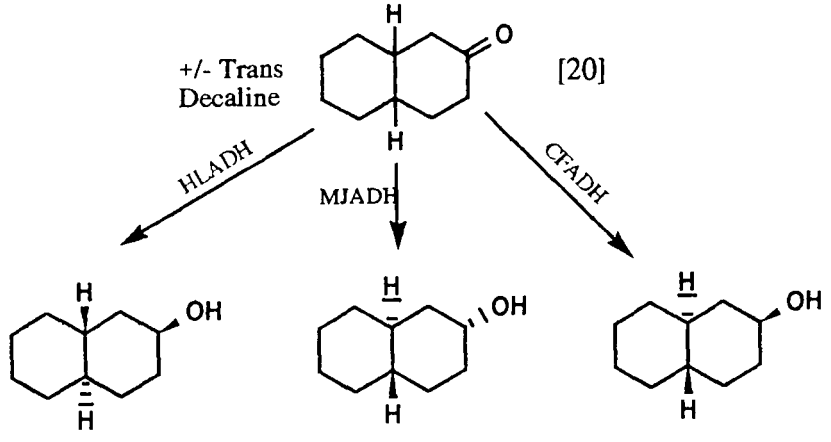
Kinetic resolution can at best only provide 50% of each enantiomer. In general the chemist is only interested in one of these, however with a prochiral substrate, face selective modification will yield up to 100%. Judicial choice of enzymes allows access to both enantiomeric series[33], eg;



[19]

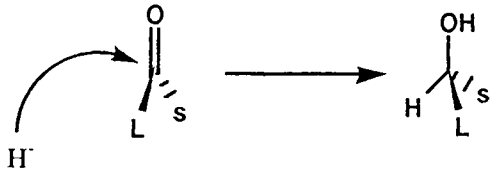
For example in case of glyoxylate [19], both enantiomeric alcohols have proved useful in biosynthetic studies of terpenes and steroids and in the synthesis of isotopically labelled mevalonic acids.

With the reduction of +/- trans decalin, [20], the required stereoisomer can be achieved using the appropriate choice of enzyme source[34]. The enantiomeric specificities of horse liver, *Mucor javanius* and *Curvularia falcata* alcohol dehydrogenases are well documented[34]. Other decalins and heterocycles are also substrates for HLADH[35-38].



1.6.1.b. Prelog Rule.

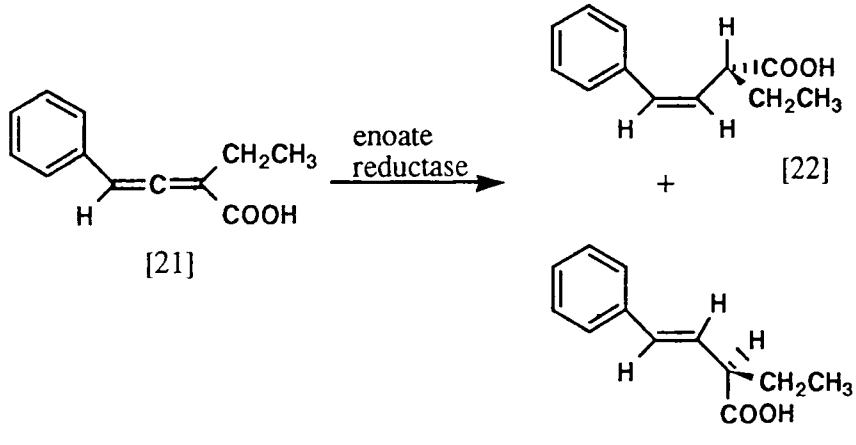
After an assessment of a range of prochiral ketones Prelog[39] developed an empirical model which has been used to predict the stereospecificity of HLADH reductions. When groups R and R' differ sterically then the hydride is generally delivered to the *Re*-face of the carbonyl.



This model, formulated twenty years ago is still useful for predicting the stereochemistry of cyclohexane and decalin substrates, but for many other systems the predictions are not reliable. The analysis was refined by Jones and Jakovac[40] who developed a cubic space model. This model is powerful and has been able to rationalise the stereochemical outcome of all the successful substrates presented to the dehydrogenase (HLADH) so far.

1.6.1.c. Hydrogenation of Double Bonds.

An area that has received considerable attention from the chemist is the reduction of double bonds. The organic chemist is much more likely to use a platinum or palladium catalyst, with a chiral auxiliary to induce asymmetry, than an enzyme or micro-organism, but the enzymatic routes provide a useful addition to the armoury of synthetic methods[41-42], eg;



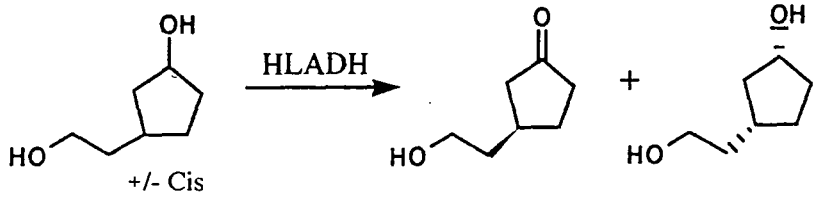
[21] [22]

The reduction of [21] was carried out on 13Kg of substrate using an enoate reductase from *Chlostridium kluyvery*. [22] Is an intermediate in the synthesis of tocopherol (vitamin E)[43].

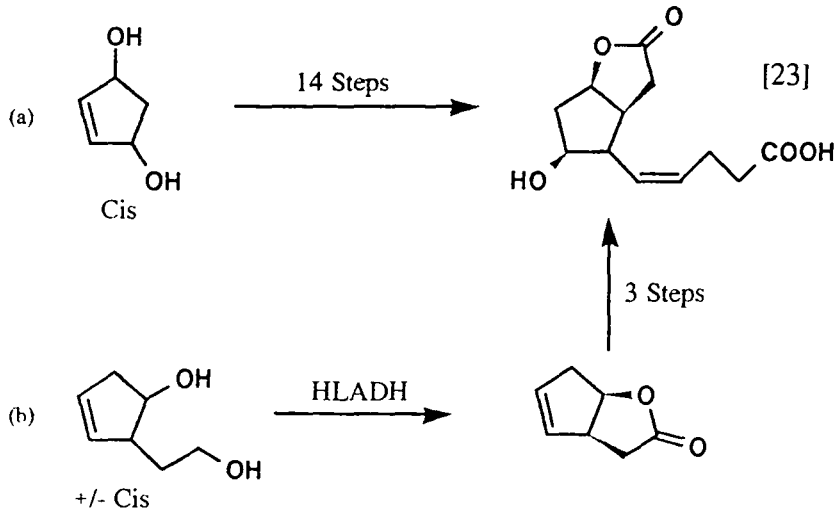
1.6.2. Oxidations.

1.6.2.a. Alcohols

Horse liver alcohol dehydrogenase (HLADH) is also capable of discriminating primary from secondary alcohols with concomitant kinetic resolution of enantiomers. This is difficult to achieve in a single step using chemical methods[40, 44], eg;

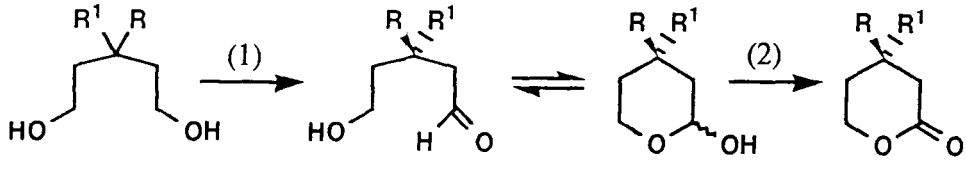


Comparison of the classical chemical synthesis of the optically pure prostaglandin precursor [23], and its synthesis using chemoenzymatic means, demonstrates the advantage of introducing the chirality by biotransformation methods[45-47].



1.6.2.b. Prochiral Selectivity.

HLADH is able to distinguish between the faces of a prochiral diol and oxidize one hydroxy group generating a homochiral product[48-49]. Since the substrate is a meso compound, potentially, it all can be converted into a single optically pure product, eg;



(1) = (2) = HLADH

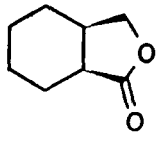
a) R = H
R^1 = Me

b) R = Me
R^1 = OH

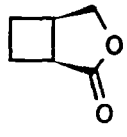
c) R = H
R^1 = OH

NAD^+ in this case was recycled using FMN and all of the compounds were oxidised in 1.5-2g amounts.

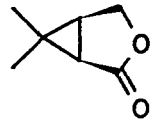
HLADH can be used to oxidize glycerol to D-glyceraldehyde[50]. Prochiral cyclic syn-diols can also be converted to their corresponding lactones with a high degree of stereoselectivity[51-53], eg;



[24]



[25]

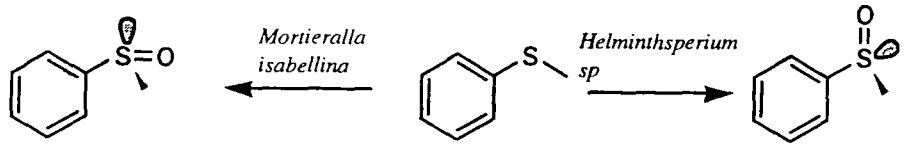


[26]

Compounds [24] and [25] are useful in the synthesis of verrucarins, a class of natural products that have attracted interest due to their antibacterial, antifungal and cytostatic activity. Compound [26] has been used in the synthesis of insecticidal pyrethroids.

1.6.2.c. Other Compounds.

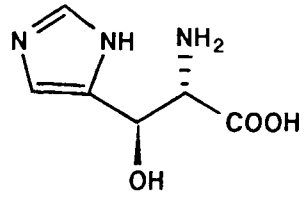
It is also possible to oxidize organic sulphides stereospecifically to chiral sulphoxides and with appropriate choice of organism both enantiomers are accessible[54], eg;



1.6.3. Other Enzymes of The EC1 Group.

1.6.3.a. Oxidations and Reductions

D-amino acid oxidase (EC 1.4.3.3) is a member of the oxidoreductase group. The amino oxidases catalyse the conversion of α-C-NH_2 to C=O to generate α-keto acids. Catalase (EC 1.11.1.6) can be used to mop up the H_2O_2 produced in this reaction to prevent further oxidation. This is an example of a linked enzyme system being used to generate the required product and prevent side reactions. Such a strategy was the basis of early laboratory preparations of enantiomerically pure amino acids. By selective destruction of one enantiomer amino acid oxidase have been used in the preparation of a range of natural product precursors eg the bleomycin precursor[61] [29];



[29]

Monoamine oxidase (EC 1.4.3.4) and diamine oxidase (EC 1.4.3.6) have broad substrate specificities eg 1,4-diaminobutane, 2-phenylethylamine, propylamine and spermidine are all substrates[62-63]. These enzymes have not been studied in detail or put to extensive synthetic use yet but their broad substrate specificities make them attractive targets for future study.

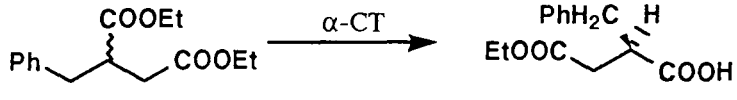
1.6.4. Hydrolases.

The use of partially purified enzymes in hydrolysis reactions is much more common than the use of whole cell systems. In addition the absence of a co-factor is attractive. There are a number of purified enzymes which have been studied in detail, eg pig liver esterase (PLE), porcine pancreatic lipase (PPL), α-chymotrypsin (α-CT), *Pseudomonas Sp* lipase and *Candida cylindracea* lipase (CCL). Most of the current literature on enzymatic resolutions use one of these enzymes although the number of available hydrolase enzymes is increasing.

1.6.4.a. Resolution of Chiral Esters.

Enzymatic hydrolysis of an ester with concomitant resolution of the acid or alcohol functionality is possibly the most straightforward method for obtaining homochiral molecules.

Early examples include the hydrolysis of chiral diesters by kinetic resolution of the enantiomers, eg [33] was hydrolysed using α-chymotrypsin in buffer to yield the (S) alcohol [34] in 45% yield and >98%ee[71].



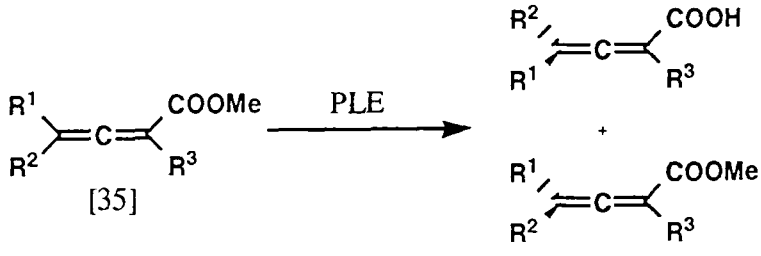
[33] [34]

Candida cylindracea lipase has been used to resolve 2-chloropropionic acid by the hydrolysis of its octyl ester. The reaction gave the ester in 46% yield and 96%ee. The same reaction carried out on the methyl ester gave no resolution of the acid[72]. The lipase is a lipid membrane active protein which works most effectively at a water/lipid interface and is optimised to bind lipophilic substrates. The octyl ester forms an emulsion in the buffer medium thus generating the lipid water interface, whereas the methyl ester is

completely soluble in the buffer medium.

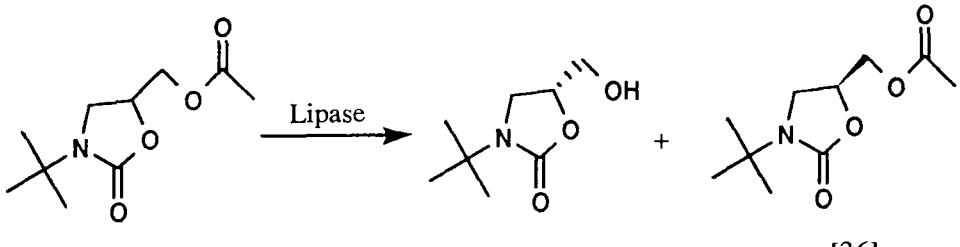
The resolution of a range of 3-hydroxy esters with PLE shows a marked dependence on structural variations. When R is ethyl, allyl or dimethoxyethyl the %ee of the alcohol is >94%, but for n-hexyl, the %ee is only 24%. R^1 can be either ethyl or methyl with no effect on the enantioselectivity[73].

It has been noted for the PLE hydrolysis of racemic allenic esters [35] that the selectivity depends on the substituents[74]. The (S) ester is hydrolysed consistently when the C4 substituents are small or acyclic. When the substituents are large or cyclic the selectivity reverses. The %ee is greatest when C2 and C4 substituents are bulky;



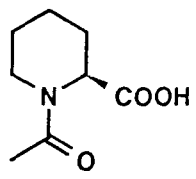
[35]

A range of racemic heterocyclic esters have also been resolved by PLE hydrolysis with good enantioselectivity[75,76]. The residual ester, [36], from the enzymatic hydrolysis of the racemate has been used as a precursor in the synthesis of β-andregenic blocking agents a useful class of cardiac drugs;

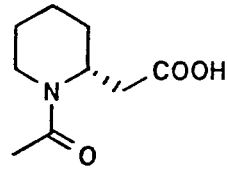


[36]

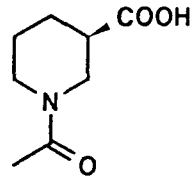
Compounds [37] to [39] were all synthesised form their methyl esters and the range of %ee's shows the effect of structure on selectivity[76]. There appears to be no predictable pattern to this variability and more data is needed before reliable predictions can be made;



[37]

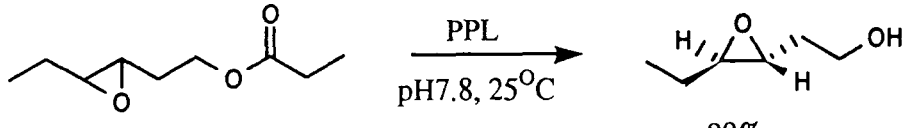


[38]



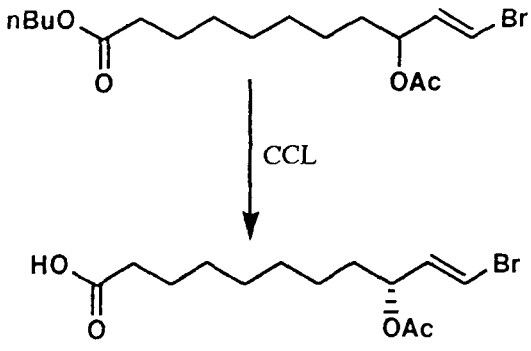
[39]

Another class of synthetically useful hetreocycles are the epoxides. The synthesis of homochiral epoxides from allylic alcohols using Sharpless methodology[77] is well known. On the other hand synthesis of homochiral epoxides from olefins more generally is limited. Synthesis of the racemate and subsequent resolution by enzymatic means affords an alternative route to these synthetically useful compounds[78], eg;



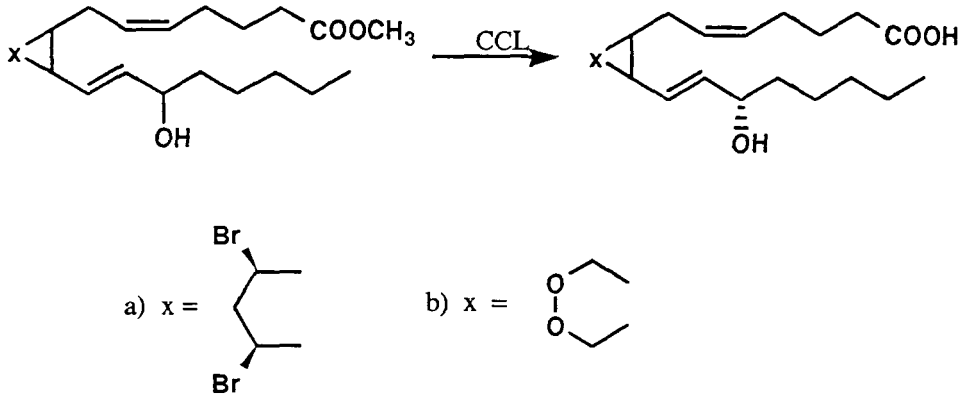
80%ee

It is possible to achieve hydrolysis of the methyl ester with resolution of a functional group several carbons along the chain. A good example of this is illustrated with compound [40][79]. CC lipase was used to hydrolyse the n-butyl ester of long chain acids. Various chain lengths were presented to the enzyme and the optimal %ee was found for n=8. This is a remarkable observation as it suggests there is a site at a significant distance from the catalytic serine residue that is able to discriminate enantiomers.



[40]

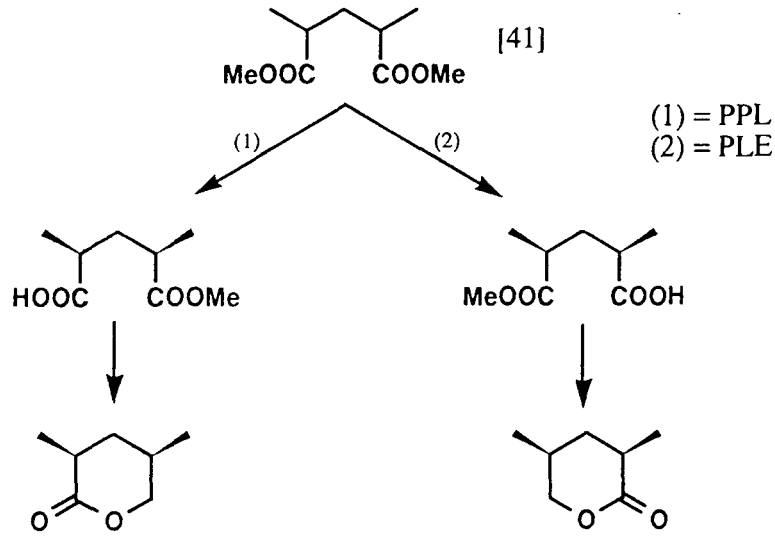
In addition, using the same lipase, a number of prostaglandin precursors can be resolved in this manner[80];



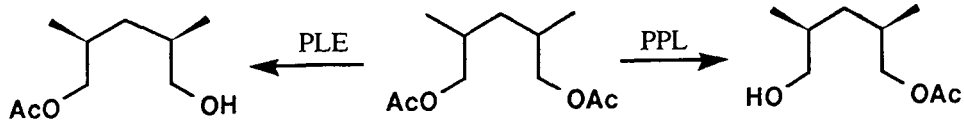
1.6.4.b. Prochiral Selectivity.

Prochiral selectivity is the most important of the two methods for overcoming the limitations of the kinetic resolution of enantiomers, ie limiting the yield to a theoretical maximum of 50%. The other method is the use of racemisation to recycle the unused enantiomer. This second method will be discussed later, in section 1.7e (page 37). Starting with the prochiral diester [41] it is possible to get selective hydrolysis of one ester to yield a chiral product. With careful choice of enzyme it is possible to access both enantiomeric series[81]. Both the lactone products

have been used in the synthesis of macrolide and polyether antibiotics.

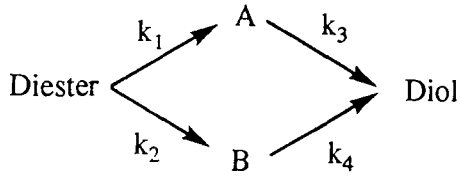


A similar reaction leading to the alcohol analogue [42] is also possible and again with judicial choice of enzyme both enantiomers are accessible[82];



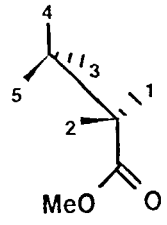
[42]

The optical purity of the lactones derived from the diol are potentially much higher. This arises from the fact that the diester hydrolysis stops at the mono ester stage. The enzyme will continue to hydrolyse the second ester but at a different rate, eg;

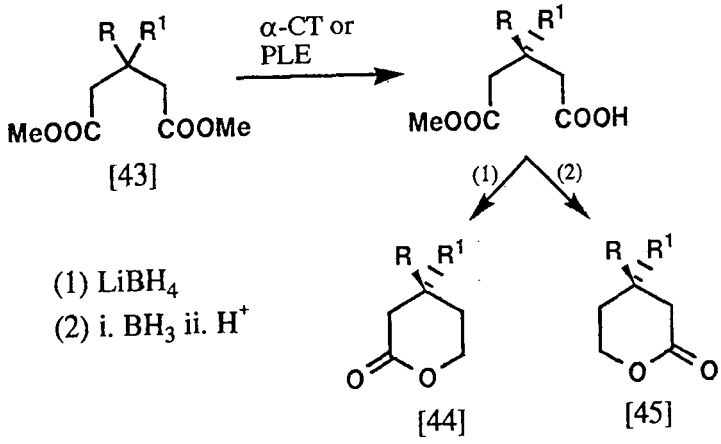


If $k^1 > k^2$ and $k^4 > k^3$ then the unwanted monoester enantiomer is hydrolysed to the diol faster than it is formed thus increasing the %ee at the expense of the maximum potential yield. If the reaction proceeds until all the diester is consumed (but with some diol) then the optical purity can be >98%ee[82].

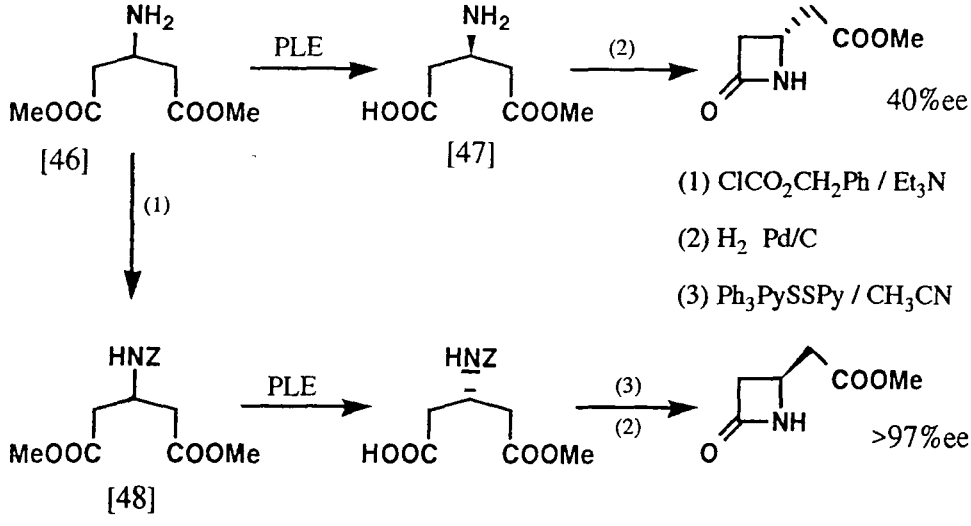
Mohr et al[83] have studied a wide range of dimethyl esters and have come up with a simple model of the stereochemical constraints of the active site of PLE[83], eg;



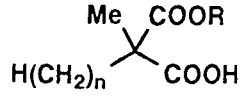
The selective hydrolysis of prochiral diesters, eg [43], gives access to a wide range of enantiomerically enriched lactones [44] and [45][84,85]. These lactones can be prepared using oxido-reductases from the corresponding prochiral diol. The oxidoreductases have the disadvantage, however, over the use of the hydrolysis methods in that they require a co-factor thus making the technology more complicated. The uses of compounds [44a] to [44c] have already been discussed (section 1.6.2b, page 28).



The same enzyme can be used to access both enantiomers of the monoester [47] by expoliting a removable protecting group to change the face selectivity of the hydrolysis[85]. The addition of the benzyloxycarbonyl protecting group (Z) results in hydrolysis to the free amine with the opposite configuration. The low optical yield of the (R) enantiomer arises due to non enzymatic hydrolysis of the diester [46], however there is no such hydrolysis with the Z protected compound [48].



This switch in selectivity is also observed during progression down the homologous series (table 1.5), eg the hydrolysis of dialkylated propanedioic acid diesters [49] reverses enantioselectivity at chain length n=5[86].



[49]

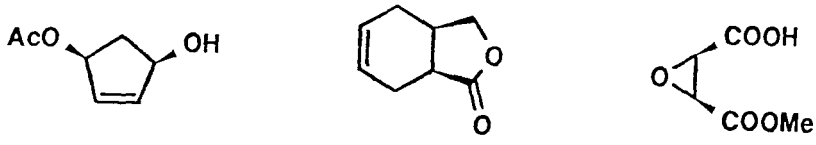
R	n	%ee	Config	R	n	%ee	Config
Me	2	75	S	Et	2	20	S
Me	3	50	S	Et	3	5	S
Me	4	55	S	Et	4	25	S
Me	5	50	R	Et	5	10	R
Me	6	90	R	Et	8	0	-
Me	7	90	R				

Table 1.5 : Effect of chain length on enantioselectivity of PLE hydrolysis

1.6.4.c. Prochiral Cyclic Diesters.

A range of cyclic[82,83,87,90] and heterocyclic[89, 91] diesters have also been assessed as substrates for hydrolase enzymes. These reactions again compare with the reductions of prochiral cyclic[22a,22b] and heterocyclic[22c,22d] ketones by the oxido-reductases.

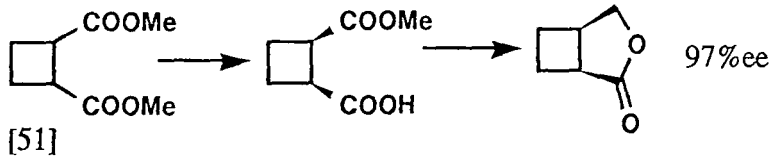
Both enantiomers of the lactone [50] are accessible from the same monoester using either a) 1. BH_3 2. H^+ or b) $LiBH_4$ to close the lactone. These lactones are useful in the synthesis of a range of natural products eg as a precursor to brefeldins and prostaglandins.



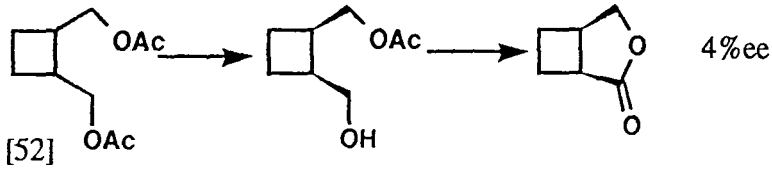
[50]

The stereoselectivity of these hydrolysis reactions depends greatly on the structure of the substrate and only minor changes can greatly effect the selectivity.

The ability of the enzyme to differentiate between prochiral faces of the molecule can depend on the whether it is the alcohol or the acid functionality that is being resolved[89]. For example the diester [51], is resolved more efficiently than the alcohol [52], leading to the same lactone product. This is contrary to the results found for the acyclic system on page 33[82];



[51]

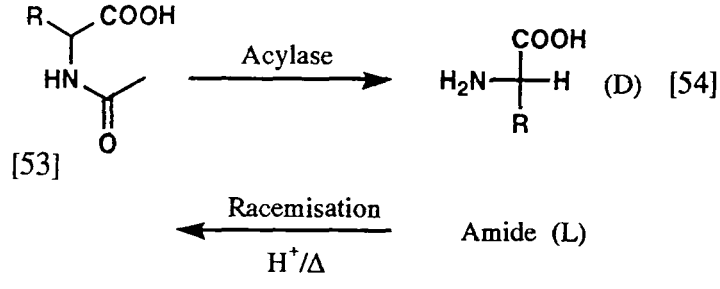


[52]

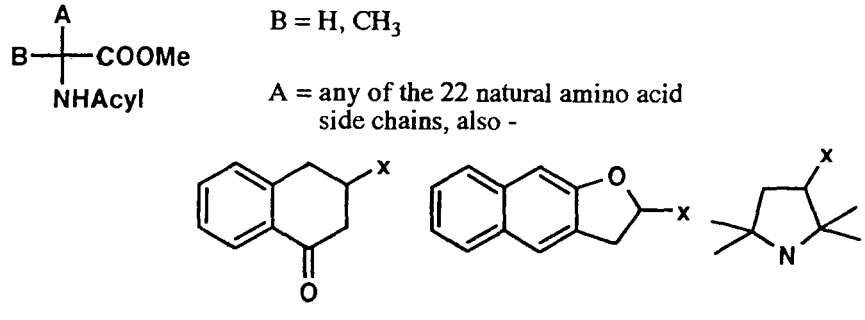
1.6.4.d. Racemisation (as a method of increasing yield)

i. Chemical Racemisation

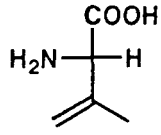
The simplest of the racemisation methods is to mediate the enzyme resolution by standard methods and then purify both the product and the residual starting material, chemically racemise the residual starting material and then re-use it. One of the earliest applications of this method was in the synthesis of optically pure amino acids [54] from corresponding racemic amides [53][92].

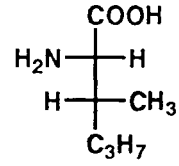


Using α-chymotrypsin a wide range of aromatic and heterocyclic amino acids can be resolved[93] with chemical racemisation of the residual enantiomer thus affording the potential of 100% yield.



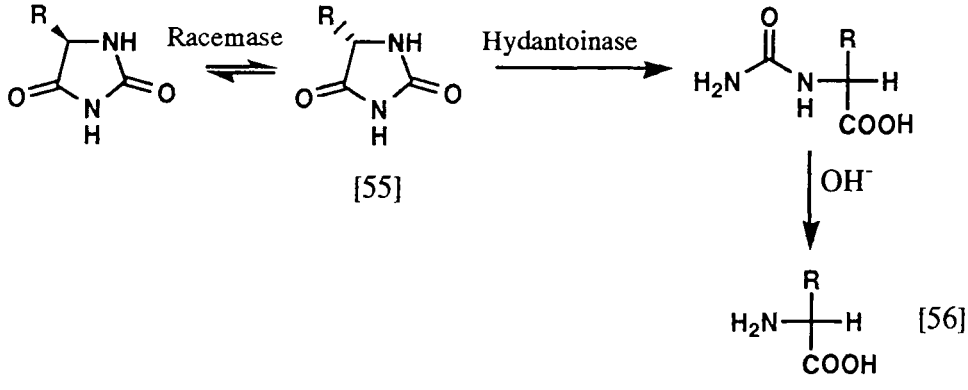
Several useful synthons have been prepared in this way from the racemic N-acyl derivatives using commercially available acylases[94], eg;





ii. Enzymatic Racemisation.

A more elegant method of racemisation is to use a racemase *in situ* to recycle the unused enantiomer. Using hydantionase, compound [55], can be hydrolysed stereospecifically. The unused enantiomer is racemised *in situ* by a racemase[95]. The product is hydrolysed chemically to afford the free, optically pure, amino acid [56].



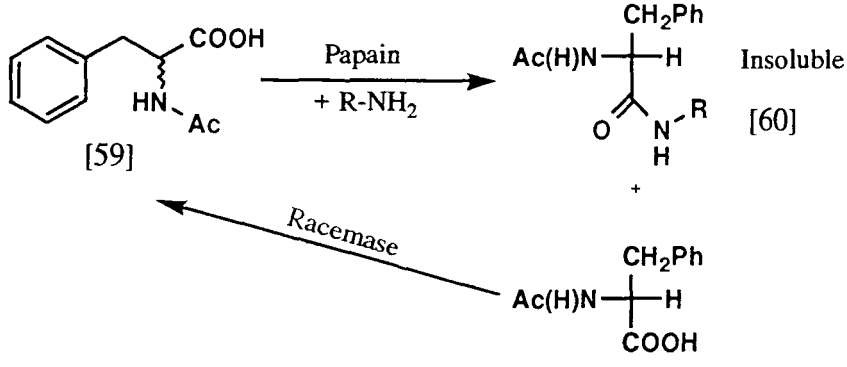
[55]

[56]

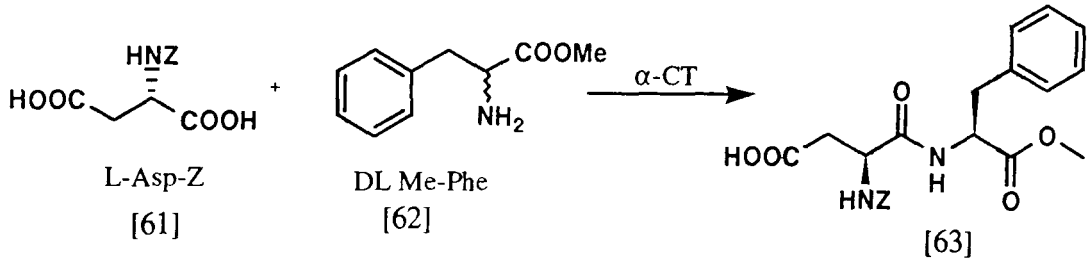
Compound [55] has been synthesised from an aldehyde *via* the Bucherer reaction. Hydantionases of both D and L specificity are available. This reaction can be used in the semi-synthetic synthesis of penicillin (synthon D-phenylglycine) and cephalosporin (D-p-hydroxy-phenylglycine).

1.6.4.e. Acylations and Transacylations.

It is possible to achieve esterification of an alcohol or an acid under appropriate conditions. In aqueous media the thermodynamic equilibrium position for the hydrolytic reaction lies in the direction of hydrolysis. If the product can be made insoluble then the equilibrium position shifts in favour of the acylated product eg- the use of papain to catalyse the synthesis of the amide of a derivative of phenylglycine, [59]. The product [60] is insoluble in the medium. Optically pure hydrazides of a range of amino acids have been prepared in this way[99]. The unused enantiomer can be racemised *in situ* using a racemase.



The commercial synthesis of aspartame, an artificial sweetner, is based on this method[100]. Aspartame is a dipeptide of L-aspartic acid and the methyl ester of L-phenylalanine.



The precursor can be generated enzymatically from Z protected L-aspartate [61] and methyl-L-phenylalanine [62]. The product [63] (Z-aspartame) is insoluble in the medium and therefore accumulates. Racemic DL-Phe-OMe is used in this process and the unused D-enantiomer recovered, racemised and recycled. The final product, aspartame, is generated by catalytic hydrogenation to remove the Z protecting group.

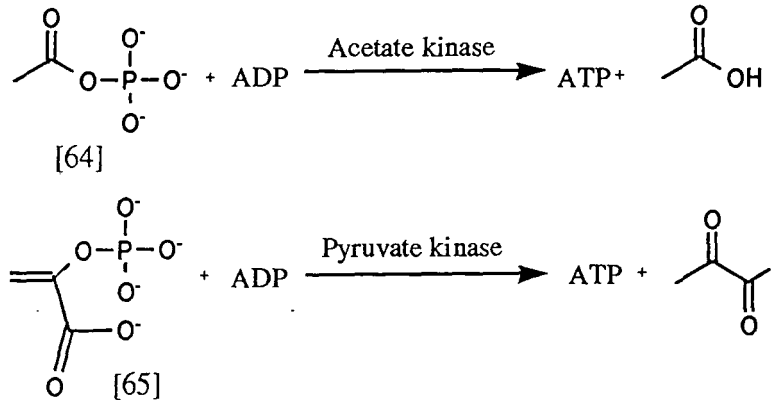
1.6.5. Other Biotransformations

So far the discussion has concentrated on the oxido-reductases (EC1) and the hydrolases (EC3) because they represent the most useful of the 6 classes of enzymes for biotransformations. The other 4 classes, transferases, lyases, isomerases and ligases account for only a small percentage of the literature.

1.6.5.a. Transferases (EC2).

i. Co-factor Synthesis.

The most commonly used group of the EC2 class are the kinases. They catalyse the transfer of phosphate from ATP to an acceptor such as a sugar. They can also catalyse the reverse reaction from a high energy phosphate donor to ADP to regenerate ATP. This has proven a most useful reaction for biotransformations as it offers a method of recycling the ATP co-factor[105].

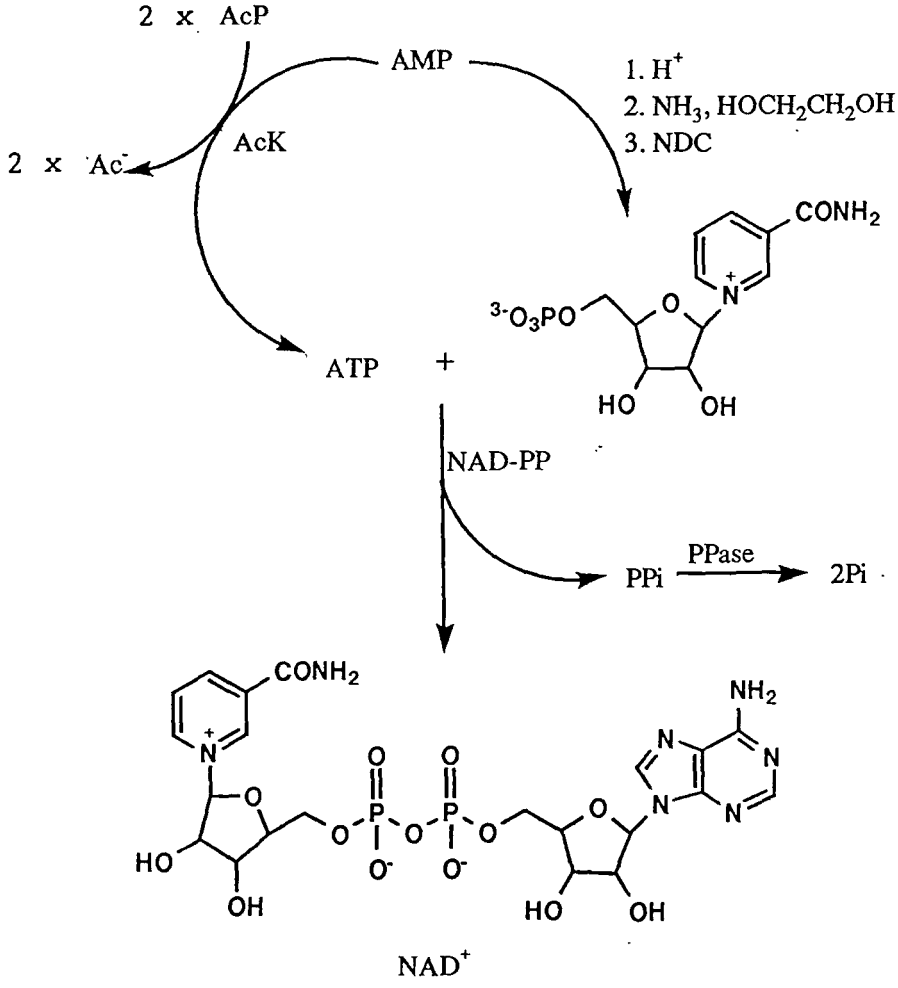


Acetylphosphate [64][106], is the more attractive of the two methods as it is available commercially, however there are technical problems in its use. It hydrolyses slowly in buffer and the inorganic phosphate then precipitates the Mg^{++} that is required for kinase activity. Phosphoenolpyruvate [65][107], is much more stable than acetylphosphate [64] in solution but it is more difficult to prepare.

Acetyl kinase will accept a range of nucleotide phosphates and diphosphates as substrates.

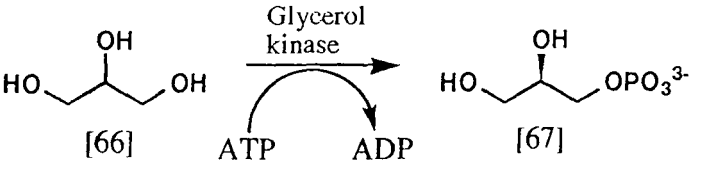
The regeneration of ATP is useful in a number of reactions that require it as a co-factor, eg- the synthesis of glucose-6-phosphate by glucose-6-phosphate kinase[108]. G-6-P is useful in NAD(P)H regeneration and has also been used for the synthesis of dihydroxy-acetonephosphate[109].

Acetylkinase (AcK) has found several uses for the synthesis of unnatural analogues of co-factors. It has been used to directly synthesis ATP-γ-S[110] and ATP-α-S[111] analogues of nucleotide triphosphates. It is also useful in the chemienzymatic synthesis of NAD(P)$^+$. This synthesis does not compete with the extraction of NAD$^+$ from natural sources but it is a very useful method of obtaining isotopically labelled and structurally modified analogues[112], eg;



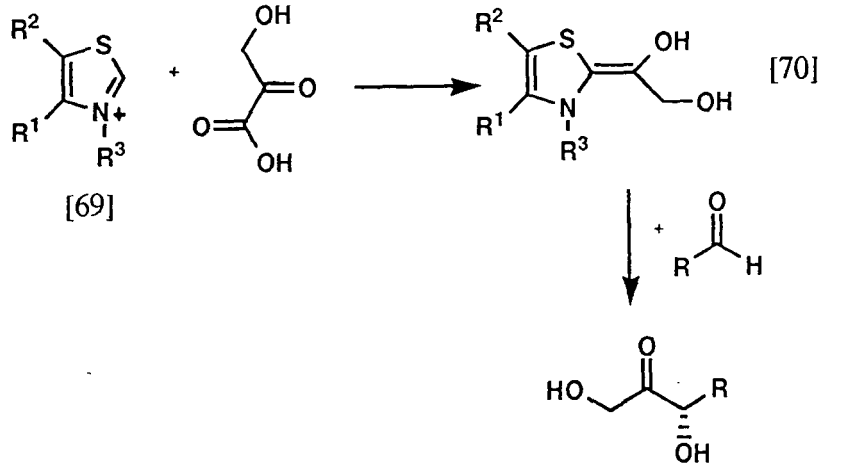
ii. Chemical Synthesis.

The transferases have found limited use in preparative synthesis. One system that has been studied in some detail, however is glycerol kinase which catalyses the stereospecific conversion of glycerol [66] to glycerol-1-phosphate [67]. This enzyme shows some versatility and will also accept a range of 3 and 4 carbon sugars[113].

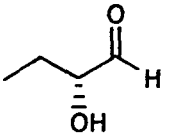


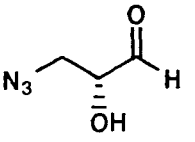
Transketolase, an enzyme of the pentose phosphate pathway, has been studied in considerable detail. It has been used for the stereospecific synthesis of sugars and sugar phosphates.

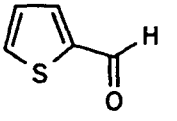
Transketolase is a thiamine pyrophosphate [69] dependent enzyme which catalyses the breakdown of the ketose donor into the TPP-hydroxyacetyl complex [70]. This complex then acts as a nucleophilic hydoxyacetaldehyde synthon which attacks the aldehyde acceptor[115], eg;

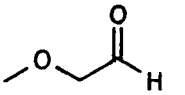


The reaction is reversible unless hydroxypyruvate is used as the donor. In this case decarboxylation drives the reaction in the desired direction. A range of aldehydes [71] to [74] are accepted by the enzyme[116-119], eg;


[71]

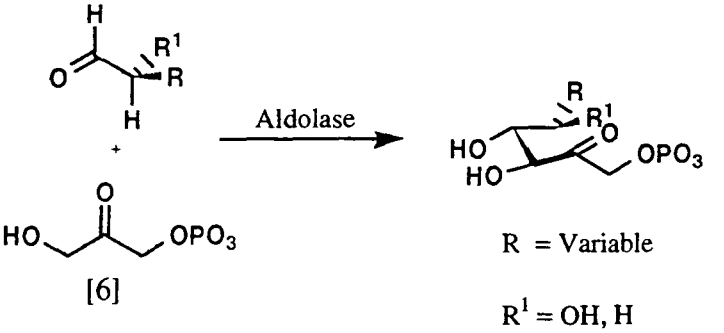

[72]


[73]


[74]

1.6.5.b. Lyases. (EC4)

The lyases provide the fourth largest group of commercially availible enzymes, and they have proven powerful synthetic catalysts. Rabbit muscle aldolase is the most studied of this class. It catalyses the addition of dihydroxyacetone phosphate [6] to a range of aldehyde acceptors[109a], eg;

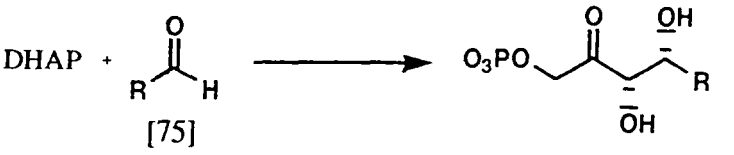


[6]

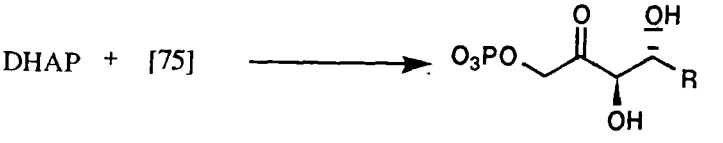
This reaction is useful in the preparation of rare, unnatural and isotopically labelled sugars[109b]. There are many readily available aldolases which should prove of valuable catalysts in the future, however few of them have been studied in detail. DHAP is an essential substrate and cannot be structurally modified but a range of aldehydes are accepted by the enzymes and with appropriate choice of

enzyme the desired stereochemistry at C3 and C4 can be generated[120-122], eg;

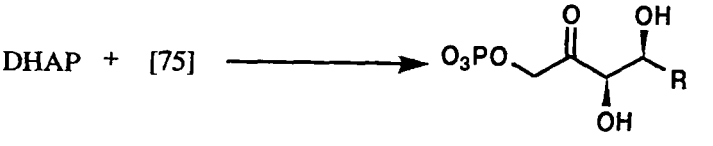
i. D-Fructose-1,6-diphosphate aldolase



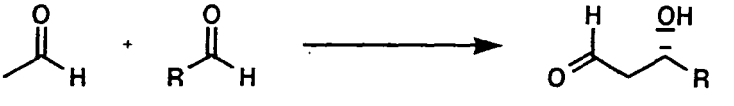
ii. L-Fructose-1-phosphate aldolase



iii. L-Rhamnulose-1-phosphate aldolase

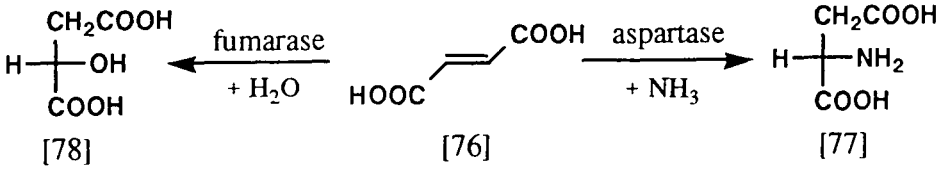


iv. Deoxyribose aldolase



The use of lyases in academia and in industry is a growing area but as yet there are few examples of their use for the synthesis of gram quantities of materials, eg. the commercial production of aspartic acid [77] uses the addition of ammonia to fumaric acid [76] catalysed by aspartase[123]. Fumaric acid can also be used as the starting

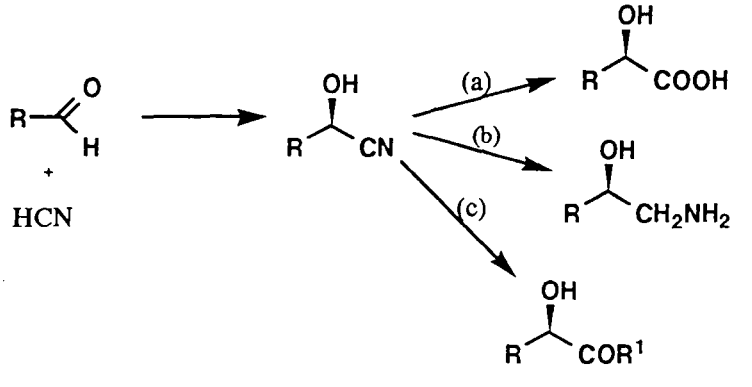
material for the production of malic acid [78] by hydration with fumarase, eg;



[78] [76] [77]

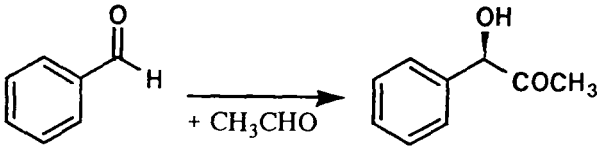
The synthesis of isotopically labelled L-phenylalanine from cinnamic acid and labelled ammonia can be achieved in a similar manner[124] with phenylalanine and ammonia lyase.

Oxynitrilase is an enzyme of the lyase group which has been successfully exploited for biotransformations. It catalyses the addition of HCN to an aldehyde to form a homochiral cyanohydrin. The enzyme is readily available from almonds. It is particularly versatile and will accept a wide range of aldehydes as substrates[126]. The best results have been achieved using immobilised enzyme in continuous flow reactors with the substrate aldehyde dissolved in a mixture of water and ethanol in which the enzyme is insoluble, eg;

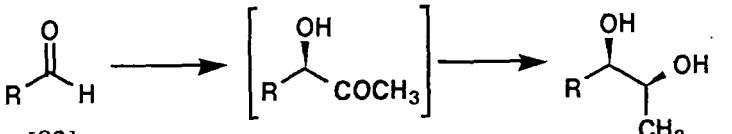


The enzyme catalysed acyloin condensation also falls into the lyase classification. This transformation is of historical significance as it was one of the first

industrial uses of a microbiological process. In 1961 a yeast was used to prepare a precursor of D-ephedrine by an acyloin condensation[127], eg;



Recently, even more important examples of this synthetically useful and sterecspecific condensation have been reported. In the yeast transformations of aldehydes, [82], to diols, [83][128], The initially formed acyloins are not isolated but are further reduced to the diol, eg to afford the pheromone synthon [83a][129], the potential pseudomonic acid precursor [83b][130] and the α-tocopherol chromanyl intermediate (vitamin E) [83c][131];

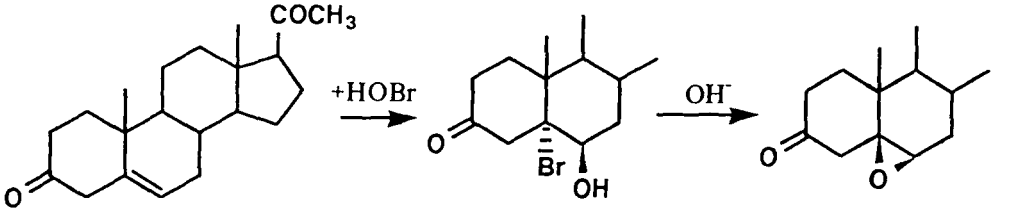


[82]

R = a) PhCHCH-
b) PhCHC(CH3)-
c) α - (Fur)CHC(CH3)-

[83]

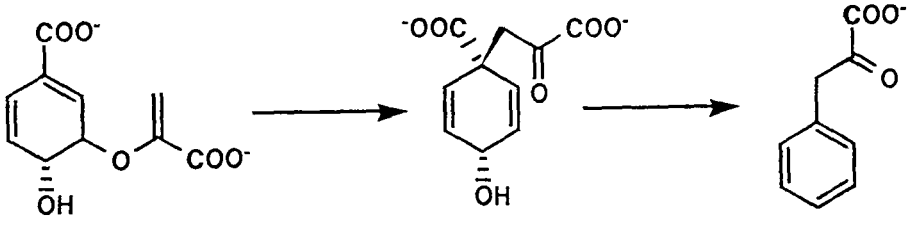
Chloroperoxidase transformations also comes under this classification. During the process HOX is added to a C=C double bond. This reaction has been most widely exploited in natural product modification[132] eg;



1.6.5.c. Isomerases (EC5).

The fifth group are the isomerases, EC 5, the migration of C=C double bonds, *cis-trans* isomerisations and racemisations are a few of the processes catalysed. The use of racemase enzymes has already been discussed (section 1.7e, page 38) for amino acid production.

Glucose isomerase is used in the food industry for the interconversion of glucose with fructose. This reaction has little preparative use, however the enzyme is available in bulk, but little is known about its substrate specificity[123]. There are only a limited number of mutase enzymes which have been studied for biotransformations. One of these is chorismate mutase, an enzyme of the shikimic pathway which catalyses a Claisen rearrangement in some bacteria and plants[134]. On acid work-up the reaction produces the prephenate synthon [84], eg;



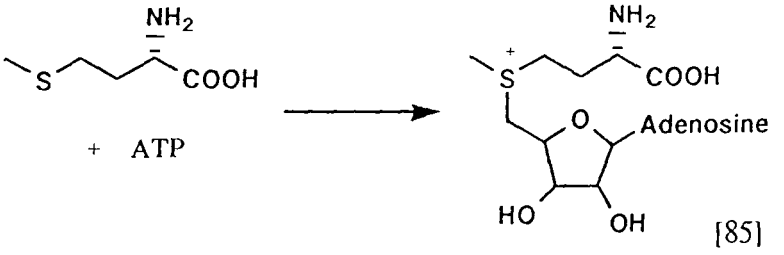
[84]

1.6.5.d. Ligases (EC6).

The sixth group of enzymes in the classification system are the ligases. These enzymes catalyse the formation (and breaking) of C-O[135-136], C-S[137], C-N[138] and C-C[139] bonds and the formation and hydrolysis of phosphate esters[140].

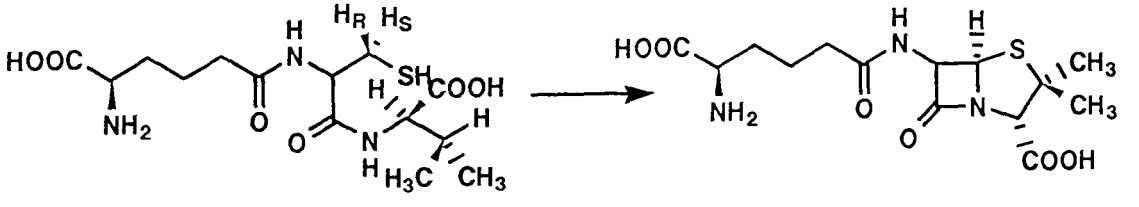
Only a few examples exist of their use in preparative biotransformations but they show several very useful reactions. S-Adenosyl-L-methionine (SAM)[85] is a co-factor in many enzyme systems but in particular methyltransferase processes. SAM is an expensive and unstable compound and an enzymatic route for its synthesis is attractive. The availability of large quantities of SAM synthetase from genetically engineered *E. coli*[141] affords the possibility of

an enzymatic synthesis. Indeed in 1982 a method employing this enzyme was reported[137];



[85]

The synthesis of the β-lactam ring in penicillin antibiotics by chemical means is difficult and synthesis with stereocontrol is even more difficult. The use of purified isopenicillin N-synthetase (isopenicillin cyclase) or *Cephalosporin acremonium* cyclase allows the stereospecific β-lactam cyclisation of a range of amino acid precursors [86] to generate new penicillin and cepham antibiotics [87][142], at present however the transformations are achieved on a very small scale.



[86] [87]

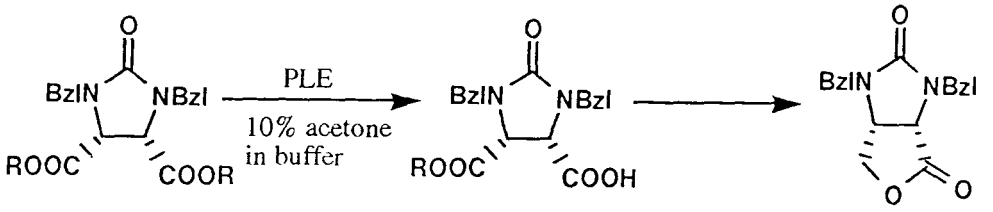
Part 2 :
Biotransformations in Organic Solvents

1.7. Introduction.

It was generally believed that enzymes would only operate in aqueous media and any addition of nonaqueous solvents would progressively lead to deactivation of the enzyme. The 3D structure is held together by non covalent electrostatic and hydrogen bonding interactions and water plays a key role. In addition to this the surface of the enzyme consists of hydrophilic amino acid residues and the interior contains predominantly hydrophobic residues. It would then be expected that in hydrophobic media the enzyme would uncoil and turn itself inside out. A fortuitous discovery, however, was the realisation that some enzymes will indeed function in predominantly non-aqueous media with little or no added water. Many reagents are unstable and have low solubility in water. Early attempts to overcome the solubility problem involved the addition of water miscible co-solvents to the reaction mixture. Later the use of biphasic water / apolar solvent systems were used as emulsions, micro-emulsions, reversed micelles or with the water and enzyme encapsulated in a porous support eg. sepharous. However a most interesting observation was the discovery that the enzymes retained their catalytic activity even if the dry enzyme powder was suspended in anhydrous nonpolar media.

1.8. Co-solvents.

The first use of organic solvents in enzymatic reactions were as co-solvents in buffer. 1-10% of water miscible solvents were added to many enzyme reactions simply to achieve the minimum level of substrate solubility[143], eg;

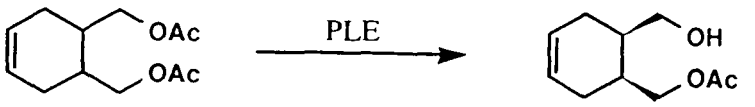


[88] [89]

For example, the resolution of the prochiral diacetate [88] in phosphate buffer with 10% methanol gave the monoester [89] in 70% yield with 92%ee. This can be compared to the hydrolysis of the dipropyl ester in buffer which only gave 75%ee[143b].

Recently a number of studies have been published on the addition of various co-solvents in ratios up to 50%. The influence of a range of protic and aprotic solvents with varying polarities on the enantioselectivity of α-chymotrypsin have been studied using methyl (N-acetyl)phenylalanine as the substrate[144]. The addition of any of the solvents reduced the overall rate with hydrolysis of the L enantiomer being affected more than hydrolysis of the D enantiomer. The enzyme reaction remained L selective overall, but with a much reduced selectivity. The solvents in order of decreasing influence on enantioselectivity are DMSO < MeOH < acetonitrile < IPA < DMF < acetone < dioxan.

A number of studies have been carried out on PLE (pig liver esterase)[145], eg the hydrolysis of [90], taken as a standard system.



[90]

Solvent	Rel Rate	Yield(%)	%ee
H_2O/DMSO (8:2)	0.70	62	59
H_2O/DMF (8:2)	0.35	74	83.5
H_2O/t-BuOH (9:1)	0.44	78	96
Albumin/H_2O	1.31	63	78
H_2O	1.00	60	55

Table 1.6 : Effect of co-solvent on relative rate of PLE mediated hydrolysis of [90].

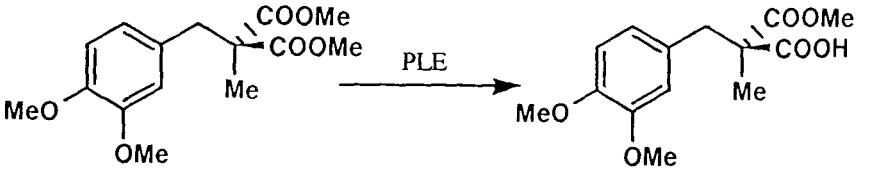
In the event there was no obvious trend with respect to enantioselectivity, and as before any addition of a co-solvent reduced the overall rate.

Another well studied enzyme is horse liver alcohol dehydrogenase (HLADH). There have been two extensive studies on the effects of co-solvents. The first focussed on the oxidation of cyclohexanol and the second on the reduction of cyclohexanone, see Table 1.7[146].

	Relative Rate		
Solvent	1%	5%	20%
MeOH	59	27	<0.1
tBuOH	44	17	4
DMSO	3	0.4	-
HMPA	104	80	55
Diglyme	75	45	18

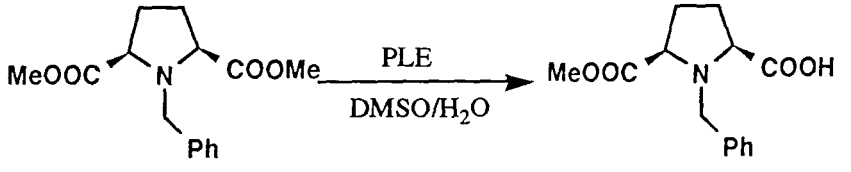
Table 1.7 : Relative rates of reduction of cyclohexanone with HLDH in the presence of various co-colvents

High ratios of co-solvent (up to 50%) have been used in the synthesis of gramme quantities of optically pure compounds. eg the hydrolysis of the pro-chiral diester [91] proceeds with highest %ee at 50% DMSO, eg. 93%ee, and with good yields, 85-100%[147], eg;



[91] 50% DMSO in buffer, 85% yield, 93%ee

In this case the concentration of DMSO had a significant effect on the %ee of the product eg. the hydrolysis of [92] proceeds well in 25% DMSO and gave [93] in 100%ee as shown in Table 1.8[148], therefore there is an optimal co-solvent concentration.



[92] [93]

%DMSO	%ee
0	17
10	61
25	100
50	97

Table 1.8 : Effect of DMSO on the enantioselectivity of the hydrolysis of [92].

1.9. Emulsions.

In biological systems lipase enzymes are generally membrane bound and operate at a lipid water interface. There is good reason to believe, therefore that they will be stable in organic solvents and process hydrophobic substrates. Indeed, consistent with this is the fact that lipases mediate esterification and transesterification reactions in organic media. This is possible as the enzyme will accept nucleophiles, other than water, to attack the acyl enzyme intermediate (see page 12 for a full discussion of the lipase mechanism).

For ester synthesis the technique is simple. The acid and alcohol are mixed in an organic solvent and the lipase powder suspended and the mixture is then shaken and assayed until the required conversion is obtained. For transesterification the acid is replaced with an ester.

Emulsion	
Hydrolysis- menthol laurate	70%ee(-), 38%yield 1h
Transesterification- Menthol laurate + pentanol	80%ee(-), 48%yield 15d
Formation- menthol + lauric acid	86%ee(l), 41%yield 10h
Organic Solvent	
Transesterification- menthol laurate + isobutanol	94%ee(-), 45%yield 15d
Formation- menthol + lauric acid	95%ee(-), 45%yield 10h

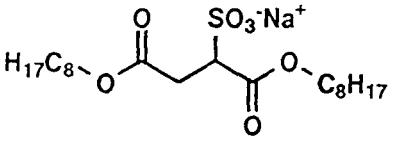
Table 1.9 : Resolution of menthol by various methods and the effect on the enantioselectivity.

One example of the comparison of hydrolysis, esterification and transesterification is the resolution of menthol. The ester synthesis and the transesterification reactions were carried out in emulsions of phosphate buffer in heptane. The transesterification reaction was also carried out in

anhydrous heptane[149]. These reactions show that lipases not only work in anhydrous conditions but in some such conditions cases can even provide improved optical purities.

1.10. Reverse Micelles.

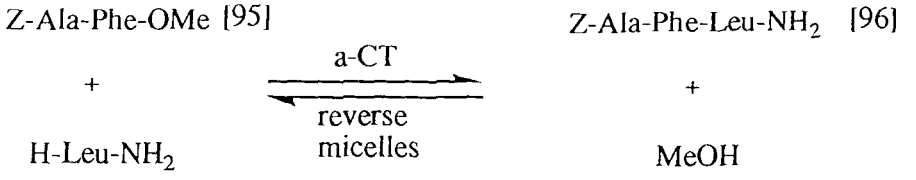
An alternative technique is the encapsulation of the aqueous phase in reverse micelles. There are a number of reviews of reverse micelles as models of biological membranes[150] and their general properties and effect on enzyme structure[151]. The creation of a reverse micellar solution involves the addition of an ampiphilic surfactant, eg sodium 1,2-bis(2-ethylhexyloxycarvonyl)-1-ethanesulfonate (AOT) [94], to a hydrophobic solvent containing water (more than the solubility limit without surfactant)[152].



[94]

This can create a homogeneous mixture of 10% water in heptane with the enzyme occupying, "water pools", or micelles with complete retention of activity and in some cases with a turnover number that is higher in the micelles than in bulk water. Another interesting effect observed on encapsulating the enzyme in the micelle is that the pH optimum can shift towards alkaline, eg for α-chymotrypsin the optimal pH shifts from pH 7 to pH 8.5[153]. Many surfactants have been studied in this regard, eg methyltrioctylammonium chloride has been used to solubilise α-CT, trypsin, pepsin and glucagone into cyclohexane with 10% H_2O and the studies seem to suggest no difference in the folding of the protein[154]. Lysozyme has been solubilised with bis(2-ethylhexyl) sodium succinate in isooctane. With as little as 0.8% water the enzyme is still active towards N-acetylglucosamine oligomers. The Km for this substrate is 0.1mM compared to 0.01mM in buffer. Again the pH optimum

shifted towards the alkaline by 2-3 units. With a water content of 1.2% it is possible to get 90% of the activity in buffer[153,155,156].

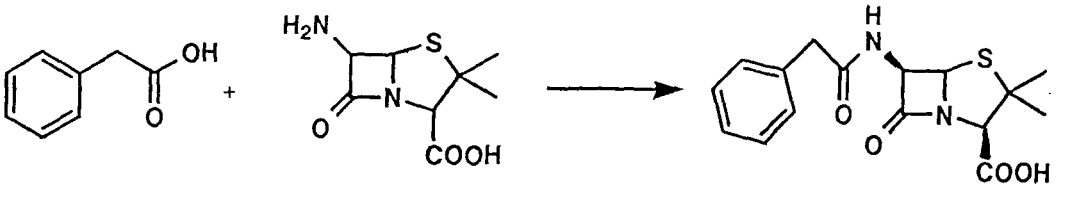


Both reactant [95] and product [96] are insoluble in the micelle thus the reaction proceeds in the desired direction as the product is expelled from the aqueous phase as fast as it is produced supressing the reverse hydrolysis reaction and leading to a 60% yield of the tripeptide in an isooctane medium. This was the first example of the synthesis of a peptide in hydrocarbon micellar solution[157]. The same synthesis carried out in water gives a very lower yield (<10%)

There are only a few examples of the use of reverse micelles in preparative scale synthesis eg;

20β-hydroxysteroid dehydrogenase was enclosed in a micellar medium using octyltrimethylammonium bromide as the surfactant. A range of apolar keto-steroids were reduced to β-hydroxysteroids using an *in situ* NADH regeneration method, with hydrogen gas as the ultimate reductant[158].

The semi-synthesis of a number of penicillin analogues have also used this method to overcome the limitations of aqueous media[159]. A range of carboxylic acids can be used to generate different amide analogues of isopenicillin H.



60% yield in $CHCl_3$ + 1%H_2O
+ tetrabutylammonium bromide

1.11. Pure Organic Solvents.

The discovery that most, if not all, enzymes will work in anhydrous media has led to a large increase in the literature in this area.

A number of reviews[160] describing this methodology outline the conclusions which have been drawn for optimal activity;

1. The enzyme powder must retain some hydrated water. That enzymes work in non aqueous solvents at all is surprising because the protein is held in its folded conformation by non covalent interactions, and water is important in maintaining their balance. However the enzyme only sees a mono-layer or so, and as long as this is preserved, then the bulk solvent can be replaced. For α-chymotrypsin it has been suggested that the water does not form a complete mono-layer, as there are only about 50 molecules of water per enzyme molecule in octane, for maximal activity. The water is probably arranged around the charged, ionophoric, groups of the protein. However, optimal activity for the oxidoreductases appears to require more or less a complete mono-layer. The requirement for this results in some solvents being more suitable than others. For anhydrous solvents there is a general trend that the more hydrophobic the solvent the better because they are less able to strip the essential water layer.

2. Often if the enzyme is precipitated from buffer at optimal pH for that enzyme it "remembers" its last ionophoric state. This is due to the ionogenic groups on the enzyme being adjusted to their optimal ionisation status.

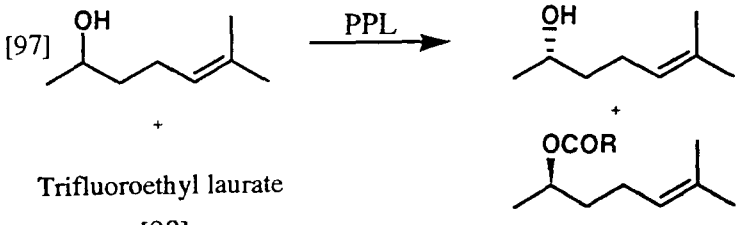
1.11.a. Synthesis in Organic Solvents.

Most of the synthetic reactions so far studied have used hydrolases (EC3) due to their low cost and availibility. In addition they do not require any co-factors. In water the reaction catalysed is that of hydrolysis of either ester or amide with water acting as nucleophile. In predominantly non aqueous media water can be replaced with an alcohol as the nucleophile, to obtain transesterification, with an amine

for aminolysis, a thiol for thiotransesterifications or with an oxime for oximolysis. In general these enzymes show quite good stability in hydrophobic organic solvents.

1.11.b. Reaction Conditions.

The reaction conditions are crucial and can greatly effect the rate, equilibrium conversion and the enantioselectivity of the overall reaction. The factors that have attracted most attention are the solvent[161], water content[162], temperature[163] and mixing conditions[164]. The results of these studies give an overview of the optimum conditions under which a hydrolase catalysed reaction should be carried out. The best solvents are those that are most hydrophobic eg hexane, cyclohexane and toluene because they are less likely to strip the essential water layer from the enzymes surface. More hydrophilic solvents can be used if the water content is increased (but still below the solubility limit) to compensate. For maximal activity the enzyme must retain a minimum percentage of water and some enzymes require more water than others. For example the reaction of [97] with [98] using porcine pancreatic lipase (PPL), the E value (enantiomeric ratio) increases two fold on drying the enzyme powder under vacuum (loss of 3-4% water).



Trifluoroethyl laurate

[98]

After the PPL has been dehydrated to 0.0015% water content the catalytic reaction using a range of primary and secondary alcohols in tributyrin as solvent and acyl donor can be carried out at 100°C[163c]. This temperature stability is due, largely, to the lack of hydrolytic reactions that are responsible for denaturing enzymes at high temperature. The lipase from *Pseudomonas cepacia* can also be made

thermostable such that it retains its catalytic activity up to 100°C for 100h. The increase in temperature ensures a higher equilibrium conversion but for this case, a decreased enantioselectivity[163a].

The mixing conditions have only been studied in a most rudimentary manner. It has been shown that if the reaction is stirred using a magnetic bar then there is a considerable reduction in reaction rate. This has been attributed to mechanical damage to the enzyme. The use of a mechanical shaker (either orbital of reciprical) leads to no damage and thus no loss of reaction rate[164].

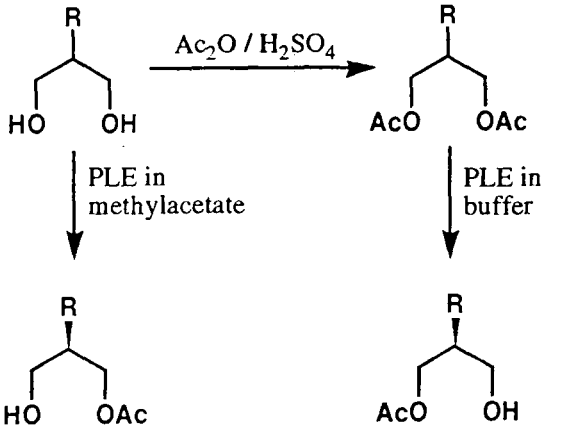
Keeping these practical concideration in mind a wide range of reactions are possible with enzymes in organic solvents.

1.11.1.a. Resolution.

The simplest modification that can be applied to reactions in organic media is to conduct hydrolytic reactions in the reverse direction, ie. to use a racemic alcohol or a racemic acid and form an ester.

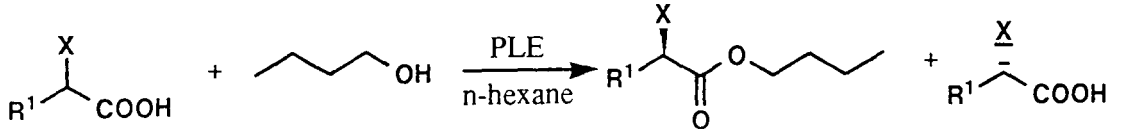
Using the same enzyme to catalyse both the hydrolysis of a prochiral diester of the diol [99] and the esterification of the diol in organic solvents it is possible in principal to access both enantiomeric series[165], eg;

[99]



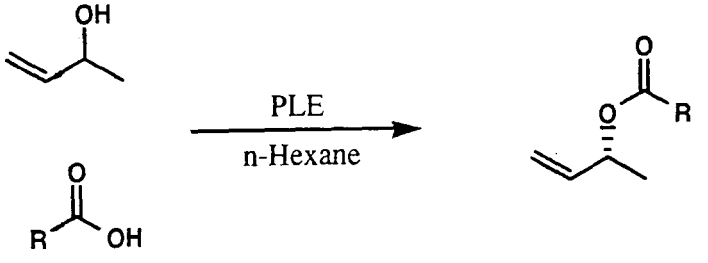
A range of alcohols and acids have been resolved using this very simple strategy[166].

eg. - resolution of racemic acids[166a]



R^1	X	Time	Yield	%ee
Me	Cl	6h	87%	95(R)
But	Br	19h	91%	99(R)
Ph	Br	216h	81%	99(R)

eg. - resolution of racemic alcohols[166f]



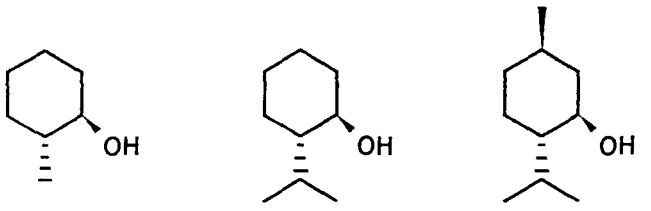
$R = (CH_2)_{10}CH_3$

Substrate	Yield	%ee
1-buten-3-ol	33.2%	50.7(R)
1-nonen-3-ol	28.4%	58.5(S)
1-penten-4-ol	35.9%	55.3(R)

Table 1.10 : Resolution of several racemic alcohols by ester synthesis mediated by PLE in n-hexane

The homologous series from 1-buten-3-ol to 1-nonen-3-ol switches the stereo-selectivity between C5 and C6 from (R) to (S) (see table 1.10). A similar switch is observed for the 1-alken-4-ols between C6 and C7.

In addition to these straight chain alcohols a series of substituted cyclohexanols have been resolved by esterification with lauric acid in hexane, at 40°C, using the *Candida cylindracea* lipase[166c], eg;

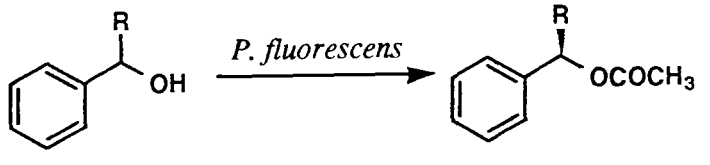


98%ee 100%ee 69%ee

1.11.1.b. Transesterification.

As an alternative to using an acid as the acyl donor an activated ester or anhydride can be employed to generate the initial acyl-enzyme intermediate. This usually results in a faster reaction and as water is not liberated the equilibrium position lies in favour of the product.

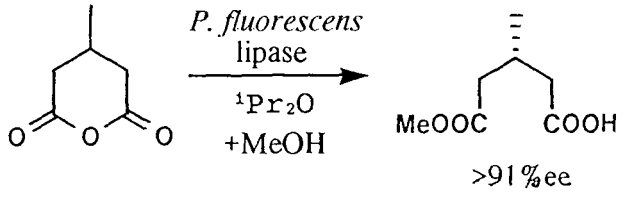
A range of anhydrides have been used as acylating agents in such esterification reactions[167], eg;



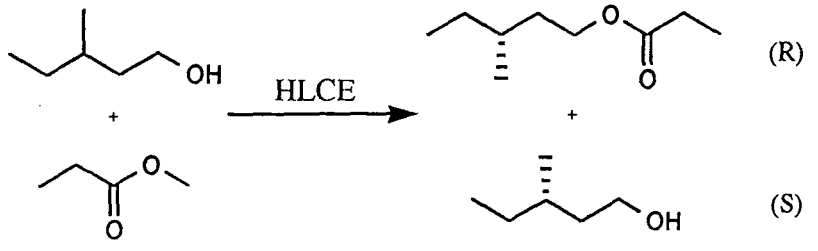
$+(CH_3CO)O$ R = Me, Et, t-But, isoProp

>95%ee (R)

Using a similar strategy prochiral cyclic anhydrides can be opened asymmetrically employing a primary alcohol as the nucleophile[168], eg;

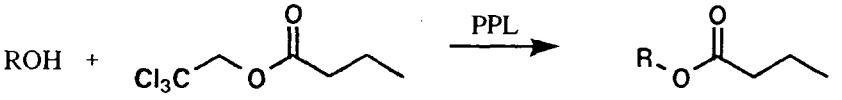


A range of esters have been studied as potential acyl donors for resolution reactions. The earliest examples were tributyrin (glycerol tributyrate) and methyl propionate[169]. Tributyrin was chosen as an analogue of the natural triacyl glycerols substrate. They were used as both solvent and acyl donor in the resolution of secondary alcohols with hog liver carboxyl esterase (HLCE), eg ;



The slow rate of reaction of transesterifications carried out with tributyrin and methylpropionate led to a series of "activated esters" as a way of increasing the rate and equilibrium conversions of transesterifications (eg. trichloroethylbutyrate (TCEB)[166a] was introduced by Klibanov). Clearly the trichloroethanol produced in the reaction is a poor nucleophile, and thus the reverse reaction is supressed. TCEB has been used as an acylating

agent for a range of racemic secondary alcohols, eg;



(TCEB)

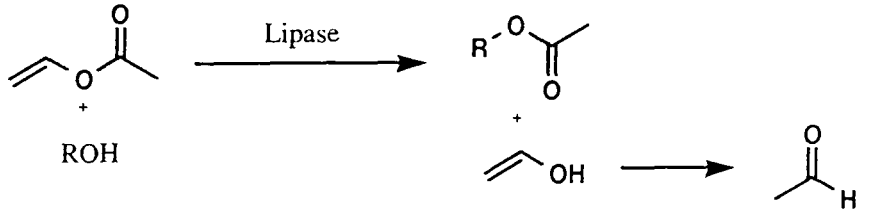
[100]

R = 2-octanol (95%ee)
2 dodecanol (95%ee)
2-methyl-2,4-pentanediol (97%ee)

The rates of the transesterification reaction of [100] with ROH are slower compared to the corresponding esterification reactions with butyric acid, but the overall conversion and yield are significantly higher without sacrificing the optical yield. Such resolutions can be carried out on multi-gramme scales. A related example is the use of trifluoroethyl laurate and butyrate as acyl donors in the resolution of branched and unbranched 2-alkanols using porcine pancreatic lipase[170]. These activated esters have also found use in carbohydrate chemistry to selectively acylate a variety of sugars[171]. Such regio-selectivity for acylation is difficult to achieve with classical chemical methods.

Sugar	Ester	Regioseletivity	Product	Yield
Glucose	TCEB	82%	6-O-ButGlu	2.1g
Galactose	TCEAc	95%	6-O-AcGal	0.5g
Mannose	TCEAc	100%	6-O-AcMann	2.4g
Fructose	TCEAc	100%	1-O-AcFruc	0.5g

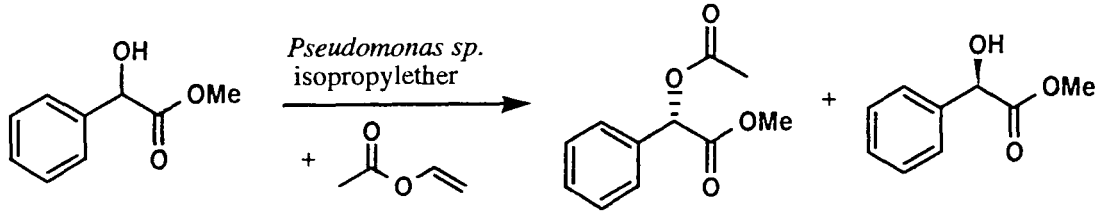
A powerful and widely used method of improving the level of conversion employs vinyl and isopropenyl acetates to prevent any reverse reaction. After hydrolysis the liberated vinyl alcohol enolises to acetaldehyde[172], eg;



Enol esters have been employed in the resolution of a wide variety of straight chain and branched alcohols[173], cyclic and heterocyclic alcohols[174], γ-hydroxy phenylsulphones[175] and organometallic species[176]. In larger scale reactions the liberated acetaldehyde inhibits the enzyme. This is believed to be by the formation of Schiff bases with free amines on the enzyme surface[177]. Faber[178] suggests this limitation can be overcome by protecting the free amine after covalent binding of the amine functional groups to a resin support.

1.11.1.c. Effect of Acyl Donor

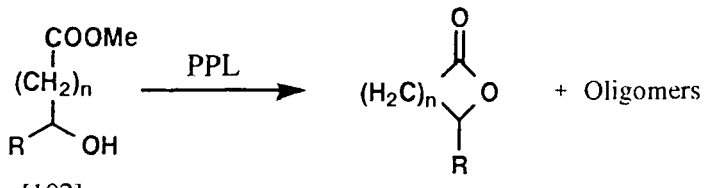
Variations in acyl donor can have a significant effect on the enantioselectivity of the transacylation reactions[179] eg. for resolution of [101] if the acylating agent is vinyl acetate, vinyl propionate, vinyl butyrate, vinyl laurate, vinyl palmitate or isopropenyl acetate it is the R ester that is formed however when vinyl chloroacetate is used then the S enantiomer is acylated, eg;



[101]

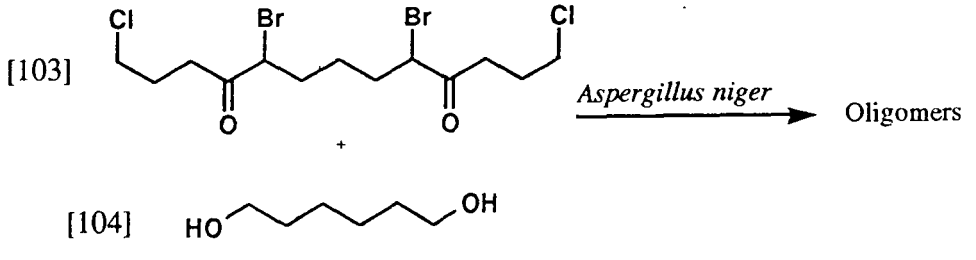
1.11.1.d. Bifunctional Compounds.

If a molecule contains both an alcohol and an acid functionality then it is possible to obtain lactones with concomitant resolution of the enantiomers[180], eg;



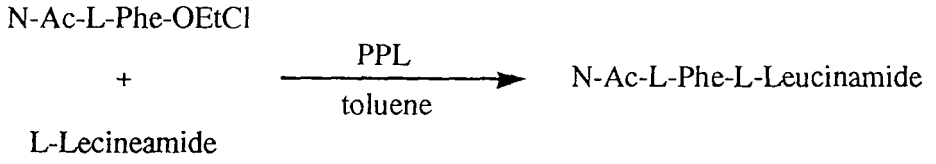
[102]

Depending on the length of the chain between the alcohol and the acid groups dimers or oligomers can be obtained. For the example shown above for [102], if n=1 then oligomers are the exclusive product. If n=3 then the β-lactone is the exclusive product. For n>3 the product depends on the substituent R. It is also possible to synthesise oligomers from a diol and a diester[181]. Incubation of bis(2-chloroethyl) (+/-) -2,5- dibromoadipate, [103], and 1,6-hexanediol, [104] with *A. niger* gave trimer and smaller quantities of higher polymers. The trimer and the pentamer were both optically active (by polarimetry) but no quantitative data was reported. This synthesis is as yet impractical but the synthesis of chiral polymers is of great interest for chiral sorbants to separate enantiomers and in the synthesis of liquid crystals.

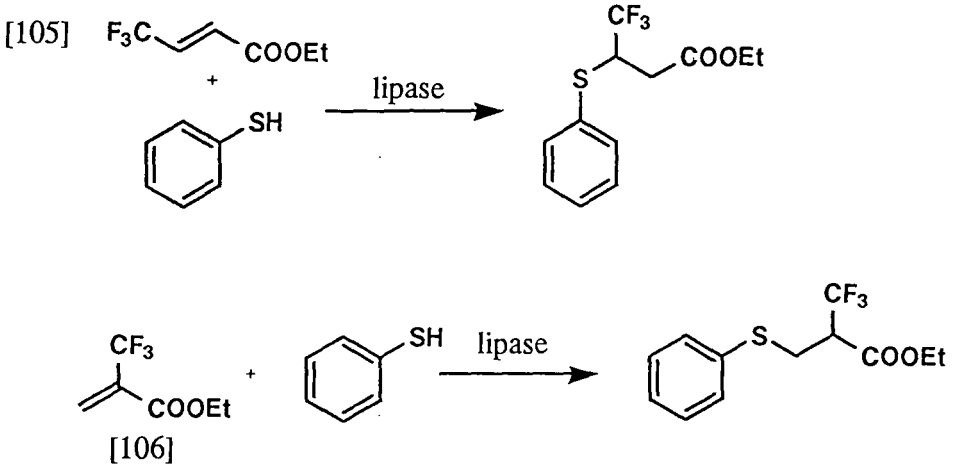


1.11.2. Other Biotransformations using Hydrolases.

Using an amine instead of the alcohol as the nucleophile allows the preparation of chiral amides (aminolysis) and peptides[182]. The organic solvent can cause a reduction in the selectivity of the protease and in one case the synthesis of peptides containing D-amino acids has been achieved. The use of the carbobenzyloxy group (CBZ) to protect the free amine can allow easier scale up of the reaction.

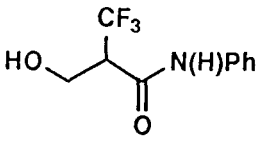


Interestingly lipases from *Alicaligenes sp* and *Achromobacter* have been used to catalyse Michael addition reactions of thiophenol to (E)-ethyl 3-(trifluoromethyl)- and 2-(trifluoromethyl)-propenoate, [105] and [106][183], eg;

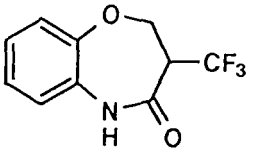


A range of nucleophiles have been employed to generate chiral addition products with [106] using CCL, PLE and α-CT, eg;

Enzyme	Nu-H	Yield(%)	%ee
CCL	H_2O	48	70(+)
CCL	Et_2NH	47	71(+)
CCL	$PhNH_2$	76	39(+)[107]
CCL	2-aminophenol	--	41(+)[108]

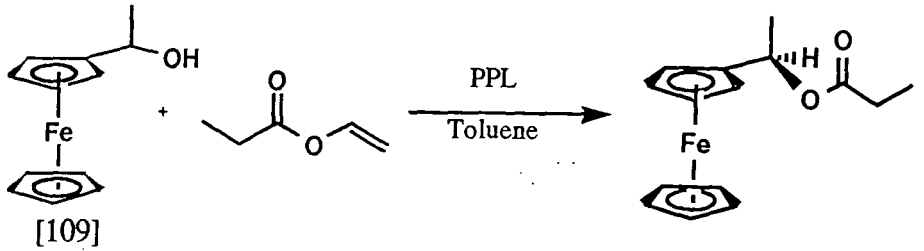


[107]



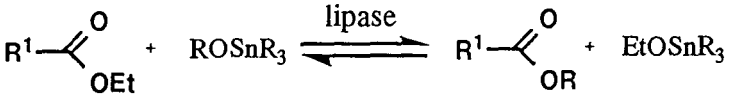
[108]

Transesterification reactions with organometallic reagents have also been studied eg. ferrocene alcohols [109] have been resolved using vinyl butyrate as the acyl donor[184,186], eg;



[109]

and stannyl ethers have been used as the nucleophile in transesterifications with a number of esters[185], eg;



1.11.3. Biotransformations with Other Enzymes.

By far the majority of the enzymes used in biotransformations in organic solvents come from the EC3 catagory ie. the hydrolases, but there are a few studies of enzymes from other groups, notably EC1 and EC4, the oxidoreductases and the lyases respectively.

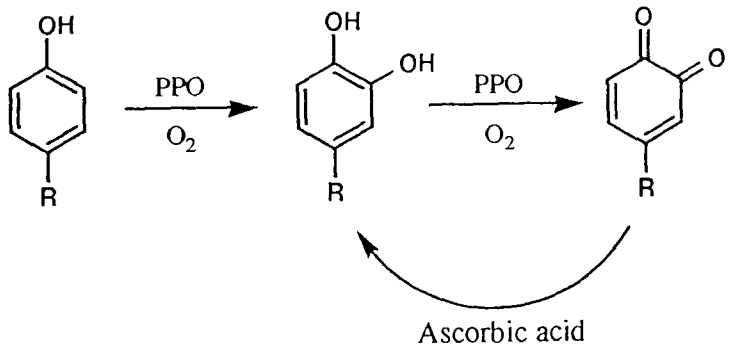
1.11.3.a. EC1 Oxidoreductases.

Oxidoreductase enzyme pose problems because of their co-factor requirement. This can, however, be overcome by depositing the enzyme and co-factor on the surface of glass beads, and then suspending the beads in a water immiscible solvent[186]. NAD^+ and NADH have been efficiently recycled with the alcohol dehydrogenase (ADH) catalysed reduction of isobutyraldehyde or the oxidation of ethanol. Using this system turnover numbers of 10^5 to greater than 10^6 have been achieved.

Using HLADH prepared in this way a number of aldehyde, ketone and alcohol substrates have been converted to optically enriched products, eg;

Substrate	Product	%ee
2-phenylpropionaldehyde	(-)2-phenylpropanol	95
2-chlorocyclohexanone	(+)*trans*-2-chloro-cyclohexanol	98
trans-3-methylcyclohexanol	(-)-3-methylcyclo-hexanone	100
cis-2-methylcyclopentanol	(+)-2-methylcyclo-pentanone	96

Polyphenol oxidase (PPO) is another enzyme that has been shown to operate in organic solvents. PPO catalyses the oxidation of substituted phenols to the diphenol and ultimately to the o-quinone product, eg;



This reaction is impractical in aqueous media as the o-quinone is unstable and quickly polymerises to polyaromatic pigments, deactivating the enzyme. The reaction is, however, achievable in dry chloroform, provided that the enzyme has been precipitated from an aqueous buffer at optimal pH. To isolate the diol the o-quinone is reduced by adding ascorbic acid as a mild reducing agent[187].

1.11.3.b. EC4 Lyases.

Oxynitrilase catalyses the addition of HCN to an aldehyde to generate a chiral cyanohydrin. This reaction has been studied in detail in aqueous systems (see page 53). For a range of aldehydes the optical purity of the product cyanohydrins is significantly improved in ethyl acetate, with the enzyme immobilised on cellulose beads[188];

	Water/EtOH			EtAc/Cellulose		
Aldehyde	Time	Yield	%ee	Time	Yield	%ee
benzaldehyde	1h	99%	86	2.5h	95%	99
3-phenoxybenzaldehyde	5h	99%	10.5	192h	99%	98
furfural	2h	86%	67	4h	88%	98.5
butyraldehyde	2h	75%	69	4.5h	75%	96

Discussion

Chapter 2

Effect of Modifying the Acyl Donor

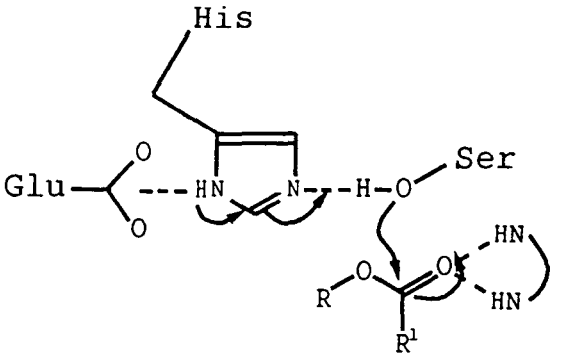
Introduction.

The hydrolases (EC2) are the most studied group of enzymes in organic solvents. Many are available as off the shelf reagents and in general they can be used without isolation or further purification.

Three have been purified to homogeneity, obtained in crystalline form and been investigated by X-ray analysis. These are human pancreatic lipase (HPL), *Geotrichum candidum* lipase (GCL) and *Mucor meihei* lipase (MML). The structure of GCL was determined to 5Å in 1979[189] and refined to 2.2Å in 1991[190]. The 2.2Å resolution crystal structure of GCL revealed a single domain of 65 x 60 x 45Å, the largest known, consisting of 9 α-helices, and 4 β-sheets. The high resolution crystal structure indicates an active site consisting of a catalytic triad of serine (217), histidine (463) and glutamate (354). Unlike many other lipases, and serine proteases, the catalytic triad for GCL has glutamate, replacing the usual aspartate residue[190]. The more usual serine - histidine - aspartate catalytic triad can be see in the diagram on page 13 (Chapter 1). The role of histidine as a general base, to increase the nucleophilicity of the serine hydroxyl group, was demonstrated by the irreversible loss of enzymic activity when it was modified by *tos*-L-phenylalanine chloromethylketone[192], a specific histidine label. The role of the carboxylate group on glutamate (354) was only discovered from crystallographic data. The diagram on page 13 can be compared to Figure 2.1 with glutamate replacing aspartate. The glutamate (or aspartate) and the

histidine together increase the nucleophilicity of the serine hydroxyl oxygen thus promoting the attack of the serine on the acyl donor.

Figure 2.1



The oxyanion hole in GLC that accommodates the carbonyl functionality of the acyl donor is formed from the backbone nitrogens of alanines 218 and 132. Adjacent to serine 217 are glutamates 216 and 466 which coordinate two water molecules close to the β-oxygen of the serine. It has been postulated that these two glutamates provide a trap for the water used in the hydrolysis reaction. The active site is inaccessible from the surface. Serine 317 is buried in a cleft which is covered by two nearly parallel α-helices. The outer surface of this cap consists of hydrophobic residues suggesting a role in the interfacial interactions of the enzyme.

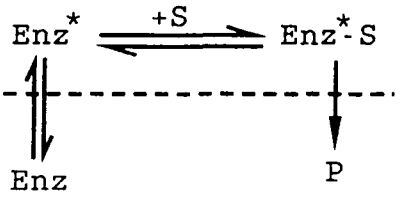
The structure of human pancreatic lipase was solved in 1990[193]. It is a single chain protein of 449 amino acids and consists of two domains. The N-terminal domain from residues 1 to 335 consists of 9 strands in the form a wound β-sheet. The C-terminal domain consists of 2 parallel β-sheets from residues 336 to 449. The catalytic triad is formed from aspartate 176, histidine 263, and serine 152 and the oxyanion hole from the backbone nitrogen of phenylalanine 77 and possibly of residue 153. Additional evidence for the position of the active site was sought by soaking the crystal in butylboronic acid (to give increased electron density at the active site) but the concentration of the

adduct was not sufficient for reliable results. The active site serine residue was identified unambiguously by treating the enzyme with phenylmethylsulfonyl fluoride. This treatment deactivated the enzyme and subsequent analysis identified serine (152) as the derivatised residue. This technique has been used to identify the catalytically active serine in a range of lipases and serine proteases[194]. The active site of HPL is buried in a similar fashion to that of GCL and is also covered by a loop of hydrophobic residues. It is not buried as deeply as that of GCL which possibly relates to the preference of GCL for long chain fatty acids. If the covering loop of the HPL is removed by partial tryptic digestion the activity of the enzyme towards water soluble substrates is unaffected, but that of insoluble substrates, eg. triacylglycerols, is much reduced suggesting the importance of interfacial interactions of the sort postulated for GCL.

The third high resolution crystal structure of a lipase is that from *Mucor miehei*. The structure was solved in 1990 to a resolution of 1.9Å[193]. It consists of a single chain of 269 residues. The protein is folded into a β-sheet of 8 strands onto an amphipathic N-terminal helix. The catalytic triad is not exposed, but is close to the surface under a lid, stabilised by electrostatic and hydrophobic interactions. Serine (144), histidine (257) and aspartate (203) constitute the catalytic triad and the oxyanion hole is formed from the backbone nitrogens of residues 145 and 146. There are no other obvious similarities to other serine proteases.

Lipases are interfacial catalysts; the general principals may be envisaged by scheme 1[195];

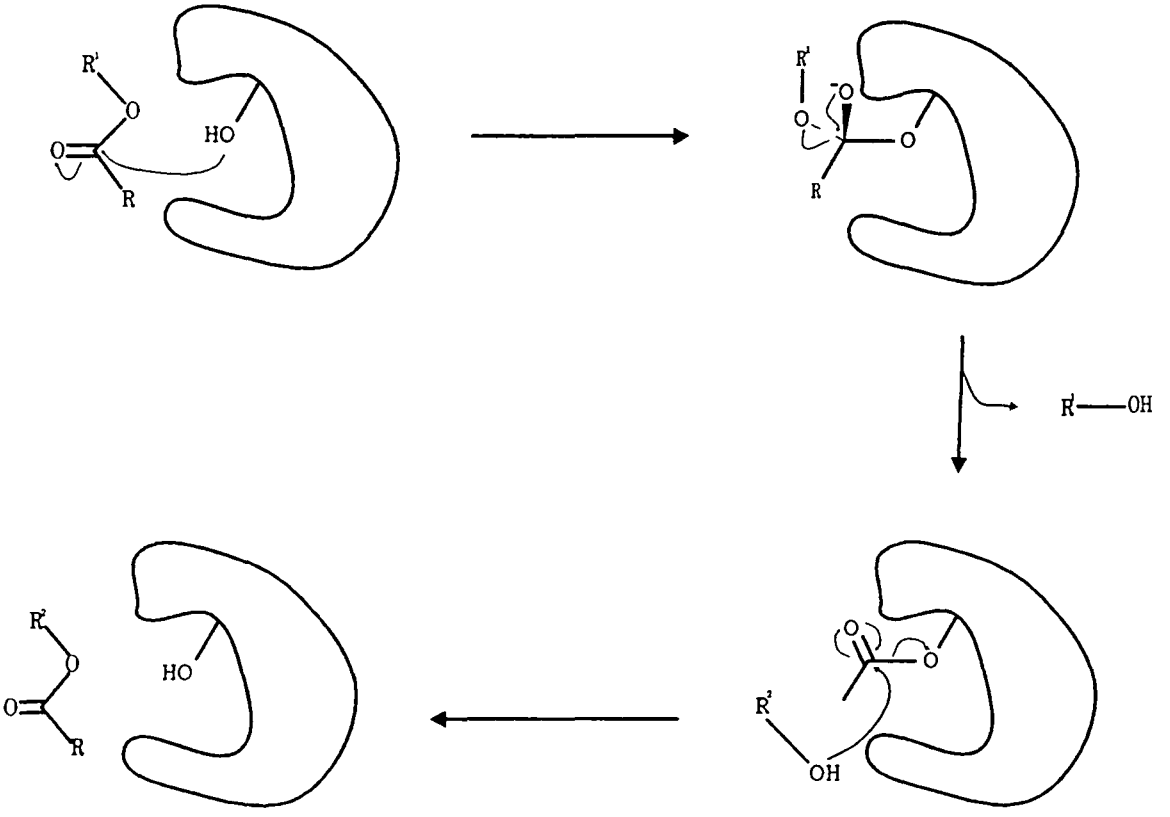
Scheme 1 -



Enz = enzyme in solution
Enz* = enzyme that has penetrated the interface
S and P are substrate and product respectively.

The kinetics of lipase catalysed acyl transfer reactions in organic media may be represented by a "ping-pong" process, where the enzyme is first acylated then deacylated, as illustrated in Scheme 2;

Scheme 2 -



Kinetic resolutions of racemic alcohols using lipases in organic solvents have been popularised by Klibanov[166a,170]. (see Chapter 1, page 61). A range of esters have been explored as acyl donor in these reactions but in each case a common mechanism can be envisaged. An acyl enzyme intermediate is generated by the attack of a serine hydroxyl group from the lipase on the ester (acyl donor). An alcohol can then replace water as a nucleophile[166a];

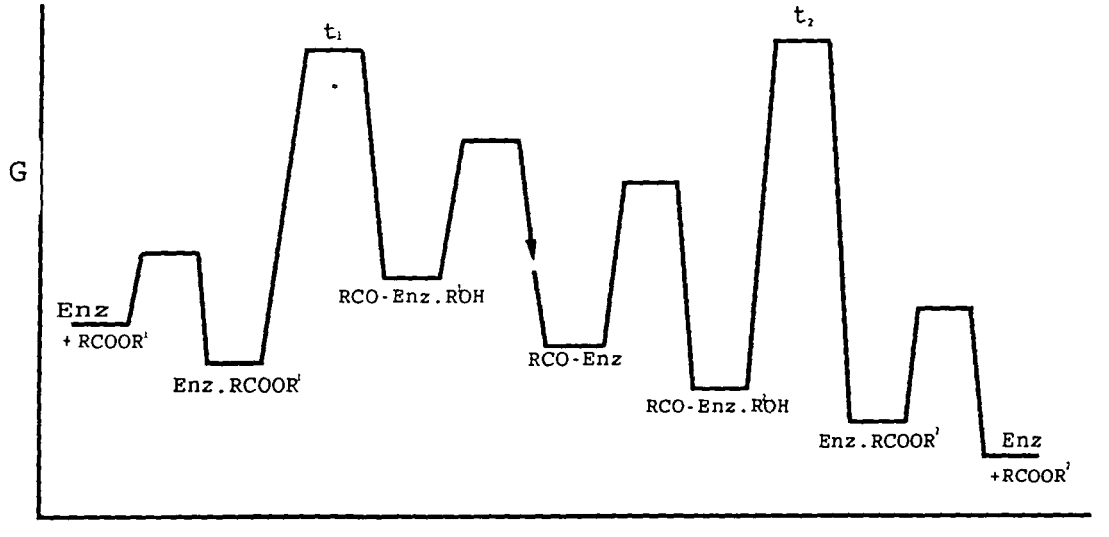
$$\text{Enz} \underset{}{\overset{+\text{RCOOR}^1,\ k_1}{\rightleftharpoons}} \text{Enz.RCOOR}^1 \overset{k_2}{\rightleftharpoons} \text{Enz-COR} \cdot \text{R}^1\text{OH}$$

$$\overset{-\text{R}^1\text{OH},\ k_3}{\rightleftharpoons} \text{RCO-Enz} \overset{+\text{R}^2\text{OH},\ k_4}{\rightleftharpoons} \text{RCO-Enz} \cdot \text{R}^2\text{OH}$$

$$\overset{k_5}{\rightleftharpoons} \text{Enz} \cdot \text{RCOOR}^2 \overset{-\text{RCOOR}^2,\ k_6}{\rightleftharpoons} \text{Enz}$$

k_1 = diffusion constant of ester onto enzyme
k_2 = enzyme acylation rate constant
k_3 = diffusion constant of alcohol off enzyme
k_4 = diffusion constant of second alcohol onto enzyme
k_5 = transacylation rate constant
k_6 = diffusion constant of second ester off enzyme

This is analogous to the hydrolytic mechanism where $R^1OH = H_2O$ These equations can be represented on a reaction coordinate diagram[195];



t_1 And t_2 are two transition states and k_1, k_2, and k_3 are constant for a particular acyl donor. When a racemic alcohol is used as the nucleophile k_4, k_5, and k_6 vary for each enantiomer and thus the reaction coordinates will not be equivalent. Each enantiomer generates distinct diastereotopic transition states and kinetic resolutions can therefore be achieved in the asymmetric environment of the enzyme.

Analysis of this mechanism demonstrates the influence of the acyl donor on the overall stereochemical properties of the biocatalytic system and may be summarised as follows[195];

1. As the resolution of enantiomers of a chiral alcohol is independent of the acylation step the choice of donor, either acid or corresponding ester, should have no significant effect on the E value (E is the enantiomeric ratio calculated as a function of %ee and %conversion of the reaction thus giving a measure of the enantioselectivity independent of conversion, see below). However, As the acyl group exerts steric and stereoelectronic effects on the deacylation step it is possible, in principle, to effect the enantioselectivity by varying the acyl donor.

2. Since the reaction is reversible the concentration and nature of the acyl donor will effect the equilibrium and therefore the stereochemical outcome of the biocatalytic process.

Calculation of E^{195}.

The enantioselectivity of biocatalytic reactions is normally expressed as the enantiomeric ratio, E. This constant is independent of substrate concentration and extent of conversion.

If the extent of conversion, c and the %ee of the substrate, $\%ee_S$ or the product, $\%ee_P$ are known the the enantiomeric ratio can be calculate from either equation 1 or 2;

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

$$\frac{\ln[1-(1+K)c(1+ee_p)]}{\ln[1-(1+K)c(1-ee_p)]} = E \qquad 2$$

The gradual accumulation of products in an enzyme catalysed reaction gives rise to reverse catalysis. Thus, the equilibrium constant, K ($K=k_2/k_1$) see equations 3 and 4, plays a pivotal role, not only in the expression of enzyme enantioselectivity, but also in the maximal obtainable chemical conversion.

$$Enz + A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} Enz + P \qquad 3$$

$$Enz + B \underset{k_4}{\overset{k_3}{\rightleftharpoons}} Enz + Q \qquad 4$$

(A and B are enantiomers)

where k_1, k_2, k_3 and k_4 are the rate constants for the forward and reverse reactions. The extent of conversion, c, the enantiomeric excess of the substrate, $\%ee_s$ and product. $\%ee_p$ and the thermodynamic equilibrium constant are related via equations 5 and 6;

$$E = \frac{\ln[(1-c)(1-ee_s)]}{\ln[(1-c)(1+ee_s)]} \qquad 5$$

$$E = \frac{\ln[(1-c)(1+ee_p)]}{\ln[(1-c)(1-ee_p)]} \qquad 6$$

These equations show that the enantioselectivity of enzyme catalysed reactions depends on a complex relationship between both kinetic (E) and thermodynamic (K) functions.

Objective.

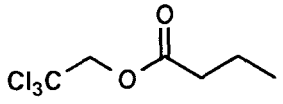
The effect of varying the acyl donor on the rate of enzymatic transesterification reactions in organic solvents has not been studied in any detail. Developments in acyl donor methodology have concentrated on improving the equilibrium conversion eg the introduction of trichloroethyl esters[166a] and of enol esters[172] to render reactions essentially irreversible (see page 66). The earliest transesterification reactions conducted in organic solvents used methyl propionate or tributyrin as both the solvent and the acyl donor. These reactions typically took several days to reach reasonable conversions (>35%). The introduction of "activated" esters as acyl donors increased the equilibrium conversion, but the reactions still took many hours to reach a useful conversion, eg the reaction of octan-2-ol with TCEB in diethyl ether, mediated by PPL, reaches 47% conversion in 5 days! The use of enol esters such as vinyl acetate affords the possibility of a potential 100% conversion, especially useful for prochiral substrates (see page 34).

There have been a number of studies probing the effect of varying the enol ester on the enantioselectivity[179]. A minor structural change in the acetate can be significant and in one case caused a reversal in the specificity, eg. for the acylation of mandelic acid using vinyl acetate, 63% conversion was reached in 90 hours affording a 98%ee of the residual R substrate. On the other hand when vinyl chloroacetate was used a 56% conversion was reached in 48h and the residual mandelic acid was enriched with the S enantiomer (23%ee). There have however, been no detailed studies of the effect of such variations on the reaction kinetics.

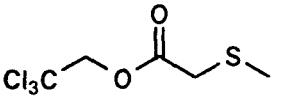
An initial objective, therefore, was to study the effect of acyl donor modifications on the initial rate and equilibrium position of a lipase mediated transesterification. At the outset a series of 2,2,2-trichloroethyl and 2-chloroethyl esters were prepared (Figure 2.2), to assess the effect of increasing the polarity of the acyl moiety. For example, it has been shown that 2-(p-chlorophenoxy)propionic acid is a

particularly good substrate, for the CC lipase, possibly due to the electron withdrawing effect of the oxygen adjacent to the carbonyl functionality[166g]. With this in mind the following series of esters were studied in a transesterification reaction with hexan-1-ol, mediated by *C. cylindracea* lipase in dry diethyl ether.

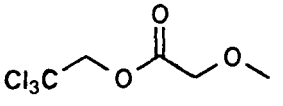
Figure 2.2



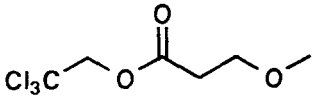
trichloroethylbutyrate (TCEB) [110]



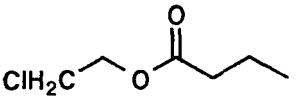
trichloroethyl methylthioacetate [111]



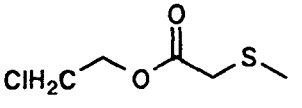
trichloroethylmethoxyacetate (TCEMA) [112]



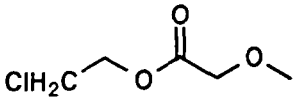
trichloroethyl methoxypropionate [113]



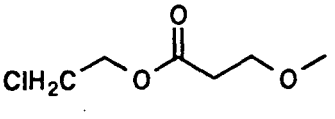
monochloroethylbutyrate (MCEB) [114]



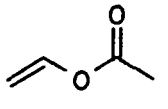
monochloroethyl methylthioacetate [115]



monochloroethylmethoxyacetate (MCEMA) [116]



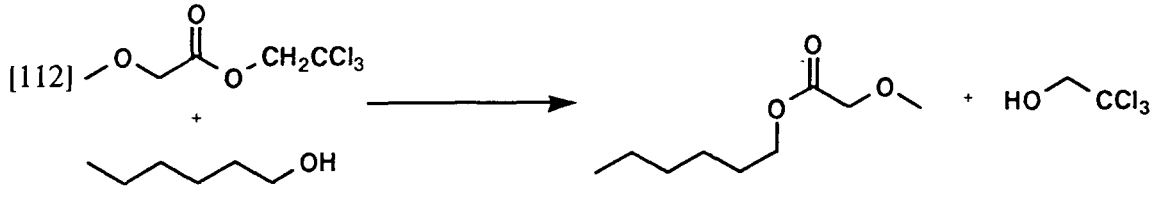
monochloroethyl methoxypropionate [117]



vinylacetate [118]

Results.

For this series of studies the reactions were conducted in diethyl ether with hexan-1-ol as the alcohol and progress was assayed at intervals by GC analysis. The levels of conversion were calculated using an, inert, internal standard (t-butylbenzene). The ratio of peak areas of the internal standard and alcohol at time, t, were compared with the ratio at time, t=0, to obtain a value for the % alcohol consumed in the reaction and therefore the overall % conversion.



$$\% \text{ conversion} = 100 - ((STD_0/ROH_0) \times (ROH_t/STD_t) \times 100)$$

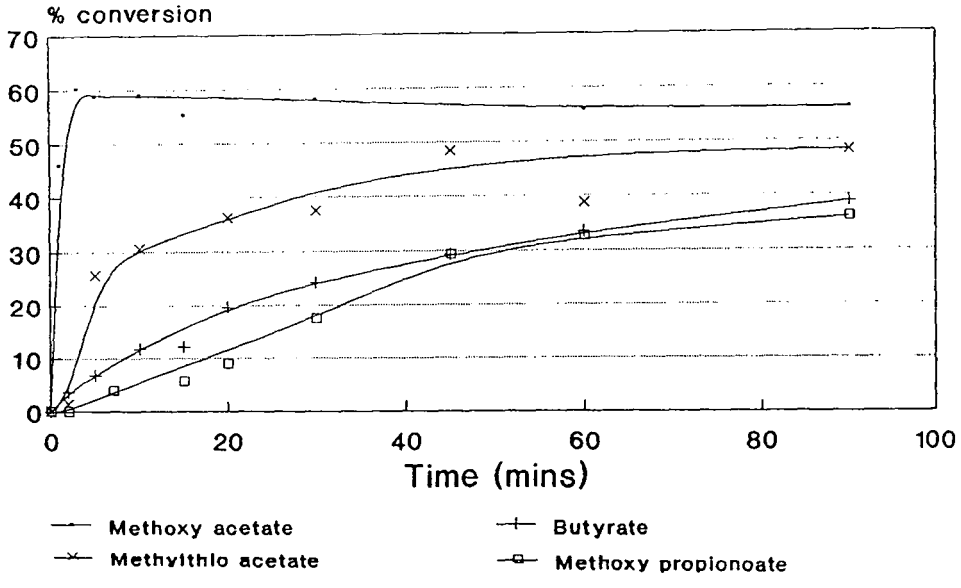
Time (min)	Peak areas (mm^2) hexanol	Int Std	conversion (%)
0	153	320	0
2	98	480	57.3
10	76	368	56.8
20	92	406	52.6
30	92	410	53.1
40	102	500	57.3
82	102	480	58.5

Table 2.1 : Reaction of trichloroethyl methoxyacetatewith hexan-1-ol in ether at 20°C

This general equation was used to calculate the conversion for all of the reactions. Graph 2.1 summarises the results for esters [120] - [123]

Graph 2.1 :

Comparison of Rates of Reaction of a Series of Trichloroethyl esters with Hexan-1-ol Catalysed by *Candida cylindracea* lipase



TCE-Ester	Initial Rate $mM.h^{-1}$	Equilibrium conversion
methoxyacetate	8.87	60%
methylthioacetate	1.12	50%
butyrate	0.33	45%
methoxypropionate	0.17	45%

Table 2.2 : Transesterification reactions with a series of trichloroethyl esters with hexan-1-ol in diethyl ether mediated by CCL.

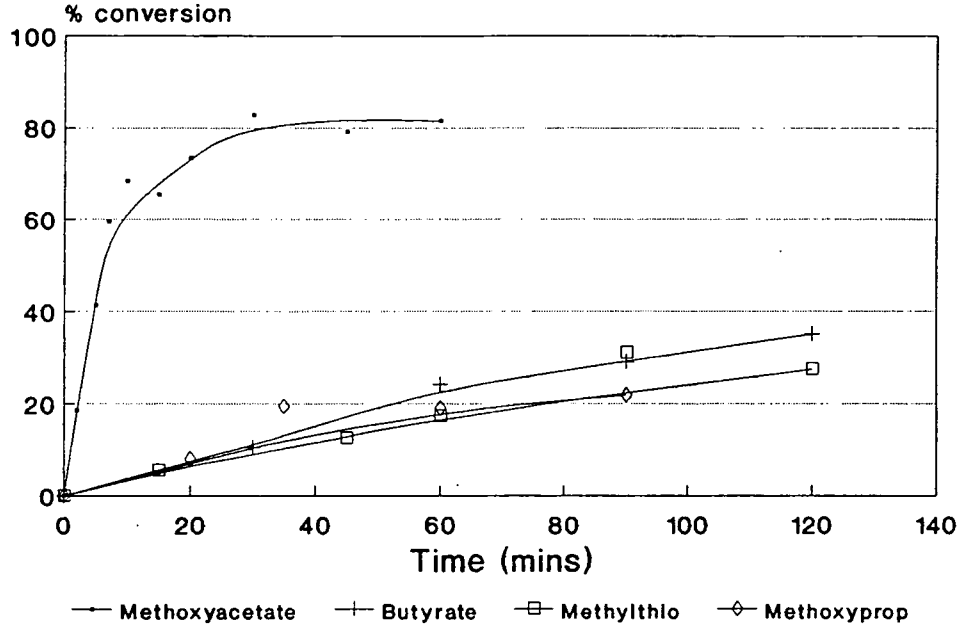
These data illustrate that trichloroethyl methoxyacetate is processed with an initial rate about an order of magnitude faster than the corresponding butyrate ester (8.87$mM.h^{-1}$ compared to 0.33$mM.h^{-1}$) with an equilibrium at 60% conversion established within a few minutes. This can be compared to several hours for all of the other systems studied. This

result is remarkable in comparison to transesterification reactions reported in the literature[166] which typically take from hours to days to reach similar levels of conversion, eg the trichloroethyl butyrate reaction with octan-2-ol mediated by PPL required 5days to achieve a 47% conversion[166a].

The same series of reactions were then conducted using the corresponding 2-chloroethyl esters as the acyl donor. Graph 2.2 summarises these results.

Graph 2.2:

Comparison of Rates of Reaction of
2-Chloroethyl esters with Hexan-1-ol
Catalysed by *Candida cylindracea* Lipase



MCE-Ester	Initial rate $mM.h^{-1}$	Equilibrium conversion
methoxyacetate	2.13	82%
methylthioacetate	0.097	45%
butyrate	0.099	45%
methoxypropionate	0.097	45%

Table 2.3 : Transesterification reactions with a series of monochloroethyl esters with hexan-1-ol in diethyl ether mediated by CCL.

In this series the methoxyacetate ester was again processed significantly faster than the other esters, all of which were comparably slower. The methoxyacetate ester was processed by an order of magnitude faster than the butyrate ester indicating that the liberated alcohol, eg ethanol from the ethyl esters and 2,2,2-trichloroethanol from the trichloroethyl esters [110] - [113] has no effect on the relative difference between the butyrate and methoxyacetate esters. A similar pattern emerges for the equilibrium conversion where the methoxyacetate ester establishes an equilibrium in 20min at 80% conversion. The butyrate reaches 40% in 3h, methylthioacetate 30% in 1.5h while the methoxypropionate reaches 20% in 1h.

The key observation from these experiments is the dramatic rate increase for both the trichloroethyl- and the 2-chloroethyl- methoxyacetate esters. It is clear that this particular acyl group has a significant effect on the rate of the transesterification reactions.

Discussion.

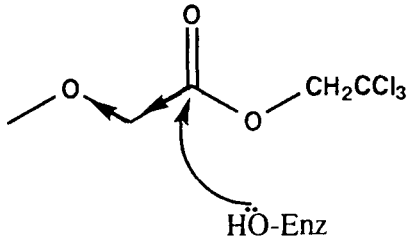
On analysis there are a number of potential causes for this variation. The polarity of an ester can have a significant effect on its non-enzymatic rate of hydrolysis. For example it has been shown[196] that the more polar esters are hydrolysed faster under basic conditions than less polar esters. In this case, where the methylene of butyrate is replaced with the oxygen of methoxyacetate, there may also be a steric component with the CH_2 being accommodated less easily that the O, although a sole steric perturbation is unlikely

Several possible rationales are listed which take into account the particular electronic properties of oxygen;

1. **A polar effect.**

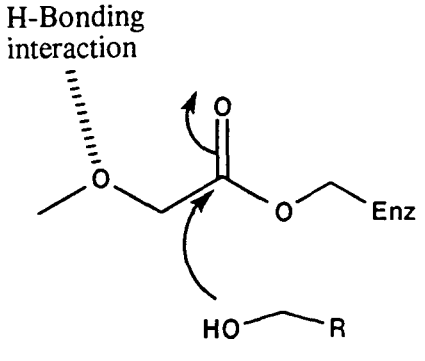
The carbonyl carbon is more susceptible to nucleophilic attack due to the electron withdrawing effect of the

adjacent oxygen. The more polar esters studied by DeTar[196] were more easily hydrolysed in non-enzymatic base catalysed processes.



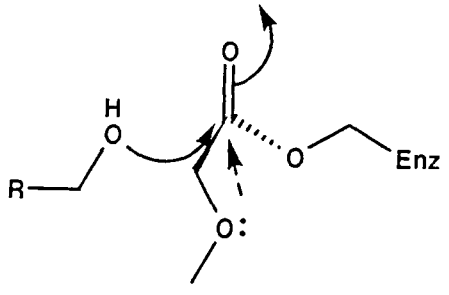
2. **H-Bonding interaction.**

The β-oxygen of the methoxyacetyl group may be involved in a stabilising H-bonding interaction with an acidic functionality at the active site of the enzyme. The methylthio analogue [111] was processed at an intermediate rate between the methoxyacetate and butyrate esters. This is consistent with a reduced capacity for sulfur to enter into hydrogen bonding. If this analysis is correct clearly the oxygen of the methoxypropionate ester is not correctly sited for the H-bonding interaction as a comparable rate enhancement was not observed.



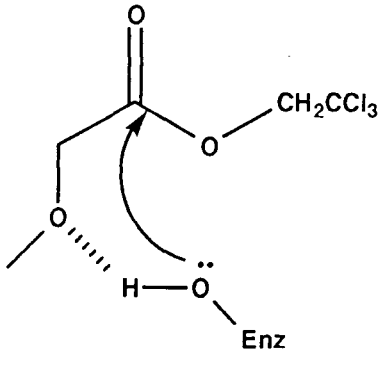
3. **Lone pair donation.**

The β-oxygen may back donate a lone pair thereby lowering the energy of attainment of the tetrahedral intermediate. Data reported by DeTar[196] indicate that the nonenzymatic hydrolysis of ethyl methoxyacetate is 10 times faster than that of ethyl butyrate. If this is the cause of the rate acceleration then the effect should be independent of enzyme source.



4. **General base.**

The β-oxygen may act as a general base correctly sited to hydrogen bond to the hydrogen of the serine hydroxyl group, thus aiding deprotonation and increasing the nucleophilicity of the serine oxygen. Following the argument from paragraph 3 above, the methylthio analogue should be less effective in this regard consistent with the experimental observation. Similarly it could be argued that the oxygen of the methoxypropionate ester is not correctly orientated for such an interaction.



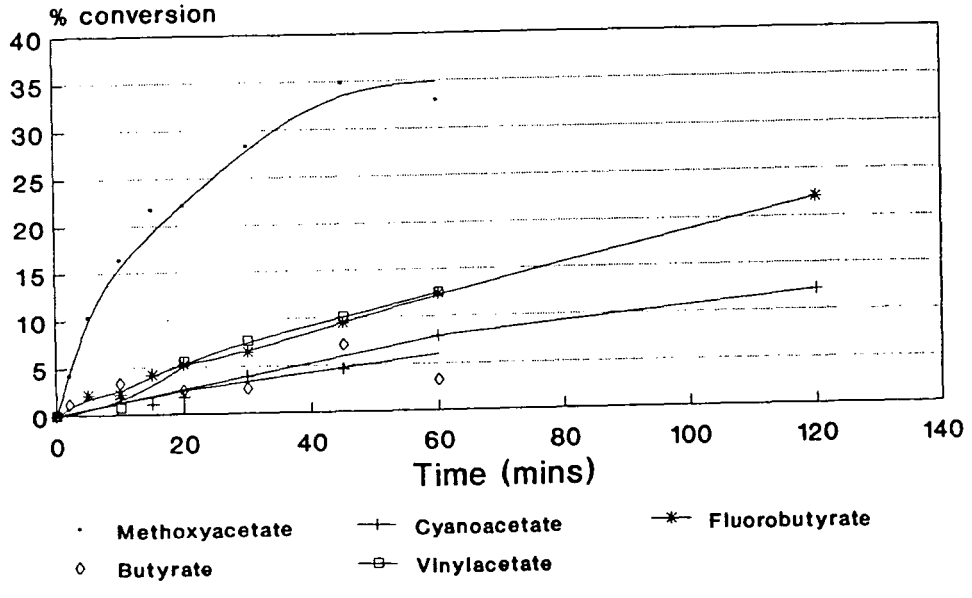
5. Diffusion rates.

The difference in initial rate may be attributed to the relative rates of diffusion of TCEMA and TCEB onto the enzyme. The esters (and alcohols) have to diffuse across the liquid / solid interface following the reaction Scheme highlighted on page 75, where the importance of the diffusion constants to the overall transesterification reaction are illustrated. It can be seen that four of the steps are diffusion controlled.

To probe, to some extent, which of these possibilities, if any, is the cause of the rate enhancement a second series of experiments was conducted with two additional polar esters, ethyl 2-fluorobutyrate and ethyl cyanoacetate. Accordingly ethyl methoxyacetate, ethyl butyrate, ethyl 2-fluorobutyrate and ethyl cyanoacetate were treated under the same conditions as before;

Graph 2.3:

Comparison of Rates of Reaction of a Series of Ethyl Esters with Hexan-1-ol Catalysed by *Candida cylindracea* lipase



Ethyl Ester	Initial Rate $mM.h^{-1}$	Equilibrium conversion
methoxyacetate	0.49	35%
2-fluorobutyrate	0.058	35%
cyanoacetate	0.035	35%
butyrate	0.033	20%
vinyl acetate	0.059	20%

Table 2.4 : Transesterification reactions with a series of ethyl esters with hexan-1-ol in diethyl ether mediated by CCL.

A key aspect involved the preparation of ethyl 2-fluorobutyrate and the presentation of this ester to the enzyme under the same conditions as ethyl methoxyacetate and ethyl butyrate. This experiment was judged important in eliminating some of the possibilities outlined above. Conventional wisdom suggests that the steric demands of the 2-fluorinated ester should not be significantly greater than those of the butyrate ester as the Van der Waals radius of a proton is 1.1Å and that of fluorine is 1.35Å. Thus the fluorine should not introduce significant steric perturbations into the system. However, electronically the fluorine renders the compound significantly more polar. If the rate enhancement of the methoxyacetate system is due simply to a polar effect then a similar acceleration would be expected for the 2-fluorobutyrate ester. The nonenzymatic rates reported by DeTar for the ethyl esters of acetic acid and fluoroacetic acid indicate that the fluoro compound is hydrolysed 100 times faster than ethyl acetate[196].

In the event the 2-fluorobutyrate ester was processed at approximately the same initial rate as the butyrate ester. The ethyl cyanoacetate judged also to be a more polar ester than ethyl butyrate was a very poor substrate. In the study by DeTar[196] the ethyl cyanoacetate was hydrolysed at a similar rate to ethyl 2-fluorobutyrate. Although useful this nitrile group offers a less rigorous comparison with

the methoxyacetate or 2-fluorobutyrate esters.

Vinyl acetate is widely used as an irreversible transacylating agent in preparative bio-transformations[172,177,179] as it leads to complete conversions, (see page 66). In this series it clearly performed very poorly by comparison with methoxyacetate. It was processed at the same rate as ethyl 2-fluorobutyrate with an initial rate of $0.059mM.h^{-1}$. These results clearly indicate that it is the oxygen atom of methoxyacetate rather than the polar effect which is responsible for the observed rate increase.

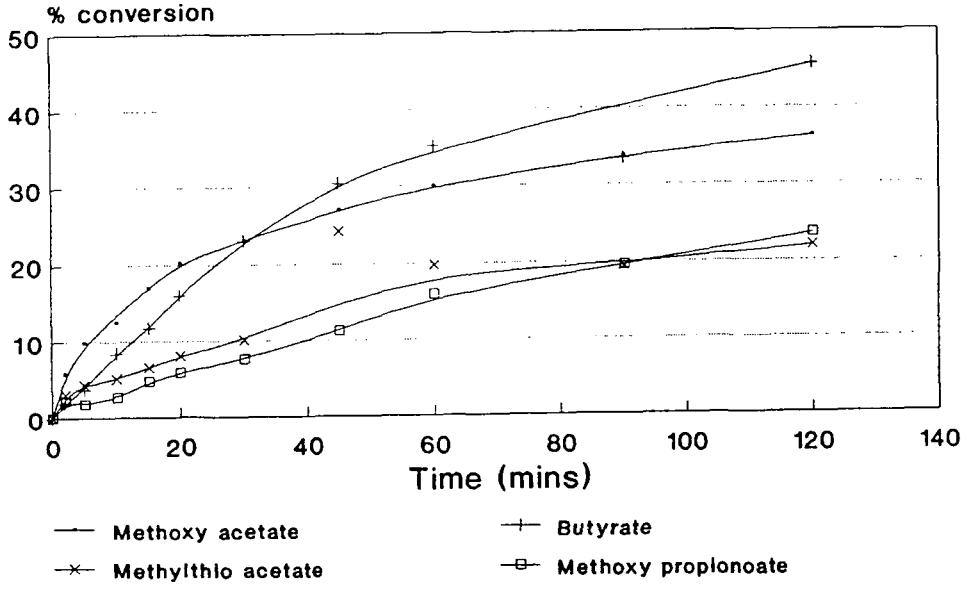
The alternative explanation involving a H-bonding interaction to an acidic functionality at the active site are strongly implicated. If this is correct then the rate enhancement should not necessarily be observed with other enzyme systems, as the active sites will vary from enzyme to enzyme.

To test this hypothesis the four trichloroethyl esters [110] - [113] were employed in a series of transesterifications with hexanol using porcine pancreatic lipase (PPL) as the catalyst. Graph 2.4 summarises these results.

Methoxyacetate was again processed with the fastest initial rate of the four esters. The relative difference with butyrate is now much smaller than for the corresponding reactions with CCL, 1:2.9 for PPL compared to 1:27.1 for CCL. The methylthioacetate and methoxypropionate esters were processed more slowly. Although the effect is not so dramatic, PPL does process methoxyacetate most readily. The active sites of PPL and CCL may have some similarities particularly in the catalytically functional region of the active site. However, this variation would tend to suggest that the rate acceleration with methoxyacetate is not a universal property of lipase catalysed reactions.

Graph 2.4:

Comparison of the Rates of Reaction of a Series of Trichloroethyl esters with Hexan-1-ol Catalysed by Porcine Pancreatic Lipase



TCE ester	Initial Rate $mM.h^{-1}$	Equilibrium conversion
methoxyacetate	0.634	45%
butyrate	0.217	50%
methylthioacetate	0.149	30%
methoxypropionate	0.075	30%

Table 2.5 : Transesterification reactions with a series of trichloroethyl esters with hexan-1-ol in diethyl ether mediated by CCL.

In addition a range of enzymes from the Amano company were screened for their ability to catalyse the reaction of hexanol with TCEMA and TCEB [112] and [110]

It is clear from Table 2.6, page 92, that they varied in their capacity to mediate these reactions. In the cases where a reaction could be detected there was no difference in initial rates between the two acyl donors.

	TCEMA		TCEB	
Enzyme[a]	Initial Rate[b]	Equlibrium	Iniial Rate[b]	Equlibrium
AP-6	Not detectable	12% in 24h	Not detectable	19% in 24h
PS	Not detectable	31% in 24h	Not detectable	54% in 24h
M-Amano-10	Not detectable	0% in 48h	Not detectable	0% in 48h
N-Conc	Not detectable	0% in 48h	Not detectable	0% in 48h
FAB-15	Not detectable	0% in 48h	Not detectable	0% in 48h
PGE-Amano	Not detectable	0% in 48h	Not detectable	0% in 48h
AY-30 (*Candida cylindracea*)	27% in 90min	40% in 24h	23% in 90min	44% in 60h

Notes a) Source AP-6 = *Aspergillus niger*, PS = *Pseudomonas sp*, M-Amano-10 = *Mucor javanicus*, N-Conc = *Rhizopus niveus*, F-AP15 = *Rhizopus oryzae*, PGE-Amano = calf tongue root and salivery gland, AY-30 = *Candida cylindracea*.

b) The reaction was monitored every 5 minutes by GC for the first 2 hours and then every 12 hours.

Reaction conditions : 0.45mM of hexan-1-ol, 0.45mM of ester and 0.3mM t-butylbenzene in dry diethyl ether (5ml). 10^6 units of enzyme was added and the reaction monitored by GC.

Table 2.6 : Transesterification reactions of TCEB and TCEMA with hexanol mediated by a range of Amano enzymes.

These results demonstrate little if any acceleration for the trichloroethyl methoxyacetate over butyrate esters for a range of enzymes. Interestingly an alternative source of the *Candida cylindracea* lipase (Amano AY-30) was also included in this study. This enzyme was indeed the best catalyst of the Amano series, however there was no significant difference between TCEMA and TCEB. This suggests that the *Candida cylindracea* lipase preparation from Sigma and that from Amano are different. In a recent study by Sih and coworkers[197] it was established that the CC lipase exists in two forms (A and B). It is perhaps reasonable to expect that the crude lipase contains both forms, and that different sources contain different ratios. This may partly explain the variations found for the two lipases.

Another possible cause of the rate acceleration highlighted on pages 85 - 88 was back donation by the β-oxygen lowering the energy of attainment of the tetrahedral intermediate. This should only depend on the structure of the ester and therefore the effect would be expected to be independent of catalyst. On analysis the screening of the range of lipases indicates this is not the case suggesting that back donation is not the cause of the rate acceleration.

Another possible cause discussed (paragraph 5, page 88) was that the diffusion rates across the liquid protein interface could account for the difference in observed rates. The diffusion rates across the interface for a given ester would be expected to be independent of the enzyme source as the bulk properties of the proteins will be similar.

These results all point towards preferential binding of the methoxyacetate ester to the CC lipase (Sigma) or alternatively that the oxygen is correctly sited to act as a general base aïding deprotonation of the catalytic serine residue.

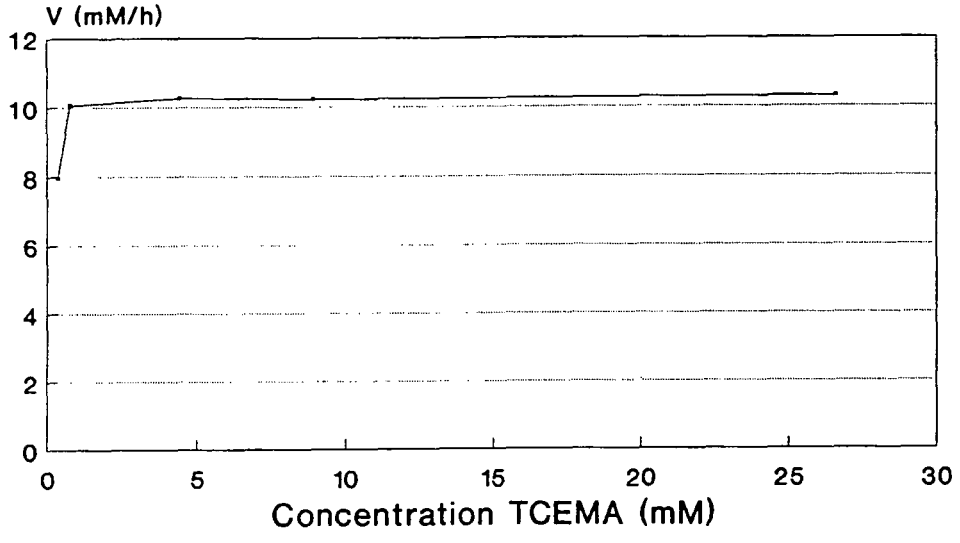
The preferential binding hypothesis would suggest that the K_M of the trichloroethyl methoxyacetate should be much lower than that of the butyrate ester as Km is a function of how well the substrate binds to the enzyme (see page 10, Chapter 1).

In an attempt to determine K_M for TCEMA under the reaction conditions, a plot of initial rate against substrate

concentration (while keeping the enzyme and the hexanol concentrations constant) was plotted. On a plot of this type the value for K_M can be calculated as the concentration, when the rate equals 0.5 x V_{max} (see Chapter 1, page 6). It was not possible to obtain data for the region of the graph where the rate is proportional to the concentration. This was due to the lack of sensitivity of the assay method employed. The data obtained, right down to the limit of detection by the GC method employed, showed the rate to be independent of concentration, ie at V_{max} eg;

Graph 2.5:

Graph of the Dependence of the Rate of Reaction of TCEMA with Hexan-1-ol on the Concentration of TCEMA



[TCEMA] mM	Initial Rate $mM.h^{-1}$
0.36	7.95
0.76	10.05
4.40	10.25
8.90	10.22
26.60	10.30

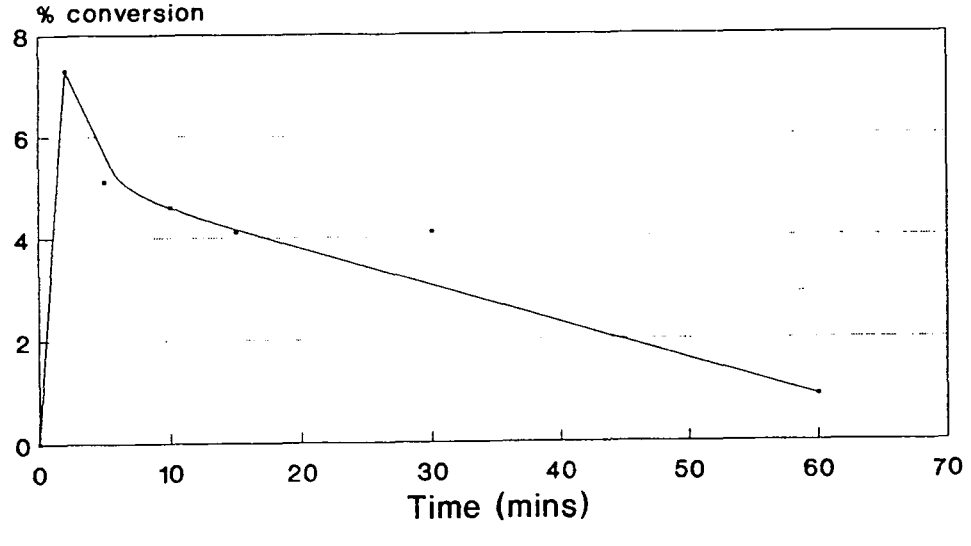
Table 2.7 : Rate dependance on substrate concentration of the transesterification reaction between hexan-1-ol and TCEMA.

From this data all that can be concluded is that the K_M for TCEMA is <0.38mM under these conditions.

An additional complication arose from participation of the bound water. At high substrate concentrations, >4mM, the reverse reaction (hydrolysis of the hexyl methoxyacetate) is not detected, but at very low concentrations of substrate, <0.4mM, hydrolysis becomes a significant factor. In Graph 2.6, which plots %conversion against time, an initially fast forward reaction is obvious, monitored by the decrease in concentration of hexanol. The reaction reaches equilibrium within 2min and then the hexanol concentration starts to rise again, suggesting that the hexanol ester is undergoing hydrolysis. The most likely explanation for this observation is that it is the hydrated water on the surface of the lipase that is participating in this hydrolysis. It is impossible to distinguish between points 2 and 4 (page 87) on these results. Similar data for TCEB proved impossible to obtain as there was no consistent variation in rate with concentration of TCEB.

Graph 2.6:

Graph of Extent of Conversion of the Transesterification Reaction of TCEMA with Hexan-1-ol at an Ester Concentration of 0.36mM



Conclusion.

The choice of the acyl donor on preparative transacylations in organic solvents has a great effect on the initial rate of the reaction and on the ultimate position of the equilibrium. The discovery that trichloroethyl methoxyacetate significantly increases the rate of transesterification reactions and improves the equilibrium conversion, opens up the possibility of much faster kinetic resolutions with the *Candida cylindracea* lipase. Typical resolutions using trichloroethyl butyrate and vinyl acetate can take between several hours to days to reach useful conversions[166,172]. However by employing trichloroethyl methoxyacetate similar conversions can be achieved in minutes to hours.

Trichloroethyl methoxyacetate, has been used in the transesterification reactions of a range of primary, secondary and tertiary alcohols and these will be discussed in later chapters.

Significant increases in rates are demonstrated for a series of secondary alcohols. The overall rate for reaction of many of the alcohol substrates affords the possibility of conducting transesterifications mediated by *Candida cylindracea* lipase at or below 0°C (see Chapter 3). Low temperature reactions are not usually an option as the rates become too slow. However, it is demonstrated that several heterocyclic alcohols, eg 2-furanethanol, 2-thiopheneethanol and 2-pyridylethanol exhibit useful initial rates and equilibrium conversions down to -15°C. These results are discussed in detail in Chapter 4.

Chapter 3

Variation of Solvent and Temperature

A. 1. Effect of Solvent on Reaction Kinetics

Introduction.

Once it had been established that lipases would function with significant activity in anhydrous organic solvents the next logical step was to explore how the nature of the solvent affected the rate, equilibrium, substrate specificity and enantioselectivity of the lipase catalysed transesterification. There have been a number of studies on the effects of solvent on lipase catalysed transesterification[160-161]. The data published so far can be summarised as follows;

For maximal catalytic activity hydrophobic solvents should be used. The enzyme requires a minimum level of water bound to its surface[162] and hydrophobic solvents are less able to strip this essential water. This has been discussed in detail in Chapter 1 (page 60). The conclusions drawn are that all enzymes require water but that the absolute level varies from enzyme to enzyme. For more polar solvents an acceptable level of activity can be achieved if a certain amount of water is added to the anhydrous solvent (still below the solubility limit). This addition of water suppresses the ability of the solvent to take up water from the protein. Halling[198] has shown that for consistent results for a range of solvents the reactions must be conducted at the same water activity, a_w, which is defined as 1 when the solvent is saturated with water. The value for water activity can be considered as the fractional saturation of the solvent. The more hydrophilic the solvent the more water is needed for a fixed water activity. Solvent profiles for a number of enzymes have been reported[160a,161a]. For example for

the reaction of N-acetyl-L-phenylalanine ethyl ester with propan-1-ol, mediated by subtilisin Carlsberg and α-chymotrypsin, Table 3.1 lists the efficiency of the enzyme (V/Km) for a range of solvents in decreasing order of hydrophobicity.

	V/Km_{ester} (min^{-1} x 10^6)	
Solvent	substilin	α-chymotrypsin
hexadecane	3900	4300
octane	2000	1700
carbon tetrachloride	340	96
butyl ether	240	48
toluene	150	120
t-amyl alcohol	2100	38
diethyl ether	97	48
2-pentanone	59	12
pyridine	97	<0.1
THF	120	7.2
acetone	810	0.6
acetonitrile	150	0.4
dioxane	9.2	0.2
DMF	19	<0.1
DMSO	<0.1	<0.1

Table 3.1 : Capacity of various solvents to support a transesterification reaction between N-acetyl-L-phenylalanine and propan-1-ol mediated by subtilisin and α-chymotrypsin[160a].

Table 3.1 clearly shows a decreasing ability of the solvent to support the reaction, catalysed by α-chymotrypsin, with increasing hydrophilicity. The reaction catalysed by subtilisin shows a similar trend but there are a significant number of exceptions eg. t-amyl alcohol supports a reaction equivalent to that of octane. Also acetone, THF and acetonitrile appear to be out of sequence.

For the transesterification reaction between phenethan-1-ol and vinyl butyrate, mediated by porcine pancreatic lipase, there appeared to be no correlation between solvent hydrophobicity or dipole moment, and the ability to support the transesterification reaction[161a], (see Table 3.2). Consistent with previous studies however the best solvent was cyclohexane. Interestingly nitromethane and acetonitrile performed as well as benzene at supporting this reaction. This is clearly an unexpected observation in view of the polarity of these solvents.

Solvent	Initial Rate $mM.h^{-1}$
nitromethane	9.7
DMF	2.3
triethylamine	4.2
t-amyl alcohol	6.9
butanone	8.6
acetonitrile	14.0
benzene	10.0
cyclohexane	43.0
decane	5.5

Table 3.2 : Effect of solvent on the initial rate of transesterification reaction of phenethan-1-ol with vinyl butyrate[161a].

Objective.

It was of clear interest to assess the effect of the solvent on reactions with *Candida cylindracea*. The transesterification of TCEB with heptan-2-ol was taken as a reference reaction and was conducted under a standard set of conditions using a series of anhydrous organic solvents.

Results and Discussion.

The reactions in each case followed standard Michaelis-Menten kinetics but the initial rate varied significantly with solvent. As expected the initial reaction rate was fastest with the most hydrophobic solvents while solvents that were completely miscible with water showed no activity at all;

Solvent	Dipole Moment	Dielectric Constant (debyes)	Polarity (logP)[a]	Initial Rate ($mM.h^{-1}$)
hexane	0.08	1.9	-	0.53
cyclohexane	0	2.02	0.0	0.53
toluene	0.37	2.38	0.55	0.53
benzene	0	2.3	0.8	0.21
diethyl ether	1.15	4.3	1.25	0.21
2-bromobutane	2.23	8.64	-	0.21
carbontetrachloride	0	2.2	0.5	0.13
chloroform	1.85	4.7	2.3	0.06
DMF	3.86	37.2	2.25	0.04
dichloromethane	1.6	36	2.35	0.02
acetonitrile	3.2	36.02	2.3	0
THF	1.63	7.3	1.45	0
acetone	2.88	20.2	-	0

notes. a) Log P = log of the partition coefficient between octanol and water.

Table 3.3 : Correlation of solvent properties with their ability to support the transesterification reaction between heptan-2-ol and TCEB mediated by CCL.

These observations agree well with those in other systems, (see Table 3.1, page 98) and these data suggest that the effect of the solvent is consistent for a range of enzymes.

In general the greater the dielectric constant the slower the reaction, however some anomalies exist. For example it would be expected that carbon tetrachloride, with a dipole moment of zero and low dielectric constant, should be higher up the Table above toluene. For the reaction catalysed by subtilisin, Table 3.1, carbontetrachloride is indeed a better solvent than toluene. THF is also anomolous. THF and 2-bromobutane have similar dielectric constants but 2-bromobutane supports an initial rate of $0.21 mM.h^{-1}$ whereas THF did not support any reaction under these conditions. This can be rationalised to some extent by taking into account the relative hydrogen bonding capacity of the two solvents. THF can hydrogen bond to water and is therefore able to strip the enzyme of its hydration layer and thus deactivate it. 2-Bromobutane, on the other hand, is less able to participate in hydrogen bonding and so supports an activity higher than its dielectric constant would suggest.

2. Effect of Solvent on Enantioselectivity

Introduction.

In the recent literature there have been a number of reports on the effect of the solvent on enantioselectivity. In some cases the selectivity can change significantly[161a], and it has been reported that the solvent can even reverse the selectivity[161b]. These changes in magnitude of enantioselectivity have been argued by Klibanov[161b] to be due to the result of a relaxation of the conformational rigidity of the enzymes 3-D structure. For example in the reaction of phenethan-1-ol with vinyl acetate, catalysed by subtilisin Carlsberg, the enantiomeric ratio E, was a function of the dipole moment, with those solvents with low dipole moment performing best[161a].

Solvent	Enantioselectivity $(k_{cat}/Km)_S/(k_{cat}/Km)_R$
dioxane	61
benzene	54
triethylamine	48
THF	40
pyridine	31
DMF	9
nitromethane	5
methylacetamide	3
acetonitrile	3

Table 3.4 : Enantioselectivity of the transesterification reaction of phenethan-1-ol with vinyl acetate mediated by subtilisin in various solvents[166a].

More recently a similar correlation has been reported for the *Candida cylindracea* lipase[161b]. The reaction of 2-(4-chlorophenoxy)propionic acid with butan-1-ol was conducted in a range of solvents and the %ee of the substrate and the product were assessed along with the %conversion. From this data a measure of the overall enantioselectivity, E (enantiomeric ratio), of the process could be calculated (see page 78 for an explanation of how this is calculated). Table 3.5 summarises the data from these experiments.

Solvent	Enantioselectivity, (E)	
isooctane	1.4	(R)
cyclohexane	1.4	(R)
hexane	1.1	(R)
toluene	1.2	(R)
dichloromethane	0.25	(S)
ethyl acetate	0.32	(S)
butanol	0.38	(S)
THF	0.25	(S)
acetone	0.24	(S)
phosphate buffer	24	(R)

Table 3.5 : Reversal of enantioselectivity of the transesterification reaction between 2-(4-chlorophenoxy)propionic acid and butan-2-ol catalysed by CCL mediated by change of solvent[166b].

The data clearly show a significant effect on the magnitude of E. Moreover, as E is calculated from the ratio of $(k_{cat}/Km)_R/(k_{cat}/Km)_S$, then those reactions with values for E greater than 1, show selectivity for the opposite enantiomer than for those where the value for E is less than 1.

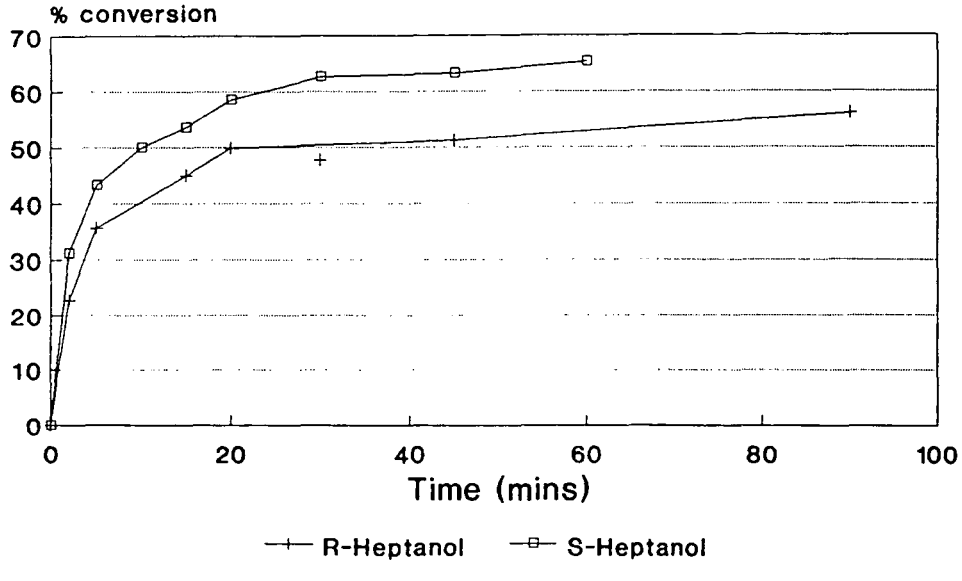
Objective.

To explore the effect of solvent on the lipase catalysed transesterification with CCL. The reactions of heptan-2-ol with TCEMA and TCEB were compared in diethyl ether and in chloroform. Optically pure samples of (R) and (S) heptan-2-ol were studied separately to assess the rates for each enantiomer. The data are summarised in Graphs 3.1 to 3.4.

Results and Discussion.

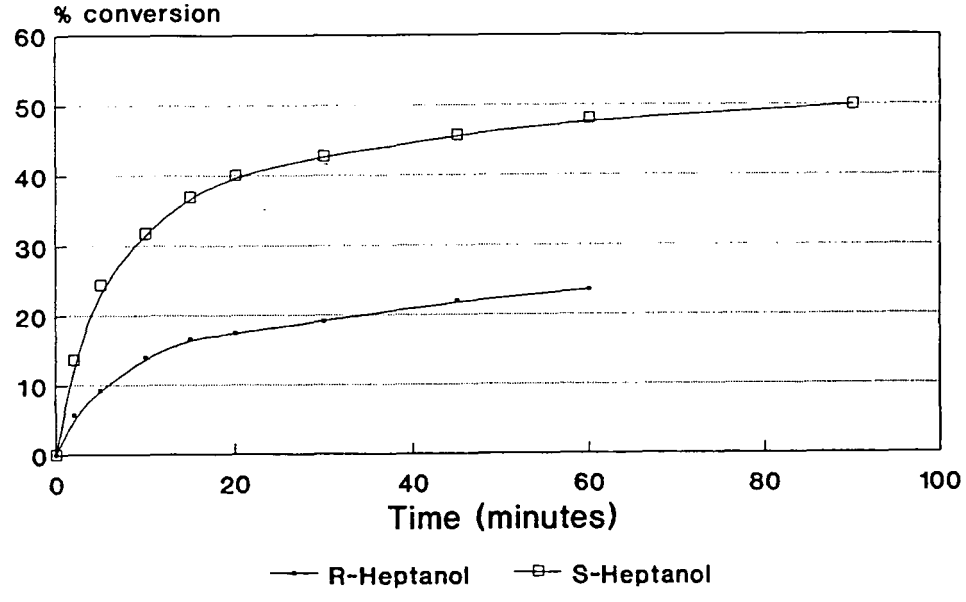
Graph 3.1:

Comparison of (R) and (S) Heptan-2-ol
Transesterification Reactions with
TCEMA in Diethyl Ether



Graph 3.2:

Comparison of (R) and (S) Heptan-2-ol
Transesterification Reactions
with TCEMA in Chloroform



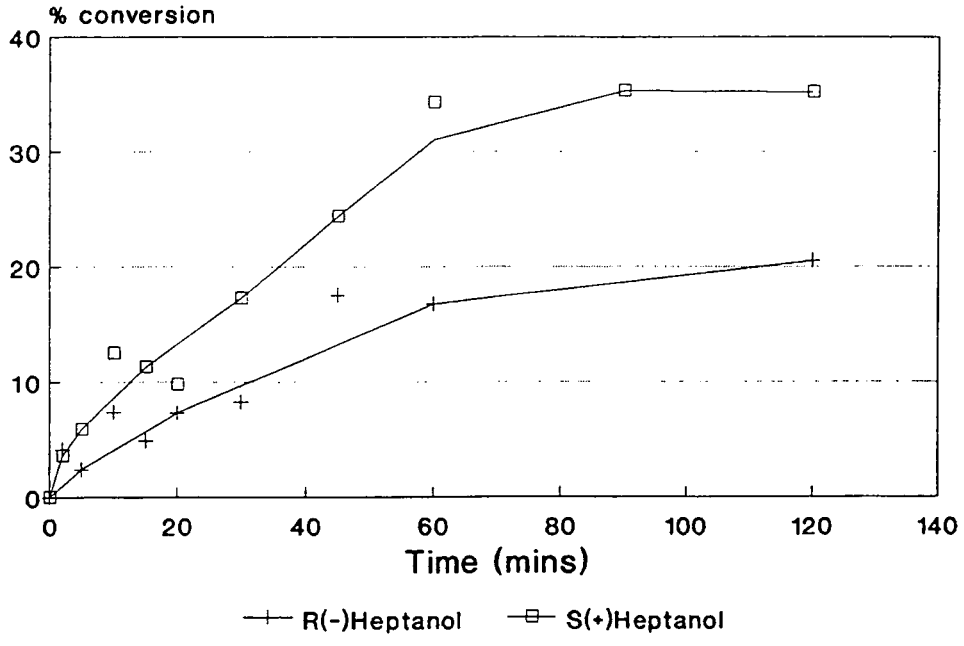
The overall rate of the reaction in chloroform is much slower than in diethyl ether for the S enantiomer. This is consistent with the solvent profile experiments, outlined in section A. The difference in rates of the two enantiomers however increases significantly ie V_S/V_R for the reaction in diethyl ether = 1, and can be compared to 3.2 for the reaction in chloroform. These results are in contradiction to those reported by Klibanov[161a] where the data suggest that solvents with a low dipole moment, support high enantioselectivity. It was suggested[161a] that this is due to a relaxation in the conformational rigidity of the enzyme.

This relaxation may reduce the steric constraints of the enzyme active site, thus reducing the energetic differences in the two diastereotopic transition states, (see Chapter 2 page 76). The dipole moment of diethyl ether is 1.15D and that for chloroform is 1.85D and so in accordance with the reported data diethyl ether should support the higher enantioselectivity. An alternative explanation is that the chloroform does indeed cause a relaxation of the conformational rigidity, but the drop in catalytic activity may be the result of the loss of activity of one of the enzyme conformers. As has been stated previously, (Chapter 2, page 93) there are at least two enzyme conformers, A and B, in a crude preparation of the *Candida cylindracea* lipase. It has been shown that one of these conformers, Lipase A, possesses the better enantioselectivity[197]. The loss of activity in chloroform may be due to differential deactivation of Lipase B with respect to A, thus accounting for the observed increase in enantioselectivity. There is no additional data to support this hypothesis and further work is required. At present the information is useful when choosing the optimal conditions for any lipase resolutions.

An analogous increase in enantioselectivity is observed with the reactions conducted with TCEB [110] with a similar marked decrease in reaction rate. The TCEB reaction in diethyl ether shows a very small difference in the initial rates of the two enantiomers, V_S/V_R = 1.27 with the same reaction in chloroform giving V_S/V_R = 2.32. The rate of the reaction in chloroform is, however, very slow and the reaction approaches equilibrium at only 18% conversion.

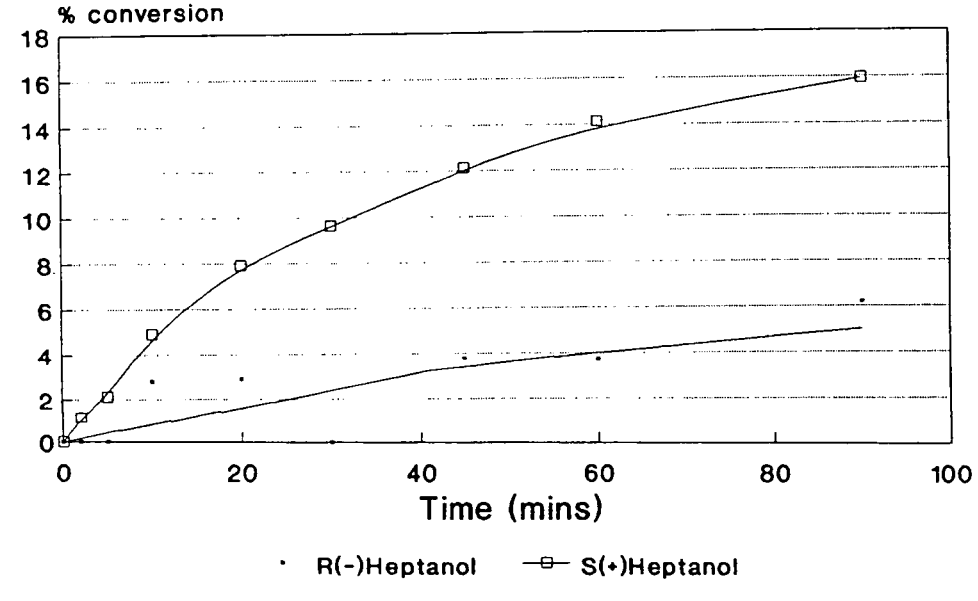
Graph 3.3

Comparison of (R) and (S) Heptan-2-ol
Transesterification Reactions
with TCEB in Diethyl Ether



Graph 3.4

Comparison of (R) and (S) Heptan-2-ol
Transesterification Reactions
With TCEB in Chloroform



Conclusion.

This solvent profile study reinforces, in a general way, the studies with other enzyme systems, and further establishes ground rules for selection of optimal the reaction conditions. The results from the solvent effects on the enantioselectivity of the transesterification reactions are more difficult to rationalise but they again allow determination of the optimal choice of solvent for a particular reaction.

The data suggest that the reaction with TCEB as the acyl donor is severely limited owing to the very slow reaction rates and the low equilibrium conversions. The use of vinyl acetate (as illustrated in Chapter 2, page 88) is also limited due to sluggish reactions and deactivation of the enzyme by acetaldehyde produced during the reaction[177,178]. The development of the methoxyacetyl acyl donor and selection of appropriate solvent has opened up a range of possibilities. For example it may be possible to conduct reactions with substrates that are too slow with other acyl donors and to use solvents that are less than ideal, but that have the advantage of increasing the enantioselectivity.

B. Effect of Temperature on Reaction Kinetics and Enantioselectivity.

Introduction.

Enzymes as biological catalysts are optimal at the environmental temperature of the organism. Therefore mammalian enzymes operate at physiological temperature and for plant, fungi and micro-organisms the metabolic enzymes function at the ambient temperature of the organisms. Anything beyond these ranges may have deleterious effects on the catalytic efficiency of the enzyme, as reaction kinetics have a temperature dependence. The substrate specificity and the enantioselectivity both depend on the conformational rigidity of the enzyme and changes in temperature may have an effect on the rigidity, by varying the strength of the non-covalent interactions that hold the protein in its 3-dimensional conformation.

For enzymes in aqueous media the range of temperatures is very limited. In general, at temperatures above 45°C the enzyme is denatured by hydrolytic processes that destroy the tertiary structure of the protein. Below 0°C the media freezes thus preventing any catalytic activity. If the media is changed to one that is largely anhydrous, eg. dry organic solvents, then both these limitations are removed and the temperature profile can be studied over a much wider range. For example lipase reactions have been studied up to 100°C in organic solvents[163]. By drying the enzyme powder before use and conducting the reaction in anhydrous tributyrin as the solvent, porcine pancreatic lipase retained its catalytic activity up to 100°C in transesterification reactions[163b]. In another study[163c], the use of dry organic reaction media rendered the lipase from *Pseudomonas cepacia* thermostable for 100h at 100°C just by drying the enzyme powder under vacuum[163c]. Not all enzymes are made thermostable by drying however, and it had been reported previously that drying of the *Candida cylindracea* lipase rendered it completely inactive[163c].

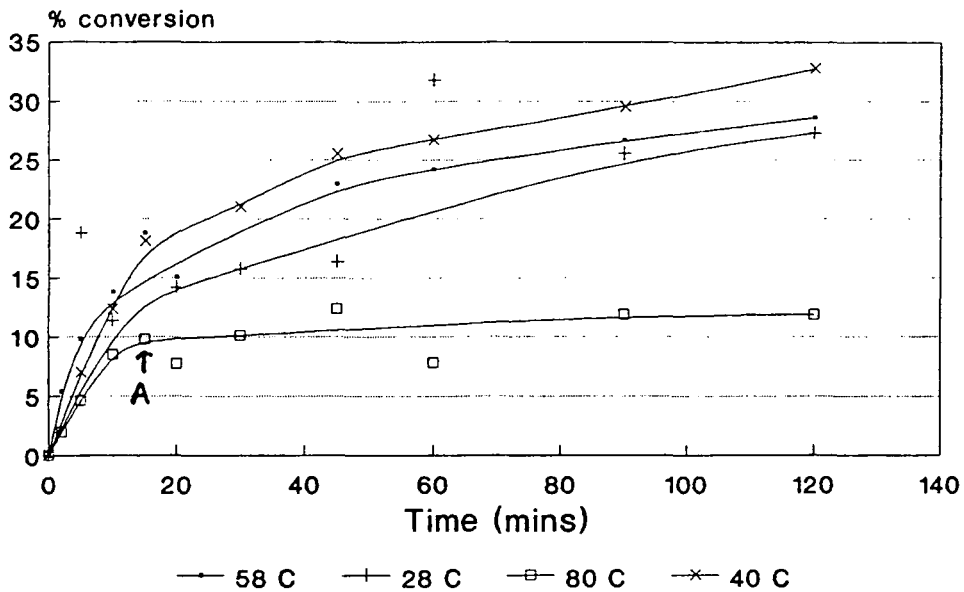
Results and Discussion.

In order to assess the range of temperatures over which the CCL will operate a standard system was chosen. The reaction between TCEB and heptan-2-ol was conducted in cyclohexane at various temperatures as illustrated in Graph 3.5. The data indicate that the enzyme can tolerate a significant increase in temperature with the change to non aqueous solvents. The initial rates of the reaction over this temperature range varied to give a temperature profile shown in Graph 3.6. The optimal temperature is 45°C.

The extent of conversion at equilibrium varies with temperature. At 80°C the reaction stops abruptly after only 12% conversion (see arrow A on Graph 3.5), however, this is probably due to deactivation of the enzyme by the hydrated surface water. It is possible that this water is participating in hydrolytic reactions which deactivate the enzyme. At 28°C, 40°C and 58°C the reactions all continue to much greater conversions, eg in 2h the reaction at 28°C reached 27%, at 40°C it reached 33% and at 58°C it reached 29%. These % conversions demonstrate that the enzyme is still active after 2h at these temperatures. At 80°C the enzyme survives for only a few minutes. In an attempt to improve this the enzyme was dried under vacuum and the reaction at 80°C was repeated, however, there was no reaction and it would appear that the enzyme had lost all its activity after dehydration. This is consistent with the results previously reported by Klibanov[163c], who observed a similar loss of activity with *Candida cylindracea* after drying *in vacuo*.

Graph 3.5

Effect of Raised Temperature on the Transesterification Reaction Between Heptan-2-ol and TCEB in Cyclohexane

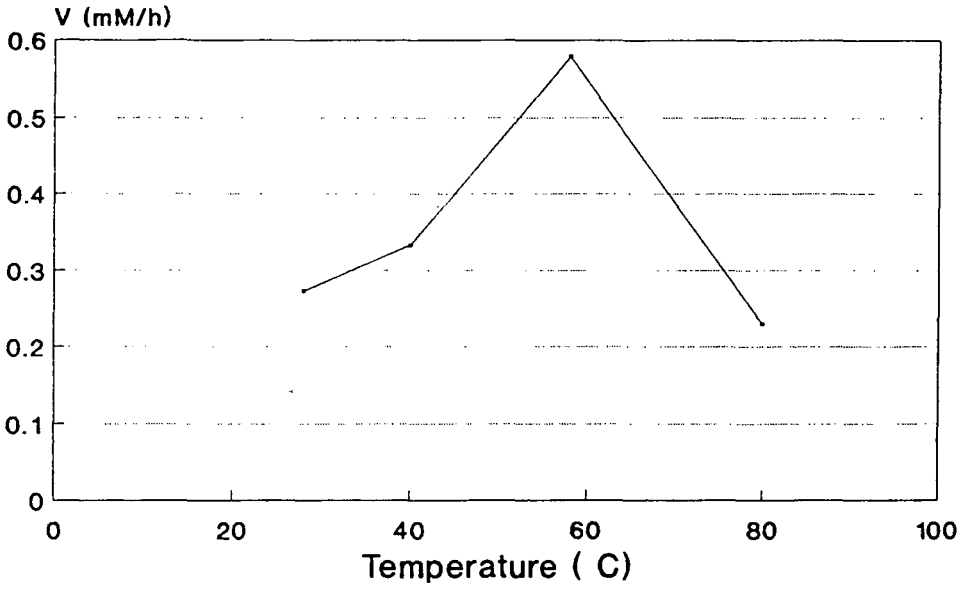


Temperature (°C)	Initial Rate $mM.h^{-1}$
28	0.273
40	0.333
56	0.579
80	0.230

Table 3.6 : Temperature profile of the transesterification reaction of heptan-2-ol with TCEB

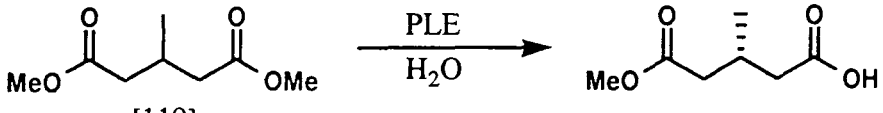
Graph 3.6

Graph of Initial Rate Versus Temperature
(From Data in Graph 3.5)



Introduction.

At low temperatures the use of TCEB as the acyl donor renders the lipase catalysed reaction rates too slow to be of any practical use for enantiomeric resolutions. Accelerations using TCEMA, the acyl donor discovered during this research, display a significant increase in rate over that of TCEB. This opens up the possibility of achieving very low temperature reactions. The low temperature reactions so far studied have been limited to 0°C. This is due to the generally sluggish rates of the transesterification reactions in organic solvents[166a]. There has been one report of a resolution conducted at a temperature below zero[163b]. Methanol was added as an antifreeze to an aqueous hydrolysis of dimethyl 3-methyl-1,5-pentanedioate [119] mediated by pig liver esterase (PLE) and the reactions conducted down to -10°C.



[119]

Solvent	Temperature	%ee
Buffer	20°C	79
Buffer + 5%MeOH	20°C	88
Buffer + 10%MeOH	20°C	92
Buffer + 5%MeOH	0°C	93
Buffer + 10%MeOH	0°C	93
Buffer + 20%MeOH	-10°C	97

Table 3.7 : Effect of addition of co-solvent and low temperature on the enantioselectivity of the PLE catalysed hydrolysis of [119][163b].

At 20°C and with no co-solvent the reaction afforded the monoester in 79%ee. The addition of a co-solvent marginally improved the enantioselectivity of the hydrolysis, eg 5% DMSO afforded the monoester in 81%ee. The addition of 5% methanol further improved the selectivity to 88%ee. Thus addition of a co-solvent allowed the possibility of studying the reaction below 0°C. With 5% methanol as an antifreeze the enantioselectivity of this reaction improved from 88%ee at 20°C to 92%ee at 0°C. The addition of 20% methanol allowed the temperature to be lowered to -10°C giving a resolution of 97%ee. This improvement in enantioselectivity was almost certainly due to the addition of the co-solvent (methanol) rather than the temperature. This can be deduced as the %ee improves from 79%ee to 92%ee when 10% methanol is added at 20°C. The lowering in temperature would appear, however, to have only a small beneficial effect on the enantioselectivity of this reaction.

Objective.

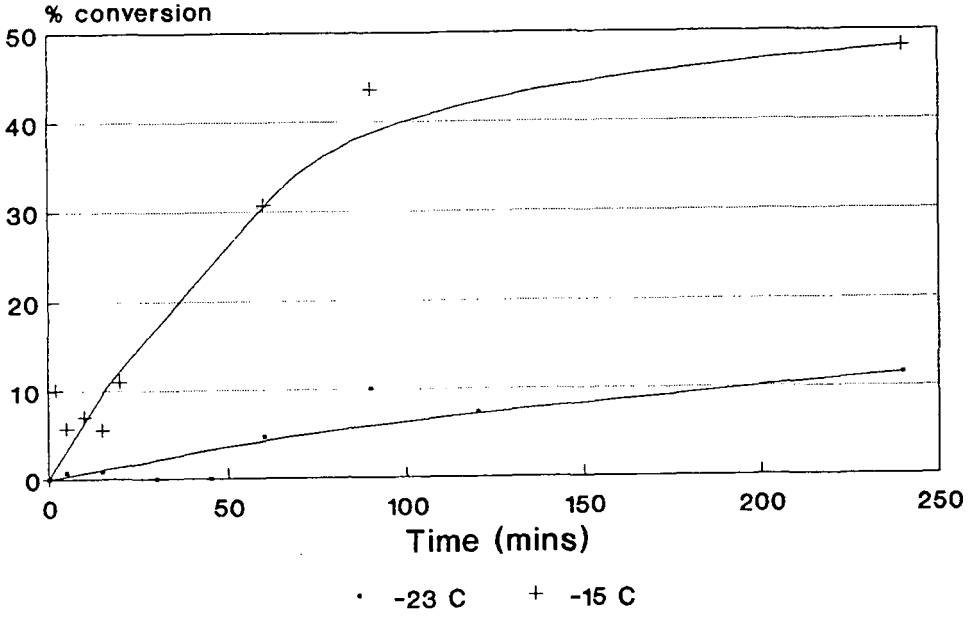
The results discussed in Chapter 2 have demonstrated that the transesterification reaction between hexan-1-ol and TCEMA is a fast reaction which reaches equilibrium in 2min. In this section the effect of lowering the temperature was assessed on the kinetics of this reaction and on that of TCEMA with heptan-2-ol.

Results and Discussion.

To study the ability of the *Candida cylindracea* lipase to catalyse reactions at reduced temperature the primary alcohol hexan-1-ol was reacted with TCEMA in diethyl ether. The reaction proceeded at a measurable rate down to -23°C, see Graph 3.7. When the substrate was changed to the secondary alcohol heptan-2-ol the minimum temperature that would allow a measurable rate was -23°C, see Graph 3.8.

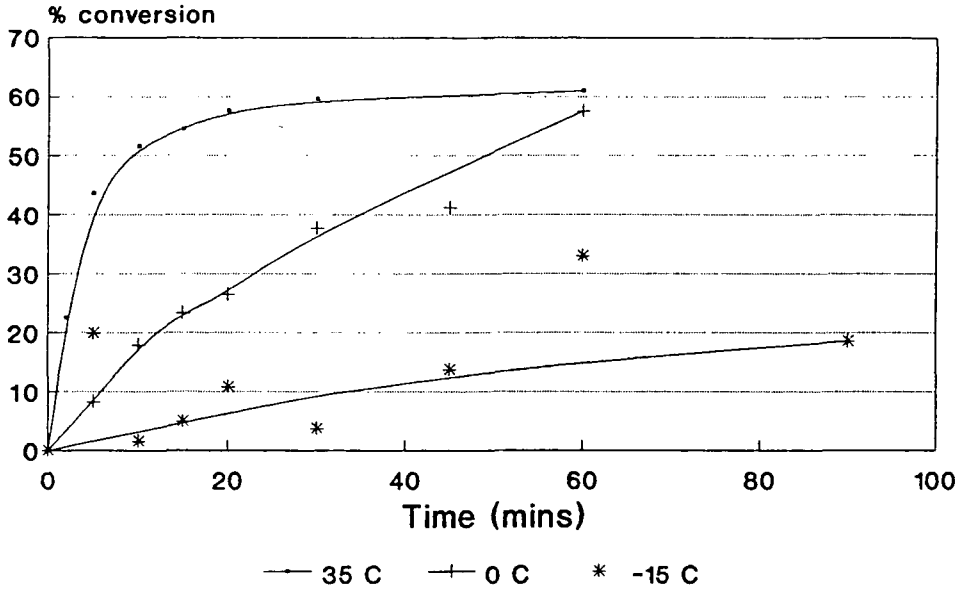
Graph 3.7

Effect of Reduced Temperature on the
Transesterification Reaction of
Hexan-1-ol with TCEMA in Diethyl Ether



Graph 3.8

Effect of Reduced Temperature on the
Transesterification Reaction of
Heptan-2-ol with TCEMA in Diethyl Ether



This is the first time that enzyme transesterification reactions have been observed at such temperatures. However they arise from a unique combination of reagents. The rate acceleration afforded by the methoxyacetate ester over the butyrate ester, in conjunction with the *Candida cylindracea* lipase, have allowed these reactions to be conducted at such low temperatures.

In general low temperature reactions are not attractive for industrial processes as the reactions have to be cooled, however there are potential advantages in conducting

reactions below zero. The possibility exists to resolve unstable chiral molecules and volatile substrates that, generally, can only be handled at low temperatures.

The notion that the enantioselectivity will be affected by varying the temperature is based on the conformational rigidity of the enzyme. It may be expected that at low temperature the enzyme will become more rigid owing to the decreased thermal motion of the system and the consequent increase in strength of the non-covalent interactions that hold the 3-D structure in place. In order to assess the effect of low (and high) temperature on enantioselectivity, two transesterification reactions were investigated. These were the transesterification of o-pyridylethan-1-ol and 2-thiopheneethan-1-ol with TCEMA and they are discussed in Chapter 4. The overall conclusion however, was that there was no beneficial effect in lowering the temperature on the enantioselectivity and indeed there was very little change in %ee over the entire temperature range of enzyme activity.

Conclusion.

The use of organic solvents as the media for transesterification reactions allows the possibility of using extreme temperatures for conducting these reactions. It has been demonstrated that *Candida cylindracea* lipase will tolerate temperatures from 80°C to -23°C when placed in anhydrous media with an optimal temperature of 55 - 60°C. The tolerance of elevated temperatures allows for the possibility of accelerating particularly sluggish substrates. The observation that the lipase catalysed reactions with CCL and TCEMA can be conducted at such low temperature is novel and of interest. The notion that enzymes will function below zero is itself surprising and opens up many interesting areas of investigation. This is the first time that enzyme mediated reactions at such low temperatures have been reported, although this affect arises from a unique set of circumstances involving a

particular enzyme and range of substrates. Having established such a system it was of clear interest to assess the effect of lowering the temperature on the enantioselectivity of the transesterification reaction with a range of secondary alcohols. This is discussed in the next Chapter.

Chapter 4.

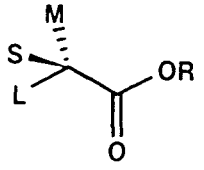
Structure Activity Relationships With the *Candida cylindracea* Lipase.

A. Reaction Kinetics.

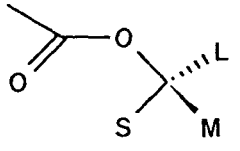
Introduction.

A wide range of chiral alcohols and acids have been resolved in transesterification reactions using the lipase from *Candida cylindracea*[166] (see Chapter 1). The majority of reports in the literature have exploited an enzymatic transformation as a means of obtaining a single enantiomer of specific classes of compounds for synthetic intermediates. It is common practice therefore to screen a range of enzymes to achieve the desired resolution and specific structure activity studies are not often highlighted.

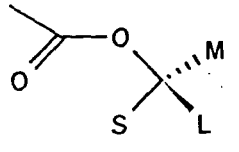
The wide range of substrates presented to the *Candida cylindracea* lipase has led to several models of the active site being proposed to predict the absolute configuration of the preferred enantiomer[199]. For example it would appear that the preferred enantiomers of chiral esters can be generally summarised on steric grounds by the following model;



On the other hand the situation is less clear for chiral alcohols. In general most resolutions conform to the steric model A. However, when a powerful electron withdrawing group such as CF_3 is involved, steric model B appears more appropriate.

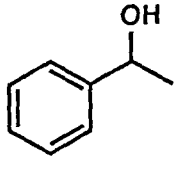


Steric Model A

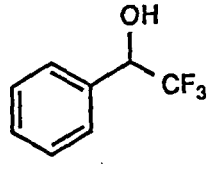


Steric Model B

Model B, proposed by O'Hagan and Zaidi[199a], was used to account for the reversal in enantiomeric preference when switching from phenethan-1-ol [120] to α-trifluoromethylbenzyl [121] alcohol. This model has also proved useful in accounting for the stereochemistry of a range of tertiary alcohols containing a CF_3 group[199a]. These models have been derived on steric grounds, however Model B may be controlled by electronic factors.



[120]



[121]

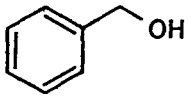
There have been a number of reports of the 3-D structure of lipases determined after crystallographic studies[189]. For the purpose of substrate specificity however, this data is limited and does not give any indication of the effects of small changes in substrate structure and the sequence of

reactivity within a homologous series. From the crystallographic study on *Geotrichum candida* lipase it would appear that the active site is buried in a cleft[189b], (see page 72 Chapter 2). In order to probe the steric limitations of the active site of CCL and probe the possible existence of a cleft, a series of phenyl alcohols was presented to the enzyme. This study was then extended to a number of heterocyclic primary alcohols. From the studies discussed earlier the presence of an oxygen in the β-position of the acyl donor, appears to have a dramatic effect on the overall rate of transesterification. It was of interest to assess if a such a phenomenon could be found on the alcohol side of the substrate. To this end o-, m- and p- pyridylcarbinols ([126], [127] and [128]) and 2- and 3-thiophene- ([129] and [130]) and furan- methanols ([131] and [132]) were presented to the enzyme under a standard set of conditions.

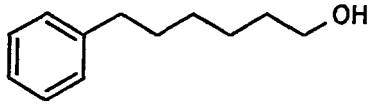
Results and Discussion.

1. Effect of Alkyl Chain Length on Reactivity.

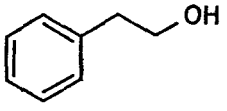
The ability of CCL to mediate hydrolysis reactions has been shown to depend on alkyl chain length[72]. In this study the hydrolysis of methyl 2-chloropropionate proceeded with no enantioselectivity, whereas the hexyl ester proceeded with an enantioselectivity >95%ee. It was therefore of interest to assess the ability of the enzyme to accept extended alkyl chains in transesterification reactions in organic media. Accordingly a range of phenyl alcohols were presented to the enzyme under a standard set of conditions and the rates for each were determined in transesterification reactions with TCEMA.



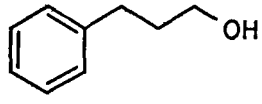
[122]



[123]



[124]



[125]

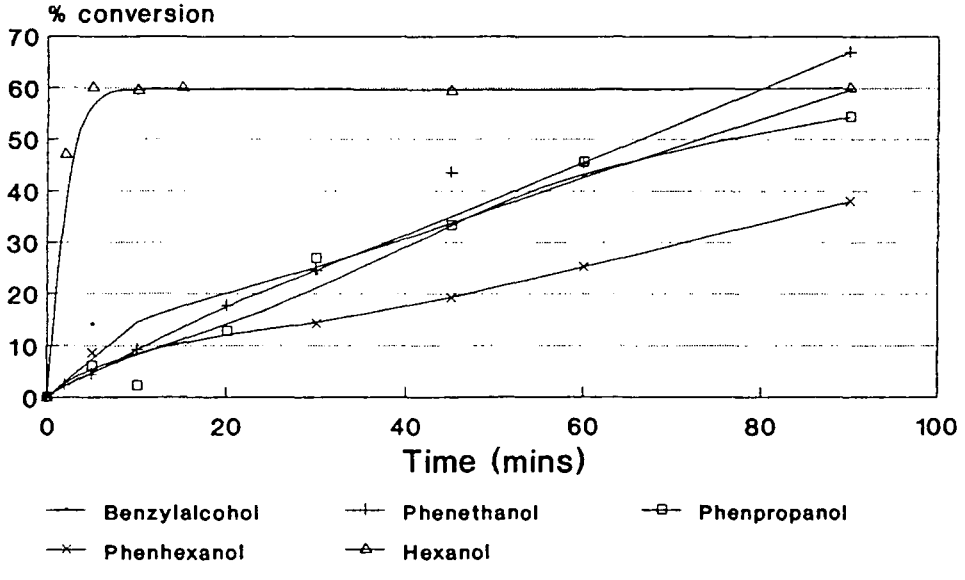
The data, which is summarised in Table 4.1 and Graph 4.1, clearly shows there is no significant difference in initial rates of the 2-phenylethanol [124], 3-phenylpropan-1-ol [125] and 6-phenylhexan-1-ol [123]. Benzyl alcohol [122] is a slightly better substrate but hexan-1-ol emerged as the best substrate and is processed an order of magnitude faster than the other alcohols.

Alcohol	Initial Rates $mM.h^{-1}$	Equilibrium conversion
hexan-1-ol	8.87	60%
benzyl alcohol	0.42	80%
2-phenethanol	0.23	80%
3-phenpropan-1-ol	0.23	80%
6-phenhexan-1-ol	0.23	60%

Table 4.1 : Effect of extending the alkyl chain on the rate of transesterification of a range of phenyl alcohols with TCEMA mediated by CCL.

Graph 4.1

Comparison of Transesterification of a Series of Phenylalcohols with TCEMA in Diethyl Ether.



It would appear that the presence of a phenyl group in the substrate has a deleterious effect on the rate but not on the equilibrium conversions. A number of reasons could account for this, the most obvious being that the substrates have difficulty penetrating the active site because of steric constraints.

If, as is suggested by the crystal structure reported by Schrag[190] for the GC lipase, the active site is buried in a cleft then it may place more steric constraints on the phenyl alcohols than on the hexanol. If, by extending the alkyl chain the hydroxyl group can react at the active site without the phenyl ring entering the interior of the enzyme then longer chain alcohols should be progressively more reactive within the same homologous series. The data from Graph 4.4 show no such progression.

Alternatively, as the natural substrates for lipase are triacylglycerols, it may be expected that the lipase has an active site configuration that will accommodate the entire length of the fatty acid chains. The active site of CC lipase has not been studied in detail, however, one study by Bhalerao[79] lends weight to the hypothesis that there is space for an extended aliphatic chain and also a hydrophilic binding site at a distance of 8 carbons from the reaction centre. It may be that the hydrophobic phenyl ring of these alcohols interferes with this binding site countering any benefit of extending the chain length.

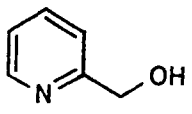
An alternative explanation which could account for the poor reactivity of the longer phenyl alcohols is that they diffuse more slowly through the hydrated water layer on the enzyme surface. Consistent with the rate data, the longer chain alcohols are more hydrophobic than for example benzyl alcohol. Thus, the reactivity sequence may be a combination of unfavourable steric effects and slower rates of diffusion onto the enzyme.

2. Reactivity of Various Primary Heterocyclic Alcohols

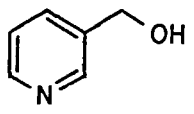
In Chapter 2 it has been shown that the effect of an oxygen atom β to the ester carbonyl increases the rate by an order of magnitude over a CH_2 in the same position. It was deduced that this effect was most likely due to a hydrogen bonding interaction with an acidic group at the active site, increasing the affinity of this ester for the enzyme, (see page 86 Chapter 2). It was of clear interest to assess the effect of similar variations in the alcohol. A range of heterocyclic primary alcohols were presented to the enzyme under a standard set of conditions, (Dig 4.2), to probe the effect of variolously sited hetero atoms on the reactivity fo the alcohol.

The initial rates of transesterification of o-, m- and p-pyridylcarbinols [126], [127] and [128] with TCEMA follow the sequence of ortho > meta = para as outlined in Table 4.2 and Graph 4.2. For comparison with non-heterocyclic systems benzyl alcohol has been added to Graph 4.2. This alcohol has an initial rate similar to o-pyridylcarbinol and a slightly lower equilibrium conversion of 80% as compared to 95% for the o-pyridylcarbinol.

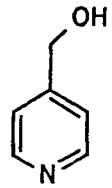
Dig 4.2



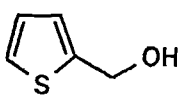
[124]



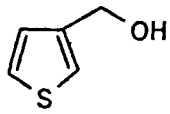
[127]



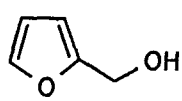
[128]



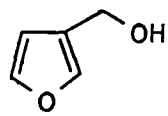
[129]



[130]



[131]



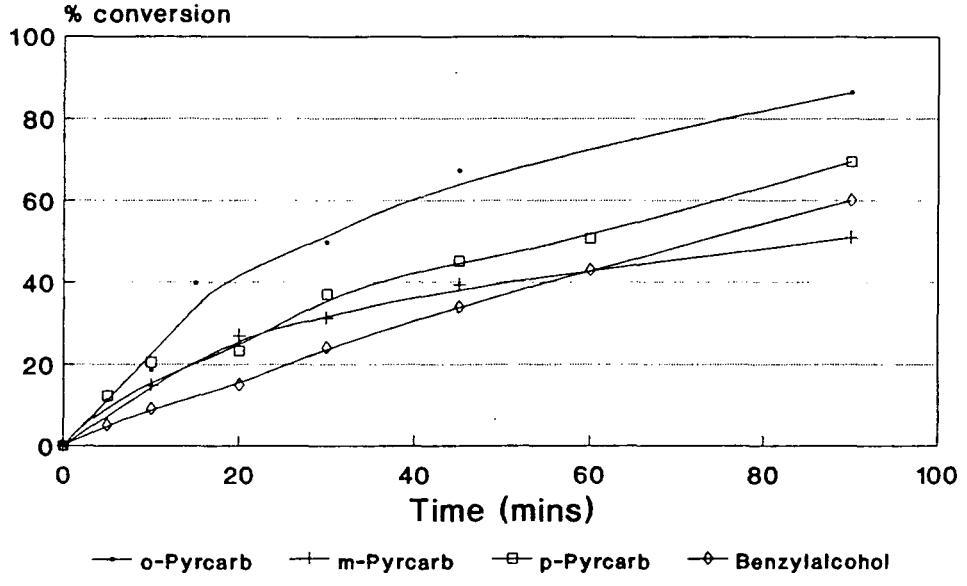
[132]

Alcohol	Initial Rate mM.h^{-1} TCEB	TCEMA	Equilibrium Conversion TCEB	TCEMA
o-pyridylcarbinol	0.91	0.60	45%	95%
m-pyridylcarbinol	0.91	0.37	45%	80%
p-pyridylcarbinol	0.91	0.37	45%	60%
benzyl alcohol	--	0.42	--	80%

Table 4.2 : Effect of position of the heteroatom of pyridylcarbinol on the rate of transesterification with TCEMA and TCEB mediated by CCL.

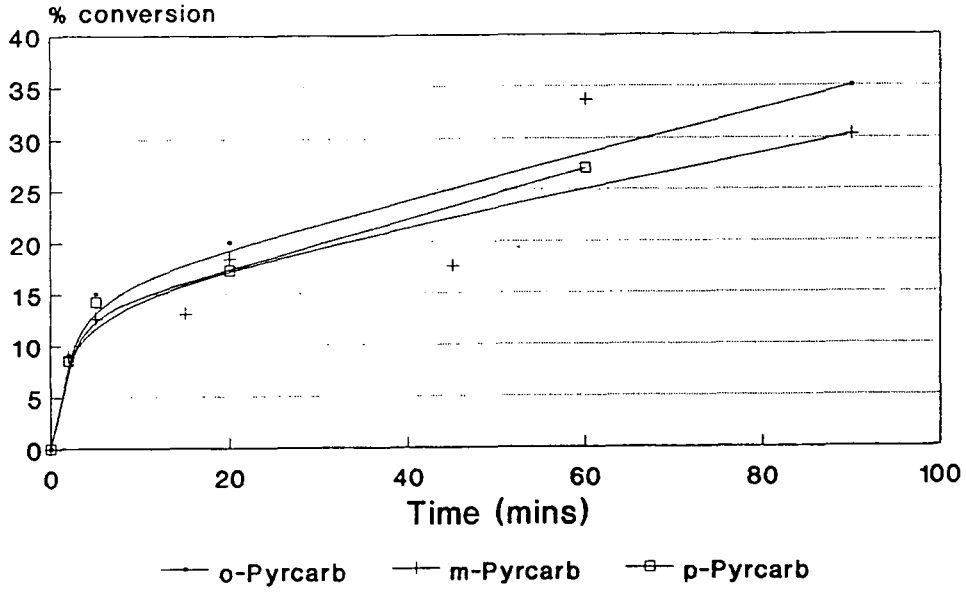
Graph 4.2.

Comparison of Transesterification Reaction of o-, m- and p- Pyridylcarbinols with TCEMA



Graph 4.3

Comparison of Transesterification Reactions of o-, m- and p -Pyridylcarbinols with TCEB



The variation in the initial rates between the o-, m-, and p-pyridylcarbinols with TCEB, are not statistically significant, (see Graph 4.3). It is noteworthy however, and a clear anomaly, that the acceleration observed for the methoxyacetate ester over the butyrate as an acyl donor is not observed for these pyridylcarbinol reactions.

The reaction of benzyl alcohol with TCEMA has a faster initial rate than that of the pyridylcarbinols however the reaction with TCEB shows no conversion after 3h. This indicates that the nitrogen *overall* has a beneficial effect on the transesterification rate under these conditions.

The extents of conversion however, are much greater for the methoxyacetate than for the butyrate reactions, (80% compared to <40%). After an initially fast rate the reactions with TCEB appear to slow down dramatically. This

seems to suggest that TCEMA is still the better of the two acyl donors for these transesterification reactions.

The absence of acceleration with TCEMA in these experiments is difficult to rationalise. The overall rate of the transestrification process is controlled by a number of factors, eg diffusion effects, nucleophilicity of the alcohol and the acyl donor. The diffusion rates would be expected to be similar as each of the substrates is similarly polar. Also the nucleophilicity of each alcohol is not likely to be a significant factor. The nature of the acyl donor will have an effect on the acylation step leading to the acyl-enzyme intermediate. If however, the acylation step is not rate limiting then it should not effect the overall rate. Thus, these results appear to result from a combination of a slower methoxyacetate reaction with a relative increase in the butyrate transesterification. There is no significant difference in the initial rates of the o-, m- and p- alcohols.

Substrates containing sulfur and oxygen heterocycles were clear candidates to extend this series of experiments. Heterocyclic alcohols 2- and 3- thiophenemethanol [129] and [130] and 2- and 3- furanmethanol [131] and [132] were presented to the enzyme. The initial rate of reaction of the 2-furanmethanol with TCEMA (1.62 $mM.h^{-1}$) is faster than that of the 3-furanmethanol (1.45 $mM.h^{-1}$). This order is consistent for both TCEMA and TCEB reactions, (see Graphs 4.4 and 4.5). The TCEMA reactions are again about an order of magnitude faster than their corresponding TCEB reactions (see Table 4.3), consistent with earlier observations (see Chapter 2, page 83).

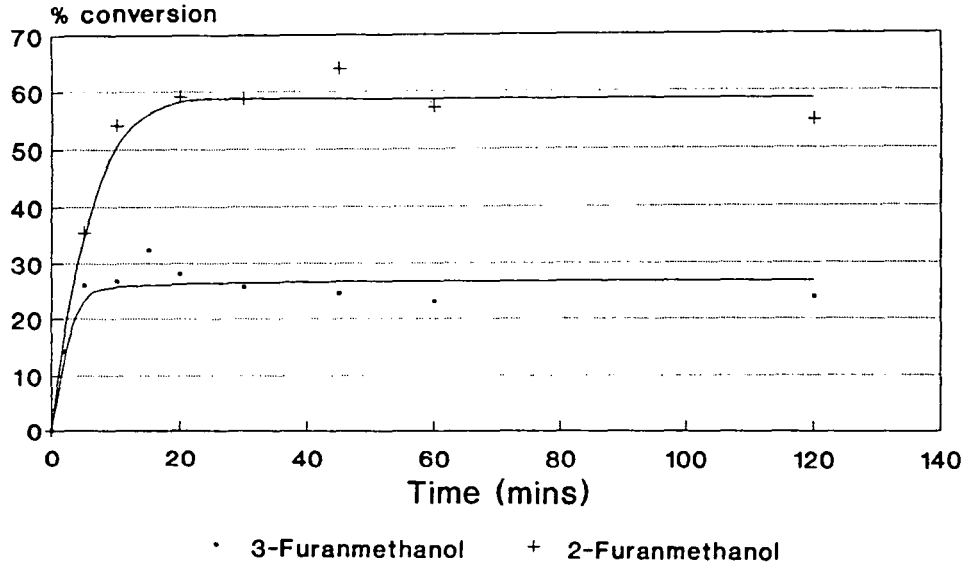
The conversion of the 2-furanmethanol proceeds to >45% for both TCEMA and TCEB whereas 3-furanmethanol stops at 25% for both reactions. Interestingly the reaction of 3-furanmethanol with TCEMA did not proceed beyond 25%.

Alcohol	Initial Rate mM.h^{-1} TCEB	TCEMA	Equilibrium Conversion TCEB	TCEMA
2-furanmethanol	0.16	1.62	45%	60%
3-furanmethanol	0.04	1.45	25%	28%

Table 4.3 : Effect of position of the heteroatom on the rate of transesterification of furanmethanol with TCEB and TCEMA mediated by CCL.

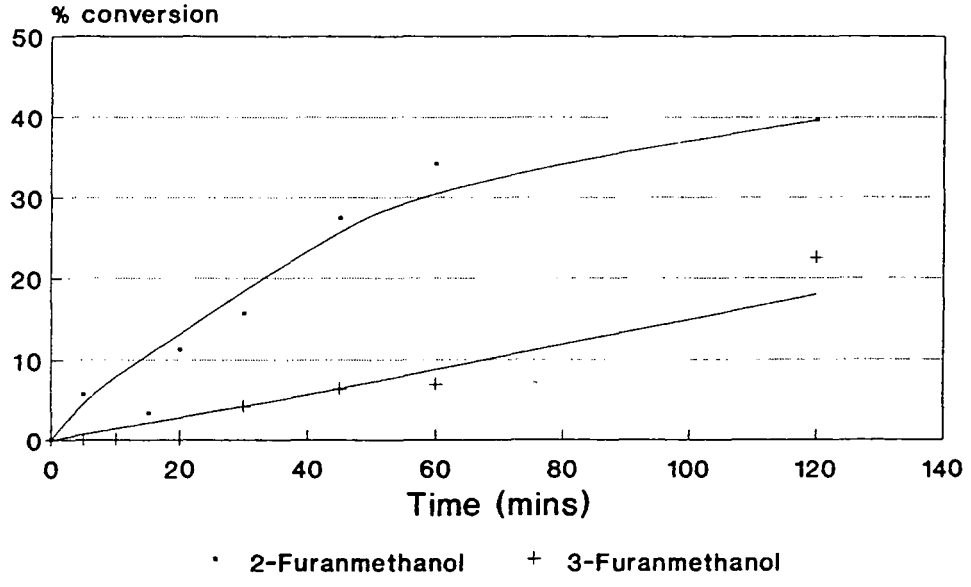
Graph 4.4

Comparison of 2- and 3-Furanmethanol Transesterification Reactions with TCEMA



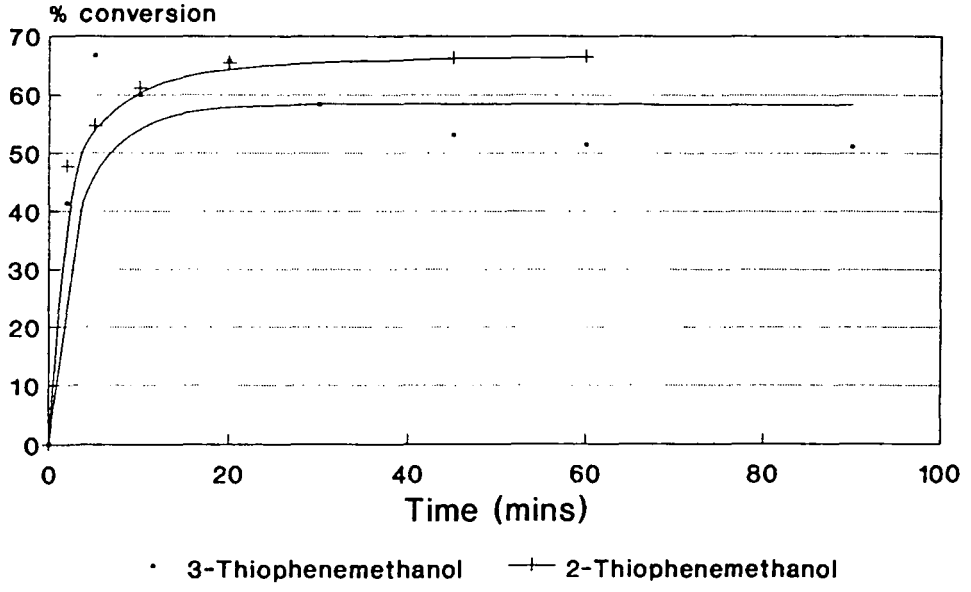
Graph 4.5

Comparison of 2- and 3-Furanmethanol
Transesterification Reactions with TCEB



Graph 4.6

Comparison of 2- and 3-Thiophenemethanol
Transesterification Reactions with TCEMA



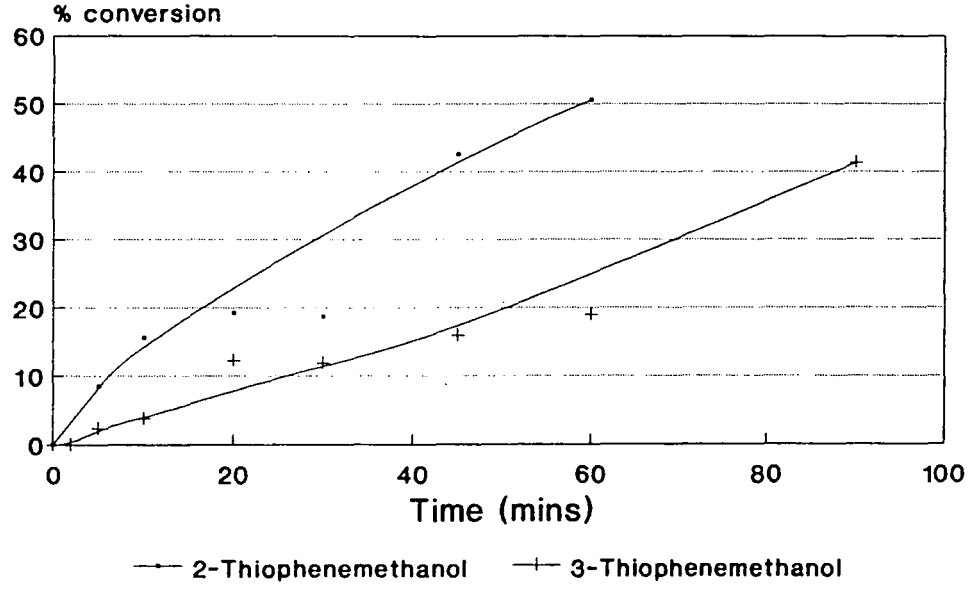
Alcohol	Initial Rates mM.h^{-1} TCEB	TCEMA	Equilibrium Conversion TCEB	TCEMA
2-thiophenemethanol	0.28	4.98	60%	68%
3-thiophenemethanol	0.12	3.06	60%	60%

Table 4.4 : Effect of the position of the heteroatom of the rate of transesterification of thiophenemethanol with TCEB and TCEMA mediated by CCL.

The initial rates for the 2-thiophenemethanol is faster than 3-thiophenemethanol with TCEMA (4.9mM.h^{-1} compared to 3.0mM.h^{-1}). For the overall slower reactions with TCEB the rate differential widens, ie 0.27mM.h^{-1} compared to 0.12mM.h^{-1}, (see Graph 4.6 and Graph 4.7)

Graph 4.7

Comparison of 2- and 3-Thiophenemethanol Transesterification Reactions with TCEB



The effect of the heteroatom on the nucleophilicity of the alcohol would be expected to be negligibile on the basis of inductive effects however, interaction of the lone pairs of the heteroatoms with the surface of the protein at the active site could clearly influence binding affinity. For the systems studied, sulfur can more readily donate its lone pairs than nitrogen which in turn is a better donor than oxygen. On the other hand however, oxygen and nitrogen will form stronger hydrogen bonds than sulfur if such interactions are significant. The increase in initial rate of the sulfur analogue over the oxygen suggests that a soft-soft electrostatic interaction may underlie this rate increase. For both the thiophene- and furan- methanols the 2- substituted compounds are better substrates than their 3-substituted counterparts. The difference in initial rates for the two furanmethanols with TCEMA is small but the relative difference increases in the TCEB reaction (see Graphs 4.4 and 4.5) although these reactions are slower. The same is true of the thiophenemethanols, Graphs 4.6 and 4.7. It would appear that there is an optimal position for the heteroatom indicative of a specific interaction at the active site.

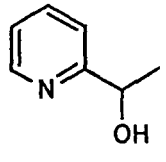
For the o-, m- and p-pyridylcarbinols the reaction sequence of ortho > para = meta suggests partly positional and partly electronic effects. The initial rate of the o-pyridylcarbinol is slightly faster than the m- and p- which are similar to the benzyl alcohol, (see Graph 4.2). This suggests that the contribution of the nitrogen to the reaction rate of the m- and p- pyridylcarbinols is negligible and lends further support to the hypothesis that there is an acidic functionality at a position to accept the lone pair donation from the heteroatom in the ortho case. Benzyl alcohol is intermediate in rate between m-, p-pyridylcarbinol and o-pyridylcarbinol. It can be argued therefore that the nitrogen does have a small but significant effect in the ortho position lending support to the lone pair donation hypothesis.

Comparison of initial rates between 2-furanmethanol [131], 2-thiophenemethanol [129] and 2-pyridylcarbinol [126] gives

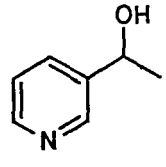
an indication of the requirements of such an interaction. For the reactions with TCEMA, the thiophene system is faster than furan, which in turn is faster than all of the pyridylcarbinols. This sequence of reactivity suggests that the binding site can best accommodate the sulfur atom. For the reactions with TCEB the sequence is N > S > O although in these cases the reactions are all generally sluggish and not a good indication of selectivity.

3. Reactivity and Resolution of a Range of Secondary Heterocyclic Alcohols.

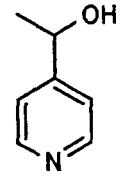
A logical extension of this work was to present a range of heterocyclic secondary alcohols to the lipase, thus o-, m- and p-pyridylethan-1-ols [133], [134] and [135] were each presented to the enzyme with TCEMA (Graph 4.8) and TCEB (Graph 4.9) as acyl donors, under a standard set of conditions. Table 4.5 shows the initial rates and the equilibrium % conversion for these reactions.



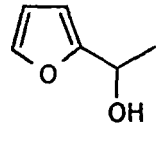
[133]



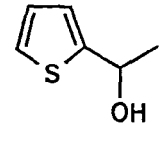
[134]



[135]



[136]



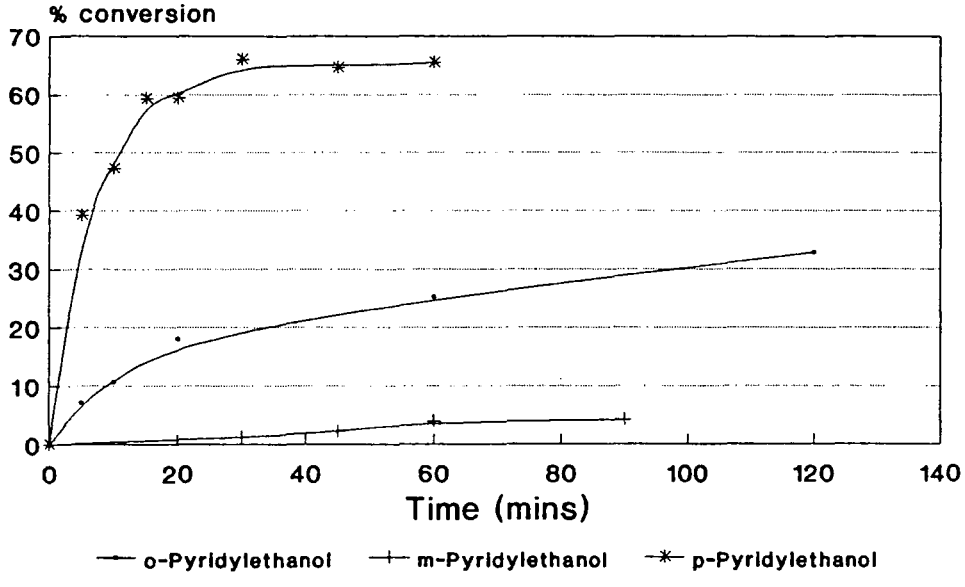
[137]

Alcohol	Initial Rate mM.h^{-1} TCEB	TCEMA	Equilibrium Conversion TCEB	TCEMA
o-pyridylethanol	0.08	1.99	55%	65%
m-pyridylethanol	0.13	0.34	55%	50%
p-pyridylethanol	0.13	2.12	55%	15%

Table 4.5 : Effect of position of heteroatom on the rate of transesterification of pyridylethan-1-ol with TCEB and TCEMA mediated by CCL.

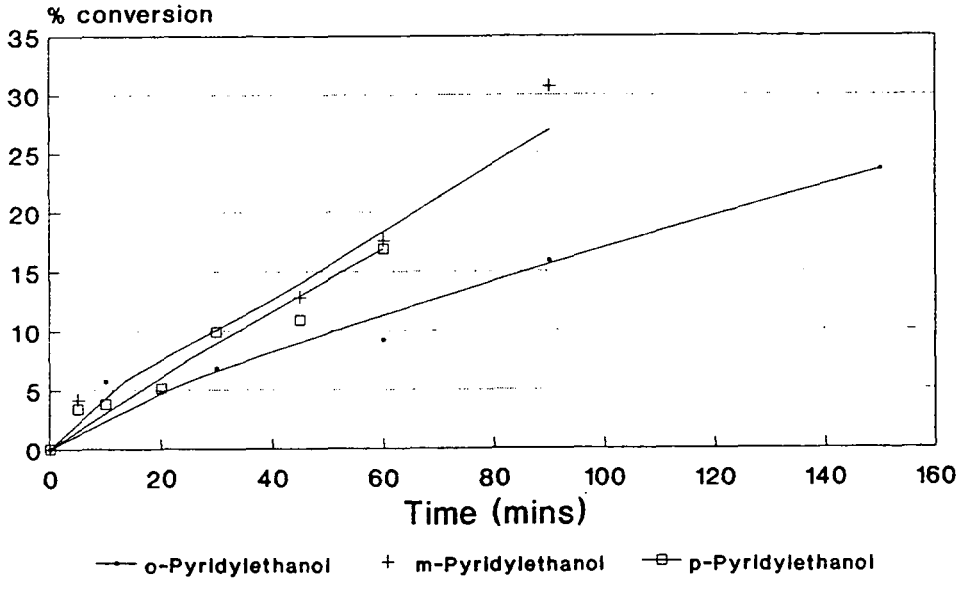
Graph 4.8

Comparison of Transesterification Reactions of o-, m- and p- Pyridylethan-1-ol with TCEMA



Graph 4.9

Comparison of Transesterification Reactions of o-, m- and p-Pyridylethan-1-ol with TCEB



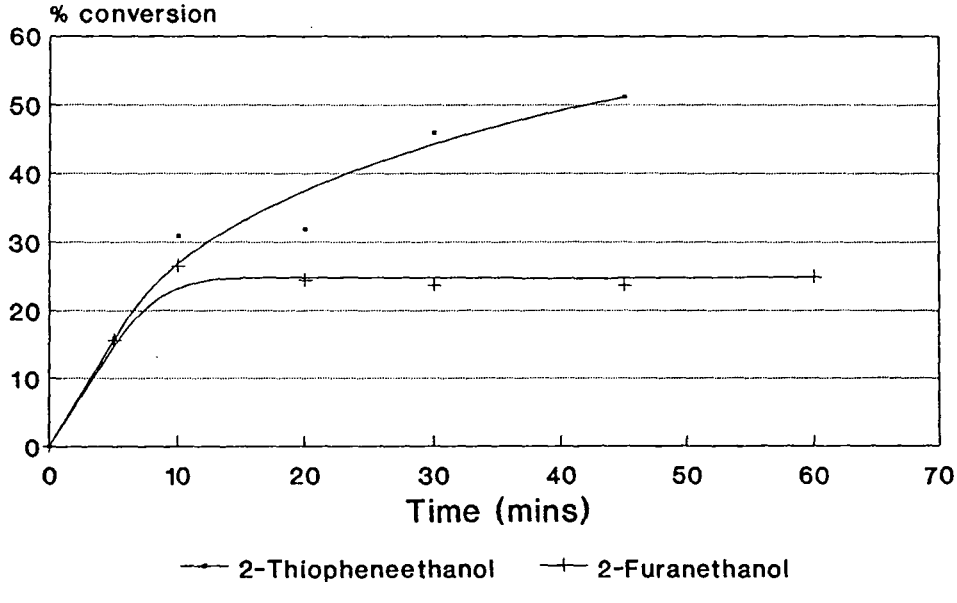
These reactions are much faster than the corresponding reaction for the analogous non heterocyclic system. 1-Phenethanol, under the same conditions, shows no reaction even after 5h. This suggests that the nitrogen has a beneficial effect overall on the ability of these alcohols to serve as substrates in the transesterification reactions. This may be due to the increased polarity of the alcohol allowing easier diffusion across the hydrated water layer, but contrasts with the analogous primary alcohols where there was no significant acceleration with the pyridines over that of benzyl alcohol, (see page 126). There is also a clear positional effect favouring the p- substituted pyridine in this series. The positional effect, with the possible donation of the nitrogen lone pair to an electrophilic site, does have a significant effect on the rate of the reaction of these secondary systems. This contrasts with the corresponding primary alcohols (pyridylcarbinols) where there is no significant effect in going from o- to m- to p-, (see page 126). Interestingly,

the corresponding reactions with TCEB show no significant difference in the three positions for either the secondary or primary systems.

2-Furanethan-1-ol [136] and 2-thiopheneethan-1-ol [137] were also presented to the enzyme under the same set of conditions. The reaction sequence found with the primary alcohols is also observed in this secondary series with the thiophene having a faster initial rate than the furan, (see Graph 4.10 for the reaction with TCEMA). Table 4.5 summarises the data for both the TCEMA abd TCEB reactions.

Graph 4.10

Comparison of the Transesterification Reaction of 2-Furanethan-1-ol and 2-Thiopheneethan-1-ol with TCEMA in Diethyl Ether



Alcohol	Initial Rate $mM.h^{-1}$ TCEB[a]	TCEMA	Equilibrium Conversion TCEB[a]	TCEMA
2-Furanethanol	21.2%	0.79	35%	25%
2-Thiopheneethanol	33.2%	0.88	35%	55%

Note- a) The TCEB reactions show no reaction after 2hours therefore the reaction was monitored every 12 hours until 48hours

Table 4.5 : Effect of switching from oxygen to sulfur heteroatom in the transesterification reaction with TCEB and TCEMA mediated by CCL.

In an effort to compare 2-pyrroleethan-1-ol with the oxygen and sulfur heterocycles the reaction was conducted in the standard manner, however immediately after addition of TCEMA the 2-pyrroleethan-1-ol decomposed. GC analysis of the reaction showed no 2-pyrroleethan-1-ol and no evidence of any transesterification reaction. In an attempt to prevent the decomposition the reaction was conducted at -25°C. Despite this the molecule completely decomposed over 30mins (monitored by GC) and there was again no transesterification reaction.

B. Studies on Enantioselectivity.

1. Resolutions of 2-Pyridylethan-1-ol at Room Temperature (25°C):

Having demonstrated that the position of the nitrogen in the heterocycle is significant and that 2-pyridylethan-1-ol [133] reacts at a relatively fast rate, it was of interest to assess the factors which effect the enantioselectivity of this reaction.

Preparative scale reactions were run with 2-pyridylethan-1-ol (1g, 8.2mM), TCEMA (or TCEB) (2g, 9mM) and CCL (2g, 1.4 $x10^5$ units) in dry diethyl ether (30ml). The reactions were shaken for 2 hours and assayed by standard methods. The absolute configuration was determined by optical rotation of the residual alcohol and reference to literature[200]. The enantiomeric excess of the 1-(o-pyridyl)-ethyl methoxyacetate was determined by ^1H NMR using a chiral shift reagent (tris[3-(trifluoromethylhydroxymethylene)- (+)- camphorato] europium (III) derivative). The %ee values at 30% conversion are shown in Table 4.6.

Acyl Donor	Optical Purity (product ester)
TCEB	62.7%ee (+)(R)
TCEMA	60.0%ee (+)(R)

Table 4.6 : Enantioselectivity of the transesterification reaction of 2-pyridylethan-1-ol with TCEB and TCEMA mediated by CCL.

Resolution of 2-Furan-1-ethanol.

The reaction of 2-furanethan-1-ol [136] with TCEMA proceeded sufficiently to afford the possibility of a kinetic resolution, therefore the racemic alcohol (1g, 8.9mM), TCEMA (2g, 9mM) and CCL (2g, 1.4 x10^5 units) were dissolved in dry diethyl ether (30ml). The reaction was shaken and stopped at 50% conversion. The residual alcohol was assayed for optical purity. This contrasts with the other reactions which were run to 30% conversion, followed by analysis of the product esters. In this case however the product ester proved difficult to purify and therefore the residual alcohol was derivatised using p-bromobenzyl chloride affording the p-bromobenzoate ester as a crystalline solid.

The optical purity of the alcohol was determined to be 37.1%ee by ^{1}H NMR anlysis using the chiral shift reagent, tris [3- trifluoromethylhydroxymethylene) - (+) -camphorato] europium (III) derivative.

2. The Effect of Temperature on Enantioselectivity.

It has already been demonstrated that the reactions in organic solvents with the *Candida cylindracea* lipase proceeded with measurable rates at temperatures well below zero (see Chapter 3, page 115). It was of particular interest now to determine how low temperatures would effect the enantioselectivity of CCL resolutions. In Chapter 3 (page 113) literature precedence was demonstrated for improvement of enantioselectivity with low temperature for pig liver esterase[163b]. It was therefore of interest to assess the effect of varying the temperature on the selectivity of the *Candida cylindracea* lipase. To assess the effect of temperature on the kinetics and the enantioselectivity, the reaction of o-pyridyl-1-ethanol [133] with TCEMA was selected. The reaction was run at various points on the temperature range 50°C to -25°C. Table

4.7 summarises the results of these reactions. In order to achieve reasonable reaction rates at low temperature hexane was selected as the solvent in this study (see Chapter 3, page 100). This is not the optimal solvent for this resolution as it has already been shown that there is a decrease in the enantioselectivity in moving from diethyl ether to hexane, however transesterifications were much faster in this solvent as established in Chapter 3, (page 100).

Temperature (°C)	Optical Purity Product ester	Time to 30% conversion
-25	56.2%ee (+)(R)	72h
-10	46.6%ee (+)(R)	48h
0	58.1%ee (+)(R)	6h
30	54.4%ee (+)(R)	2h
50	47.6%ee (+)(R)	5mins

Table 4.7 : Effect of temperature on the enantioselectivity of the transesterification reaction of 2-pyridylethan-1-ol with TCEMA mediated by CCL in hexane.

The increase in initial rate allows for a larger temperature range to be studied. In the event however there was no significant variation in the enantioselectivity of the reaction over the range -25°C to 50°C.

Lowering of the temperature might be expected to increase the conformational rigidity of the enzyme structure and thus the difference in energy of the diastereomeric interactions with the enzyme (see Chapter 2, page 76). Therefore the enzyme should be able to differentiate enantiomers more readily. Conversely at high temperatures the enzyme might be expected to be less able to resolve enantiomers owing to a relaxation in rigidity. However in this system this does not

appear to be the case for either high or low temperatures. Although, in this study there was no significant variation in the enantioselectivity, there are other obvious advantages in being able to conduct such reactions at temperatures outside the environmental range of the enzyme. At 50°C the reaction of TCEMA with [133] reaches 30% conversion in 5min and still has an enantioselectivity of 47.6%ee. This is a remarkable observation and one that could possibly have significant practical value in speeding up very sluggish resolutions. Also, there are potential applications for large scale resolutions which can become very sluggish on scale up.

Resolution of 2-Thiopheneethan-1-ol.

2-Thiopheneethan-1-ol was resolved with a respectable enantiomeric excess under the standard set of conditions. In diethyl ether at 25°C, with TCEMA as the acyl donor, a 28% conversion was reached in 4h, affording the methoxyacetate ester of (R)-2-thiopheneethan-1-ol in 70%ee and 35.8% yield. The corresponding reaction with TCEB as the acyl donor afforded the butyrate ester of the (R) alcohol in 43.5%ee after 72h (27.3% conversion).

The fast initial rate of the reaction with TCEMA made it another ideal candidate to study the effect of temperature on enantioselectivity. Accordingly reactions were conducted over the range -40°C to 80°C. There was no reaction at 80°C probably due to the denaturation of the enzyme. This is consistent with the study in Chapter 2 (page 111) where it was concluded that the hydrated water layer was entering into hydrolytic reactions and denaturing the enzyme. The reaction of TCEB with heptan-2-ol proceeded to 12% conversion before stopping abruptly. This indicates that the enzyme has a short half-life at 80°C. The reactions conducted at 50, 30 and -10°C, however, showed respectable initial rates and moderate %ee's, (see Table 4.8). The reaction at -40°C proved too slow with no appreciable reaction after 5 days.

Temperature (°C)	Optical Purity product ester	Time to 30% conversion
-40	No Reaction	--
-10	42.5%ee (+)(R)	15h
30	57.0%ee (+)(R)	1.5h
50	54.0%ee (+)(R)	30secs
80	No Reaction	--

Table 4.8 : Effect of temperature on the enantioselectivity of the transesterification reaction of 2-thiopheneethan-1-ol with TCEMA mediated by CCL in hexane.

Again, over a broad temperature range, these reactions achieve a measurable % conversion but without significant variation in the enantioselectivity. The rate of reaction of the TCEMA reaction with 2-thiophene-1-ethanol in hexane at +50°C is again remarkable. The observed 54%ee in 30secs, for 7.8mM, is an extraordinary result with obvious possibilities for large scale processes. With careful choice of solvent the reactions can be conducted over a wide temperature range. Extremely slow substrates can be accelerated by raising the temperature with no significant loss of enantioselectivity. In addition potentially unstable substrates can be resolved at very low temperatures.

Conclusion.

It appears from these results that heterocyclic alcohols are good substrates for the *Candida cylindracea* lipase. Both primary and secondary alcohols react with respectable initial rates. In general the 1° alcohols are faster than the 2° alcohols. For the 1° alcohols the reactivity sequence

is S > O > N, ie. the thiophenemethanol has a faster initial rate than the furanmethanol which in turn is faster than the pyridylcarbinols. For the 2° systems the sequence changes to N > S > O, (pyridylethanol > thiopheneethanol > furanethanol)

It appears that CCL catalysed resolutions can be achieved from -25°C to 50°C. There is no significant variation in the enantioselectivity of the resolutions with *Candida cylindracea* lipase over the complete temperature range available to this enzyme.

Resolutions conducted at elevated temperatures afford the possibility of increasing the reaction rate of particularly sluggish substrates without significant loss of enantioselectivity. Although low temperature reactions have more limited exploitable advantages, reactions below zero degrees do provide the possibility of carrying out enzyme catalysed resolutions of unstable substrates. This remains to be explored.

Chapter 5

Strucure Activity Relationships of The *Candida cylindracea* Lipase. Part 2.

A. Structure Activity Relationships for a Range of Secondary Alcohols.

Introduction.

The literature on *Candida cylindracea* has shown it to be useful as a catalyst in the hydrolysis and the transesterification of a range of primary, secondary and tertiary alcohols[202c]. Chapter 1 and Chapter 4 give an indication of the range of alcohols that have been resolved by the *Candida cylindracea* lipase.

In Chapter 4 the stereochemical constraints of the enzyme active site were probed with a variety of heterocyclic alcohols. It had been demonstrated in Chapter 2 that an oxygen atom β to the carbonyl of the ester in the acyl donor had a dramatic accelerating effect on the overall rate of the transesterification reaction. It was therefore of clear interest to assess the affect of a heteroatom in the alcohol moiety. The conclusion drawn was that a heteroatom, oxygen, nitrogen or sulfur has a significant effect on the rate of transesterification mediated by CCl. This suggests that an acidic funcionality at the active site can accept the donation of lone pairs from the heteroatom. Several steric models have been proposed to account for the stereochemical behaviour of the CC lipase[199], (see Chapter 4, page 124). The more general steric model, Model A is consistent for a wide range of secondary substrates whereas Model B appears more appropriate for secondary systems containing a strong electron withdrawing group, eg CF_3 (see Chapter 4, page 125) or tertiary alcohols. It was therefore of interest to assess

the substrate specificity of the *Candida cylindracea* lipase and to relate the specificity to the models proposed in the literature[199]. In this Chapter experiments are reported for a range of secondary and tertiary alcohols presented to the enzyme.

Results and Discussion.

The series of acetylenic, vinylic and methyl alcohols shown in Figure 5.1 were prepared and presented to the enzyme under a standard set of conditions.

Figure 5.1

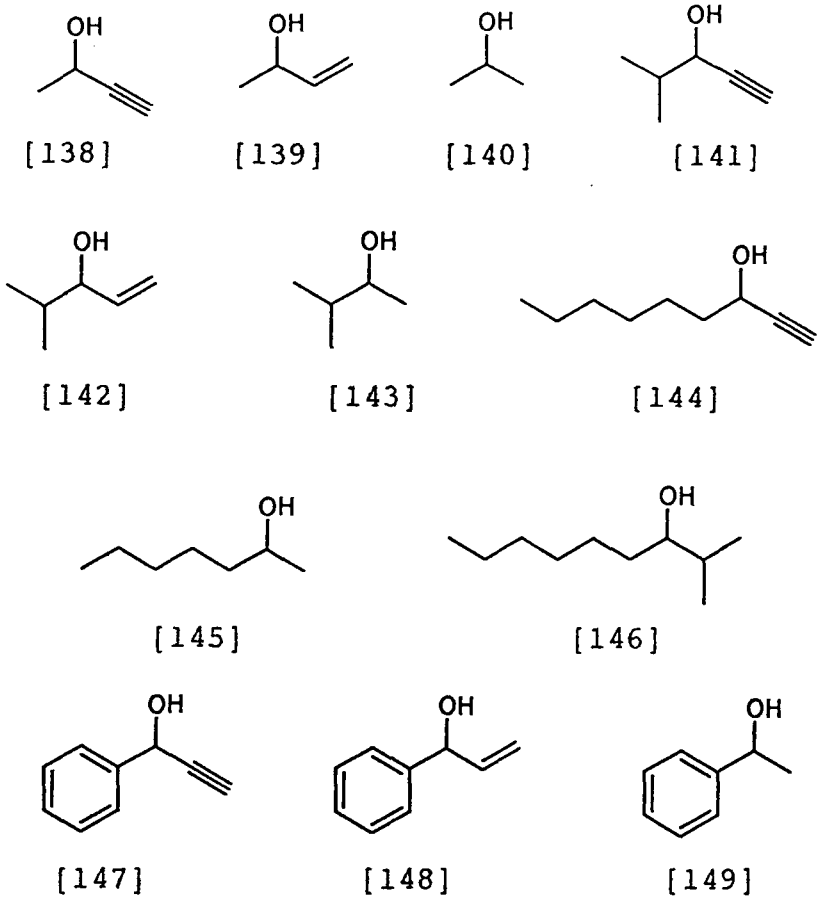


Table 5.1 shows the comparison of alcohols [138] to [149]. These results give an indication of the steric constraints at the active site of the *Candida cylindracea* lipase. From this study emerges some common trends.

The general order of reaction is the actylene > vinyl > methyl. Graph 5.1 and 5.2 illustrate the reactions of each of the 2° alcohols with an α-isopropyl group ([141] to [143]) with TCEMA and TCEB. Alcohols [141] and [142] proceed to respectable conversions (45% and 23% respectively for the reactions with TCEMA) whereas alcohol [143] is a very poor substrate and only proceeds to 5% conversion even when the reaction is left for 24h.

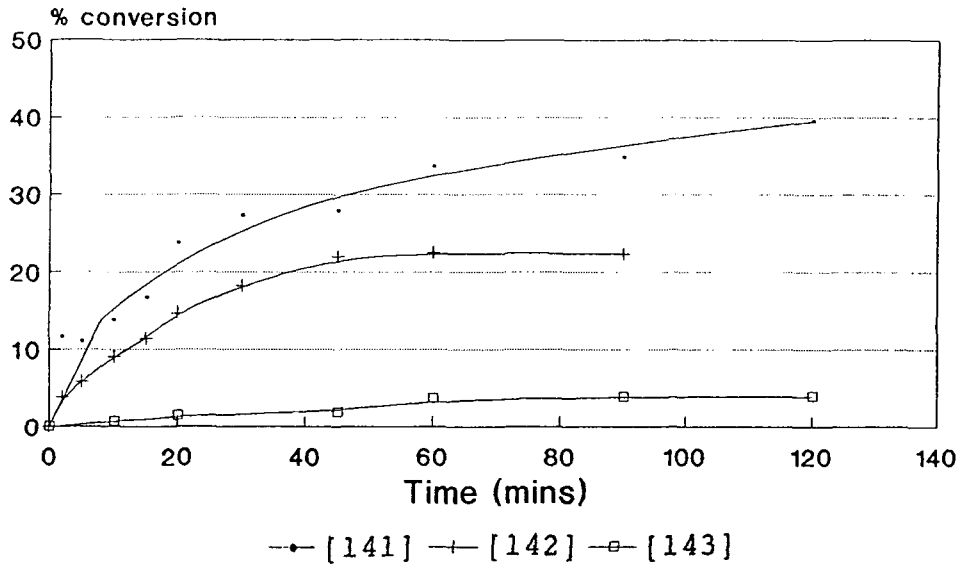
α-Funcionality[a]	Alcohol[a]	Initial Rate ($mM.h^{-1}$) TCEMA	TCEB	TCEMA %conversion	TCEMA Time (min)	TCEB %conversion	TCEB Time (min)
acetylene	[138]	2.57	0.47	55%	45[c]	20%	40
vinyl	[139]	0.42	0.01	15%	80	5%	60
methyl	[140]	0.06	--[b]	25%	100	--	--
acetylene	[141]	0.46	0.09	45%	100	35%	120
vinyl	[142]	0.42	0.10	23%	40	20%	80
methyl	[143]	0.02	0.02	5%	100	5%	100
acetylene	[144]	2.55	0.11	65%	20	35%	60
vinyl	[145]	0.11	0.08	40%	90	40%	90
isopropyl	[146]	0.27	0.07	10%	100	10%	100
acetylene	[147]	2.02	0.32	80%	120	35%	45
vinyl	[148]	--	--	--	--	--	--
methyl	[149]	--	--	--	--	--	--

Notes a) see dig 5.1 for alcohol structures.
b) no detectable reaction after 24hours
c) time for the reaction to reach equilibrium

Table 5.1 : Structure activity relationship for a range of secondary alcohols with the Candida cylindracea lipase.

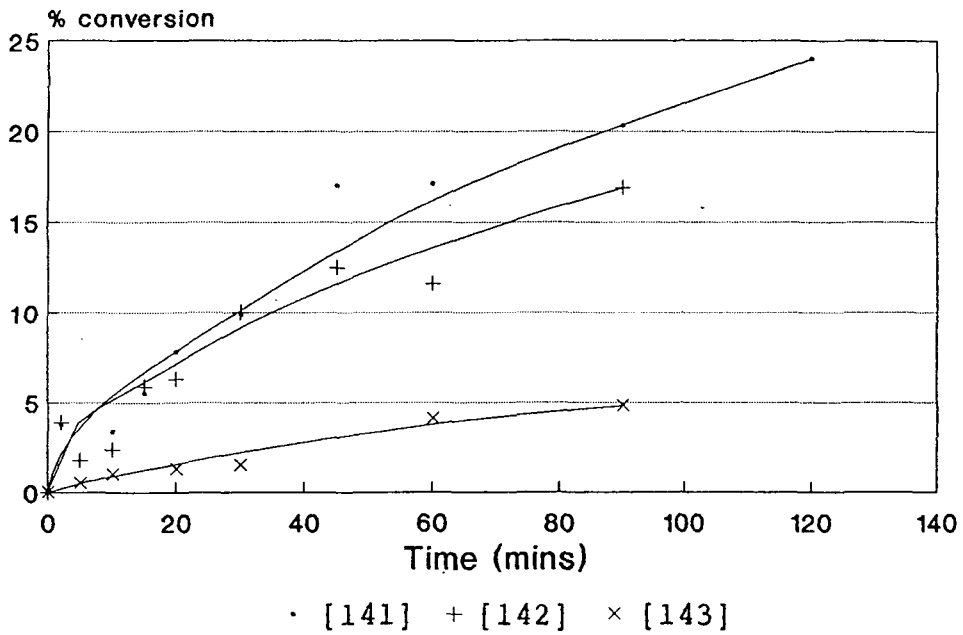
Graph 5.1

Comparison of Alcohols [141], [142] and [143]
Transesterification Reactions with TCEMA



Graph 5.2

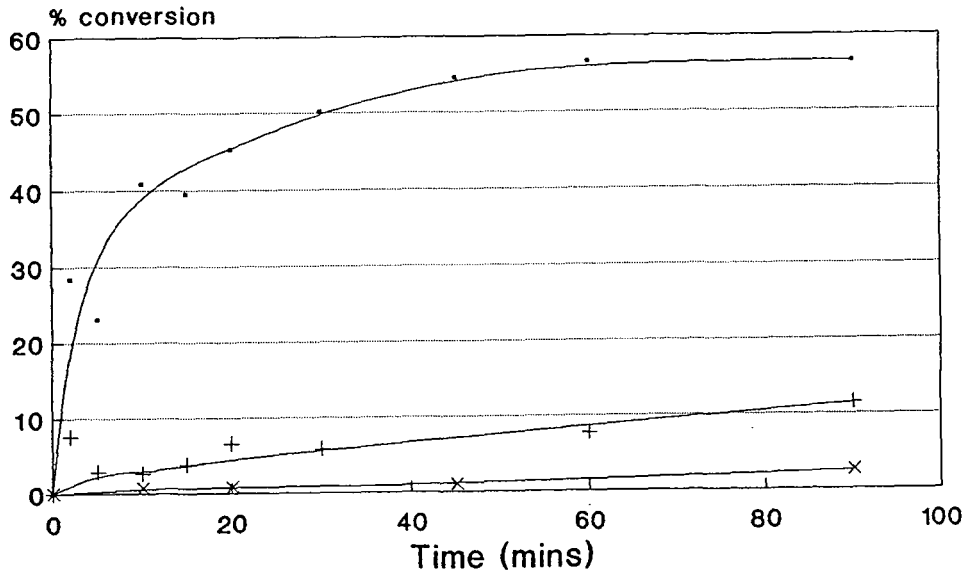
Comparison of Alcohols [141], [142] and [143]
Transesterification Reactions with TCEB



A similar order is observed for the α-substituted ethanols, [138], [139] and [140] as shown in Graphs 5.3 and 5.4. For the α-substituted phenylethanols, [147], [148] and [149] only 2-hydroxy-2-phenylprop-1-yne, [145], was processed at a moderate rate (see Graph 5.5). For example 1-phenethan-1-ol [149] proceeds to only 10% conversion after 48h

Graph 5.3

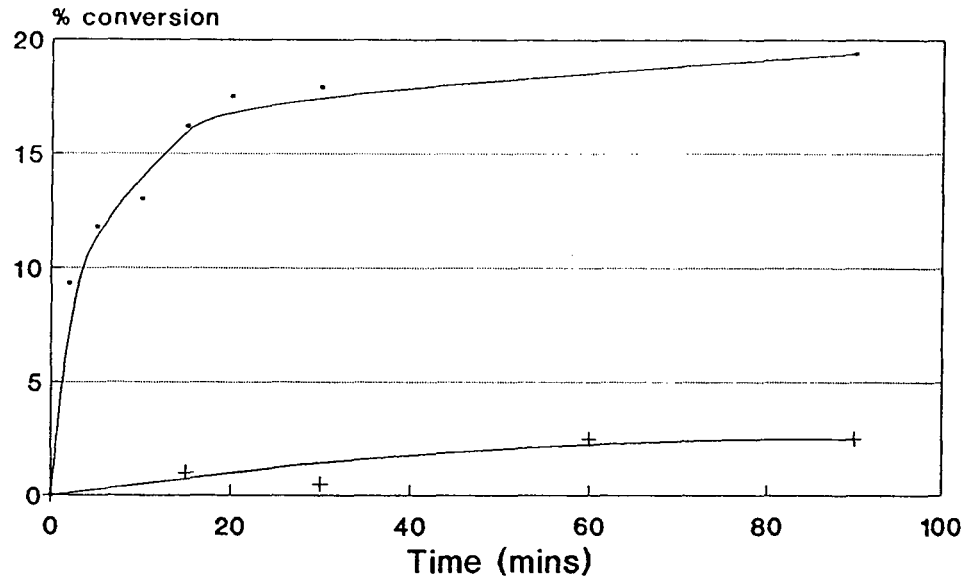
Comparison of Alcohols [138], [139] and [140]
Transesterification Reactions with TCEMA



· [138] + [139] × [140]

Graph 5.4

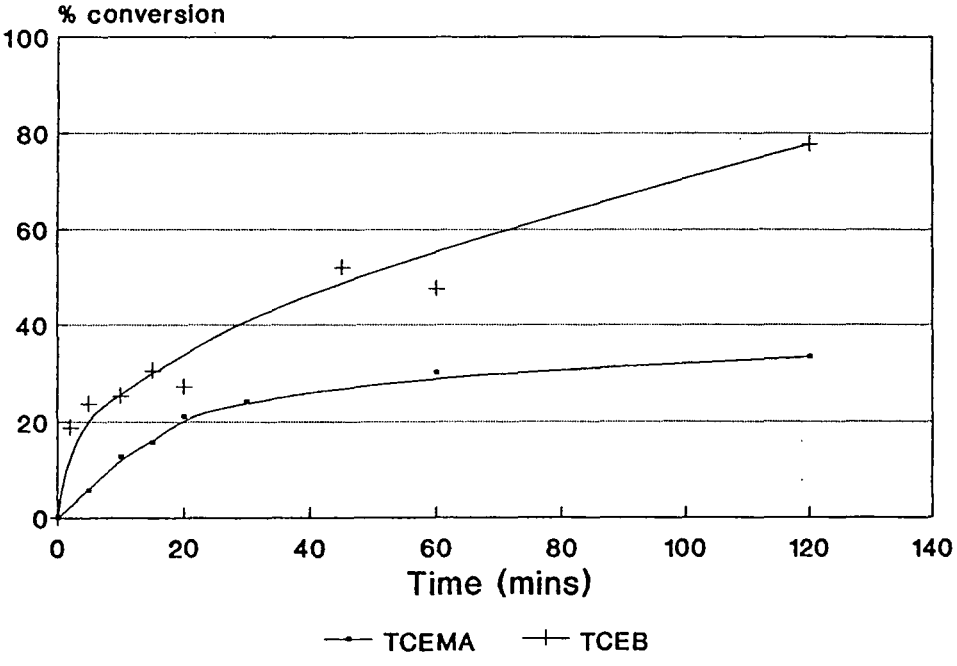
Comparison of Alcohols [138], [139] and [140]
Transesterification Reactions with TCEB



·[138] +[139]

Graph 5.5

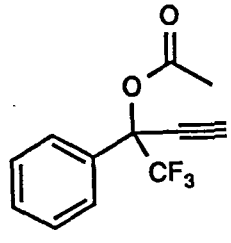
% Conversion of Alcohol [147]
Versus Time (mins)



The one notable exception to this reactivity trend is the long chain series, [144], [145] and [146]. In this series heptan-2-ol [145] is the fastest with initial rates of 2.55mM.h^{-1} and 0.11mM.h^{-1} for TCEMA and TCEB respectively. The acetylene derivative, 3-hydroxynon-1-yne [144], has an initial rate of only 0.11mM.h^{-1} with TCEMA and 0.08mM.h^{-1} with TCEB, and the 3-hydroxy-2-methylnonane [146] had initial rates of 0.27mM.h^{-1} and 0.07mM.h^{-1} for TCEMA and TCEB respectively.

The natural substrates for lipase enzymes are triacylglycerols and the enzyme is therefore designed to accept substrates with long lipophilic chains. This may explain to some extent why heptan-2-ol is a better substrate than expected.

This result aside the sequence of acetylenic > vinylic > methyl is suggestive of steric constraints at the active site of the enzyme. These observations are also consistent with those from the studies on tertiary alcohols[199a], where it was observed that the acetylene funcionality was required for maximal activity[199a]. In the study, the presence of an acetylenic funcionality at the α-chiral centre was a prerequisite for substrate turnover. For example 1,1,1-trifluoro-2-hydroxybut-3-yne acetate [150] was hydrolysed in buffer to 40% conversion in 23h to afford the corresponding free alcohol in 87%ee[199a].



[150]

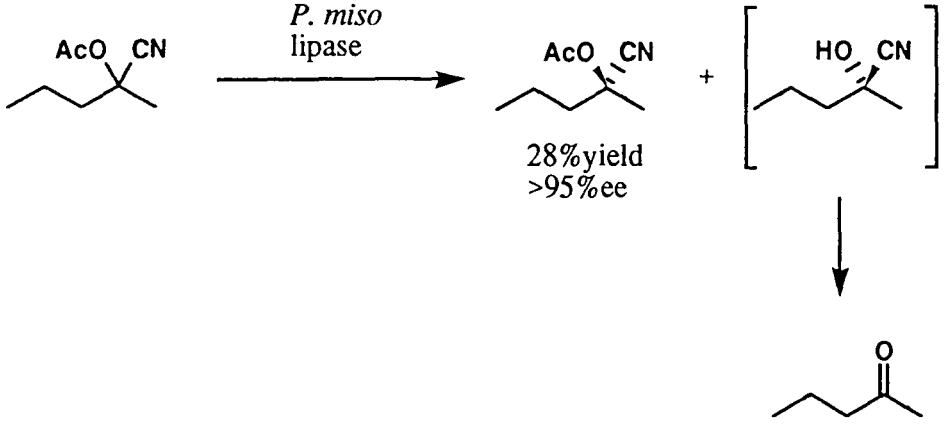
In contrast if the acetylenic funcionality is replaced with methyl, vinyl or nitrile then the acetate is inert to lipase hydrolysis even after extended periods of time (>48hours). It would appear therefore that the acetylenic funcionality

is sterically small, smaller than for example the methyl, vinyl or nitrile groups. In the steric model describing tertiary systems model B (see Chapter 4, page 125)[199] it has been rationalised that the acetylene occupies the same site as hydrogen in the corresponding secondary systems. The rate enhancement of the secondary acetylenic alcohols is therefore most likely a steric effect in the same way as the sequence of rates is 1° > 2° > 3° alcohols.

B. Structure Activity Relationships For a Range of Tertiary Alcohol Esters.

Introduction.

The nucleophilicity of a tertiary alcohol is significantly less than that of a secondary alcohol for steric reasons and it was not expected that these alcohols would behave as good nucleophiles for transesterification. Instead, as a direct comparison with previous work[199a] the 3° substrates were converted to their corresponding methoxyacetate or butyrate esters for transesterification reactions with hexan-1-ol such that the equilibrium would be in favour of the free 3° alcohol. The enzymatic hydrolysis of tertiary alcohol esters is an interesting area as there are only three reports in the literature[199a,199b,199d] concerning this class of substrate, eg.



Results and Discussion.

Scheme 5.1

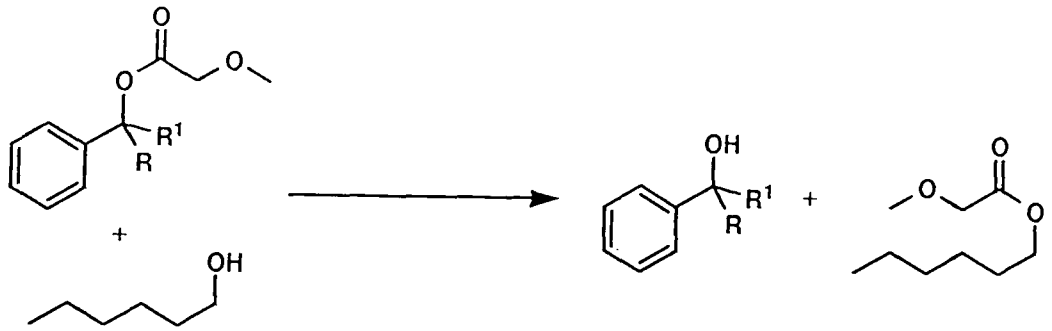
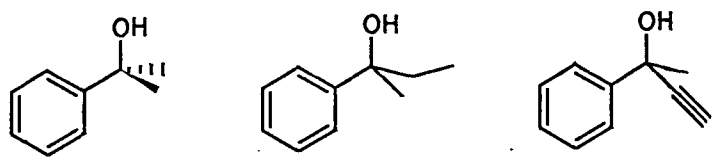
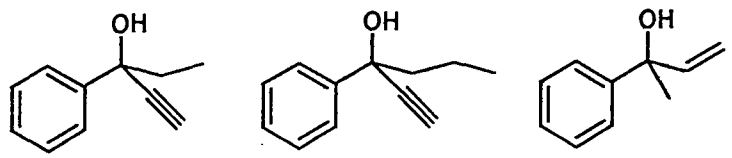


Table 5.2 summarises the data obtained from the transesterification experiments with the esters of [151] - [156]. As anticipated all of the transesterification reactions were very slow by comparison with heptan-2-ol. It is interesting that for the tertiary series the butyrate esters are processed faster that the methoxyacetate esters for many of the substrates. The fastest was the butyrate ester of 2-phenyl-2-hydroxypropanol [151b] at 0.18 $mM.h^{-1}$.



[151] [152] [153]



[154] [155] [156]

Alcohol[a]	Initial Rate mM.h^{-1} MA[b]	BUT	Equilibrium conversion MA	BUT
[151]	0.01	0.18	10% 24h	10% 24h
[152]	--[c]	0.02	--[c]	7% 24h
[153]	0.05	0.01	55% 24h	5% 24h
[154]	0.02	0.03	15% 24h	8% 24h
[155]	0.02	--	10% 24h	--
[156]	0.04	0.03	36% 24h	10% 24h

Notes a) see Fig 5.2 for structures of these alcohols.
b) MA = methoxyacetate ester, BUT = butyrate ester
c) no detectable reaction after 48h.

Table 5.2 : Results of trsnsesterification reactions with a range of tertiary alcohol esters (see figure 5.2) mediated by CCL in diethyl ether.

The butyrate ester of alcohol [151] has a faster initial rate than the corresponding methoxyacetate ester. This is further evidence that the acceleration of the methoxyacetate is not a general property of this enzyme and is subject to steric and/or conformational constraints. Similarly the study of o-, m- and p- pyridylcarbinol in Chapter 4 (page 131) displayed faster reactions with TCEB than with TCEMA. The tertiary system puts more steric demands on the enzyme than the previous secondary alcohols and so it might be expected that they behave significantly differently. It is noteworthy that under these conditions that the α-acetylene functionality is not required for substrate turnover, a feature which appears to be essential in the corresponding hydrolytic reactions in buffer.

Resolution of 3-Hydroxy-3-phenylbutyne [153].

3-Hydroxy-3-phenylbutyne [153] proved to be the best substrate in the previous series of experiments. The methoxyacetate ester of [153] was transesterified with the fastest initial rate, $0.05mMh^{-1}$, and the reaction proceeded to 55% conversion after 24h. The methoxyacetate ester of this alcohol was studied to assess the enantioselectivity of the reaction. This was of interest because aqueous hydrolyses of this compound with the same enzyme it gave only racemic material[199a] and this was judged to arise from a facile non-enzymatic hydrolysis of the acetate substrate.

In organic media however there should be no such non-enzymatic hydrolysis and therefore enzymatic selectivity can be studied.

A preparative scale reaction was conducted on 7.5mM of the butyrate ester of [153] and the reaction was allowed to proceed to 30% conversion, (48h). The product alcohol [153] was converted to the corresponding acetate ester. On analysis the product proved racemic both by optical rotation and ^1H NMR analysis with chiral shift reagent. A further reaction was conducted at 0°C in an attempt to improve the enantioselectivity. This reaction proceeded to 30% conversion in 100h but again the reaction product was racemic. These results are disappointing but can be rationalised to some extent if the product alcohol can easily racemise. The alcohol may not racemise under the enzymatic conditions but it is possible that racemisation occured during derivatisation.

Under aqueous acidic conditions the hydroxy group can be protonated leaving a relatively stable tertiary carbocation, which will rehydroxylate scrambling the stereochemistry at the chiral centre. Experiments using $H_2{}^{18}O$ on the acetate have since demonstrated that an O-alkyl cleavage does indeed occur during aqueous hydrolysis[199a]. This scrambling is suppressed when a strong electron withdrawing group eg CF_3 is attached to the chiral centre. This has been shown to be effective for the lipase mediated aqueous hydrolysis of tertiary alcohol acetates[199a], where the enantioselectivity increased from 0%ee for the CH_3 compound [153] to 87%ee for the CF_3 compound [150].

Conclusion.

For the *Candida cylindracea* lipase transesterifiactions, the secondary alcohols show a sequence of reactivity, acetylenic > vinyl > methyl. This is argued to be due to steric effects with the acetylene being sterically less demanding than vinyl or methyl groups. It has been demonstrated, in this study, that tertiary alcohols are processed by the *Candida cylindracea* lipase under transesterification conditions in organic media and in two cases [151] and [152] that an α-acetylene functionality is not required. The overall rate of reaction is sluggish and the use of methoxyacetate appears not to accelerate the reaction. Moreover it appears that the butyrate esters are processed with a faster initial rate than their corresponding methoxyacetate esters.

In the tertiary alcohol series, resolutions were difficult to achieve. This difficulty is most probably due to the stability of the tertiary carbocation generated by loss of OH. This may occur during the diffusion of the free alcohol through the hydrated water layer of the enzyme or during the derivatisation process.

Chapter 6

Experimental

General.

All ^{1}H NMR spectra were obtained on Bruker AC 250 instrument operating at 250.13MHz or a Varian Gemini 200 instrument operating at 199.97MHz. ^{13}C Spectra were obtained on a Varian Gemini instrument operating at 50.3MHz. ^{19}F spectra were obtained on a Bruker AC 250 instrument operating at 235.34MHz or a Varian VXR 400S instrument operating at 376.29MHz. Chemical shifts for ^{1}H are quoted in ppm relative to TMS in $CDCl_3$, ^{19}F shifts are quoted in ppm relative to $CFCl_3$ (fluorotrichloromethane). Chiral shift experiments were obtained on a Bruker AC 250 in CCl_4. IR spectra were recorded on Perkin Elmer 577 and 377 grating spectrometers as a neat liquid between KBr discs. Mass spectra were obtained using a VG Analytical 7070E mass spectrometer operating at 70eV. Optical rotations were obtained using a Optical Activity AA-10 automatic polarimeter.

All solvents were dried and distilled before use[208] and petrol refers to the boiling fraction 40-60°C. Reagents were used as purchased without further purification. Chromatographic separations were conducted under flash conditions using silica gel 60 (particle size 0.035-0.070mm).

GC analysis of enzyme reactions were conducted using a Perkin Elmer packed column Gas Chromatograph with a 10% OV101 (prepared in the department in Durham).

The Candida cylindracea lipase, Type on VII, was purchased from the Sigma Chemical Company.

General analytical Gas Chromatography Conditions.
Column = 10% OV101 (packed column)
Oven temp = 140°C
Detector temp = 250°C
Injector temp = 200°C

General GC analysis method (all reactions were run at least twice).

To follow the enzyme reactions aliquots were taken at intervals. The reaction was allowed to settle for a few seconds by stopping the shaker and a 100µl sample of the supernatant, without enzyme powder, was taken and placed on dry ice until needed. A 0.5µl of the sample was injected into the GC. Peak areas from the chromatogram were measured assuming that they were isoceles triangles and calculated from half base times height. Table 6.1, page 159, shows the retention times of the various substrates presented to the enzymes in this study.

Analytical, ambient temperature;

To a solution of hexan-1-ol (46mg, 0.45mM), trichloroethyl methoxyacetate (100mg, 0.45mM) and t-butylbenzene (28mg, 0.3mM) (internal standard) in diethyl ether (5ml) was added *Candida cylindracea* lipase (Sigma)(250mg) and the suspension shaken. Aliquots were taken at intervals and assayed directly by GC. Extents of conversion were calculated by comparison of the hexanol peak area to that of t-butylbenzene (see Chapter 2 page 87).

		Retention Time (mins)
TCEB	[110]	36.0
TCEMTA	[111]	40.5
TCEMA	[112]	43.0
TCEMP	[113]	51.0
MCEB	[114]	15.5
MCEMTA	[115]	17.0
MCEMA	[116]	21.0
MCEP	[117]	22.5
VA	[118]	5.0
hexan-1-ol	[120]	3.0
benzyl alcohol	[122]	21.5
6-phenhexanol	[123]	95.5
2-phenethanol	[124]	33.0
3-phenpropanol	[125]	52.5
o-pyridylcarbinol	[126]	13.0
m-pyridylcarbinol	[127]	17.5
p-pyridylcarbinol	[128]	20.5
2-thiophenemethanol	[129]	23.0
3-thiophenemethanol	[130]	23.5
2-furanmethanol	[131]	8.5
3-furanmethanol	[132]	9.5
o-pyridylethanol	[133]	27.5
m-pyridylethanol	[134]	26.5
p-pyidylethanol	[135]	28.0
2-furanethanol	[136]	18.0
2-thiopheneethanol	[137]	23.0
2-hydroxybutyne	[138]	4.0
2-hydroxybutene	[139]	3.5
2-hydroxypropane	[140]	ND
3-hydroxy-4-methylpentyne	[141]	7.0
3-hydroxy-4-methylpentene	[142]	5.5
2-hydroxy-3-methylbutane	[143]	2.0
3-hydroxynonyne	[144]	25.5
2-heptanol	[145]	9.0
3-hydroxy-2-methylnonane	[146]	36.0
3-hydroxy-3-phenylpropyne	[147]	33.5
3-hydroxy-3-phenylpropene	[148]	45.5

Table 6.1

Table of retention times in minutes of the various substrates under the standard conditions given on page 158

Analytical, elevated temperature;

To a stirred suspension of CC lipase (250mg) in cyclohexane (5ml) at 80°C was added a solution of heptan-2-ol (52mg, 0.45mM), trichloethyl butyrate (100mg, 0.45mM) and t-butylbenzene (28mg, 0.3mM) in cyclohexane (1ml). An aliquot was taken after 30s and assayed directly by GC.

Analytical, reduced temperature;

A solution of hexan-1-ol (46mg, 0.45mM), trichloroethyl methoxyacetate (100mg, 0.45mM) and t-butylbenzene (28mg, 0.3mM) in hexane (5ml) was cooled to -10°C. An aliquot was taken and assayed directly by GC. CC lipase (250mg) was added and the reaction monitored as before.

Preparative scale.

Resolution of o-pyridylethan-1-ol, [133];

To a solution of o-pyridylethan-1-ol (1.0g, 8mM), trichloroethyl methoxyacetate (1.8g, 8mM) and t-butylbenzene (250mg, 1.9mM) in diethyl ether (30ml) was added CC lipase (1g, 1 x10^5 units) and the suspension was shaken at 250rpms. The reaction was monitored by GC. At 30% conversion (6h) the enzyme was filtered off and the solvent removed under reduced pressure. The residue was chromatographed over silica gel and eluted with petrol:ethyl acetate (50:50) to afford the residual alcohol (448mg, 44% yield, 3.6mM); $[\alpha]_D^{20}$ = -2.02 (c14.9, $CHCl_3$) indicating an absolute stereochemistry of (S)-o-pyridylethan-1-ol[200]. (lit[200] $[\alpha]_D^{20}$ = -14.7 (c4.35, ethanol), 99%ee).

The product ester, o-pyridylethyl methoxyacetate, was

recovered as a colourless oil in 10% yield (17mg, 0.81mM). GC Retention time = 65mins.

^{1}H NMR ($CDCl_3$, 250MHz): δ1.78 (d, J=7.5Hz, 3H, CH_3), δ3.57 (s, 3H, CH_3), δ5.16 (s, 2H, CH_2), δ6.10 (q, J=6.75Hz, 1H, CH), δ7.36 (m, 1H, CH), δ7.48 (m, 1H, CH), δ7.82 (m, 1H, CH), δ8.70 (m, 1H, CH)

$[\alpha]_D^{20}$ = +39.8 (c0.83, $CHCl_3$) (R), chiral shift/ ^{1}H NMR in CCl_4 = 60%ee, using the δ1.78 doublet for the determination.

o-Pyridylethyl butyrate [133b];

To a solution of o-pyridylethan-1-ol (1.0g, 8mM), trichloroethyl butyrate (1.8g, 8mM) and t-butylbenzene (250mg, 1.9mM) in diethyl ether (30ml) was added CC lipase (250mg, 2.5 x10^5 units) and the reaction was monitored by GC.

The residual (S) alcohol was recovered as a colourless oil in 36.0% yield (360.6mg), $[\alpha]_D^{20}$ = -1.46 (c12.02, $CHCl_3$) (lit[200] $[\alpha]_D^{20}$ = -14.7 (c4.35, ethanol), 99%ee), and the product ester, (R) o-pyridylethyl butyrate was also recovered as a colourless oil in 12% yield (185mg, 0.93mM), GC Retention time = 48mins.

^{1}H NMR ($CDCl_3$, 250MHz) : δ1.30 (t, J=7.23Hz, 3H, CH_3), δ1.73 (m, 2H, CH_2), δ2.46 (t, J=7.25Hz, 2H, CH_2), δ1.60 (d, J=6.75Hz, 3H, CH_3), δ5.89 (q, J=6.73Hz, 1H, CH), δ7.24 (m, 1H, CH), δ7.32 (m, 1H, CH), δ7.69 (m, 1H, CH), δ8.60 (m, 1H, CH).

$[\alpha]_D^{20}$ = +7.95 (c6.2, $CHCl_3$), chiral shift/^{1}H NMR = 62.7%ee, using the δ1.6 doublet for the determination.

Resolution of 2-thiopheneethan-1-ol [137];

A solution of 2-thiopheneethan-1-ol (1.0g, 7.8mM), trichloroethyl methoxyacetate (1.7g, 7.8mM) and t-butylbenzene (250mg, 1.9mM) in ether (25ml) was treated in a similar manner to o-pyridylethan-1-ol [131]. At 30% conversion (2h) the enzyme was filtered off and the solvent removed under reduced pressure. The residue was chromatographed over silica gel and eluted with 70:30 petrol : diethyl ether to afford the residual (S) alcohol in 23.7% yield (237mg); $[\alpha]_D^{20}$ = -6.8 (c7.9, $CHCl_3$) (lit[200] (S)-thiopheneethan-1-ol $[\alpha]_D^{20}$ = -8.33 (c9.6, $CHCl_3$)).

The product ester, (R) 2-thiopheneethyl methoxyacetate, was recovered as a colourless oil in 30% yield (471mg, 2.3mM). GC retention time = 43mins

^1H NMR ($CDCl_3$, 250MHz) : δ1.78 (d, J=6.5Hz, 3H, CH_3), δ3.49 (s, 3H, CH_3), δ4.02 (s, 2H, CH_2), δ6.31 (q, J=6.5Hz, 1H, CH), δ7.03 (m, 1H, CH), δ7.13 (m, 1H, CH), δ7.32 (m, 1H, CH).

$[\alpha]_D^{20}$ = +28.7 (c15.7, $CHCl_3$), chiral shift/^1H NMR in CCl_4 = 70%ee, using the δ1.78 doublet and δ4.02 singlet for the determination.

o-Thiopheneethyl butyrate [137b];

CC lipase (250mg) was added to a solution of o-thiopheneethan-1-ol (1.0g, 7.8m), TCEB (1.7g, 7.8mM), t-butylbenzene (250mg, 1.9mM) in diethyl ether (30ml) and the reaction was carried out as above. After workup the residual (S) alcohol was isolated in 25.6% yield (256mg, 1.99mM), $[\alpha]_D^{20}$ = -12.6 (c8.5, $CHCl_3$), (lit[200] (S)-thiopheneethan-1-ol $[\alpha]_D^{20}$ = -8.33 (c9.6, $CHCl_3$)). The product ester was recovered as a colourless oil in 14% yield (218mg, 1.07mM), GC retention time = 40mins.

^1H NMR ($CDCl_3$, 250MHz) : δ0.64 (t, J=7.43Hz, 3H, CH_3), δ1.19 (d, J=6.5Hz, 3H, CH_3), δ1.29 (m, 2H, CH_2), δ1.93 (t, J=7.43Hz, 2H, CH_2), δ4.67 (q, J=6.25Hz, 1H, CH), δ6.54 (m, 2H, 2CH), δ6.80 (m, 1H, CH).

$[\alpha]_D^{20}$ = +4.7 (c6.3, $CHCl_3$), chiral shift/^1H NMR = 43.5%ee, using the δ1.19 doublet for the determination.

Resolution of 2-furanethan-1-ol [136];

CC lipase (250mg) was added to a solution of 2-furanethan-1-ol (1.0g, 8.9mM), trichloroethyl methoxyacetate (2g, 8.9mM) and t-butylbenzene (250mg, 1.9mM) in diethyl ether (25ml) and treated as above. At 50% conversion (15h) the enzyme was filtered off and the solvent removed under reduced pressure. p-Bromobenzyl chloride (500mg, 2.27mM) was added to a solution of the residue in diethyl ether (5ml) and pyridine (2ml) and the mixture refluxed for 2h. Diethyl ether (50ml) was added and the mixture washed with saturated sodium bicarbonate solution, (3 x 50ml). The organic layer was dried over $MgSO_4$ and the solvent removed under reduced pressure. The crystalline residue was chromatographed over silica gel and eluted with 50 : 50 petrol : diethyl ether to afford (S) 2-furanethan-1-yl p-bromobenzoate as an off white crystalline solid in 16% yield (411mg, 1.4mM), chiral shift/^{1}H NMR = 37.1%ee, using the δ1.47 doublet for the determination.

^{1}H NMR ($CDCl_3$, 250MHz) : δ1.47 (d, J=7.7Hz, 3H, CH_3), δ4.81 (q, J=8.1Hz, 1H, CH), δ6.15 - δ6.27 (m, 4H, Ar), δ7.29 (m, 1H, CH), δ7.49 (m, 1H, CH), δ7.83 (m, 1H, CH).

^{13}C NMR ($CDCl_3$, 50.3MHz) : δ21.75, δ64.19, δ105.59, δ110.61, δ131.6, δ132.13, δ132.21, δ132.42, δ142.40.

Resolution of 3-hydroxy-3-phenylbutyne [153].

CC lipase (1g, 1.4 X10^5 units) was added to a solution of 3-hydroxy-3-phenylbutyne methoxyacetate (500mg, 2.3mM), hexanol (250mg, 2.5mM) and t-butylbenzene (100mg, 0.75mM) in diethyl ether (25ml) and the reaction shaken at 250rpm. At 30% conversion (24h), the enzyme was filtered and the solvent removed under reduced pressure. The residue was chromatographed over silica gel and eluted with 50 : 50 petrol : diethyl ether affording the residual ester in 50% yield (250mg, 1.15mM) and the alcohol, 3-hydroxy-3-phenylbutyne, as a colourless oil in 27% yield

(90mg, 0.63mM). The optical rotations of both alcohol and residual ester were zero, indicating racemic material.

To a solution of DCC (200mg, 0.96mM) acetic acid (100mg, 1.66mM) and DMAP (5mg, 0.04mM) in diethyl ether (10ml) was added a solution of the product alcohol [153] (90mg, 0.63mM) in diethyl ether (10ml) and the mixture stirred for 2h. The solution was filtered and the solvent removed under reduced pressure. The residue was chromatographed over silica gel and eluted with 50 : 50 petrol : diethyl ether. The recovered acetate was judged racemic after chiral shift analysis in CCl_4.

Synthesis.

Trichloroethyl butyrate [110];

To a stirred solution of butyryl chloride (4.8g, 45.2mM) in methylene chloride (30ml) was added 2,2,2-trichloroethanol (6.7g, 45.2mM) and pyridine (3.6g, 45.2mM) and the reaction was heated under reflux for 3h. The reaction mixture was washed with brine, (4 x 50ml), dried over $MgSO_4$ and the solvent removed under reduced pressure. The residue was distilled, (28°C at 0.02mmHg), to afford [110] as a colourless oil in 80% yield (8g, 36.3mM).

^1H NMR ($CDCl_3$, 250MHz) : δ0.99 (t, J=4.46Hz, 3H, CH_3), δ1.73 (m, 2H, CH_2), δ2.45 (t, J=4.84Hz, 2H, CH_2), δ4.74 (s, 2H, CH_2CCl_3), lit[166a]

IR(neat):ν_{max}/cm^{-1} 2980 (C-H), 1760 (C=O), 720.

MS (CI) : m/z = 219 (M^+)

Trichloroethyl methylthioacetate [111];

A stirred solution of methylthioacetic acid (3.0g, 28.3mM) and thionyl chloride (3.4g, 28.3mM) in methylene chloride (25ml) was refluxed for 3h (until the evolution of SO_2/HCl had ceased). 2,2,2-Trichloroethanol (4.2g ,28.3mM) and then pyridine (2.2g ,28.3mM) was added and the mixture heated under reflux for a further 3h. The cooled solution was washed with brine (4 x 50ml), dried ($MgSO_4$) and the solvent removed under reduced pressure. The residue was distilled, (58°C at 0.01mmHg), to afford [111] as a colourless oil in 66% yield (4.7g, 18.7mM).

^{1}H NMR ($CDCl_3$, 250MHz) : δ2.27 (s, 3H, CH_3), δ3.33 (s, 2H, CH_2), δ4.81 (s, 2H, CH_2CCl_3).

IR (Neat) ν_{max}/cm^{-1} : 2920 (C-H), 1750 (C=O).

MS (CI) : m/z = 237 (M^+)

Trichloroethyl methoxyacetate [112];

A solution of methoxyacetic acid (5.0g, 55.5mM) and thionyl chloride (6.6g ,55.5mM) in diethyl ether (25ml) was refluxed for 3h (until the evolution of SO_2/HCl had ceased). 2,2,2-Trichloroethanol (5.27ml) and pyridine (4.5ml) were added and the reaction was refluxed for a further 3h. The reaction was cooled and washed with brine (4 x 50ml), dried ($MgSO_4$) and the solvent removed under reduced pressure. The residue was distilled, (44°C at 0.1mmHg), to afford [112] as a colourless oil in 78% yield (9.4g, 43.5mM).

^{1}H NMR ($CDCl_3$, 250MHz) : δ3.54 (s, 3H, CH_3), δ4.26 (s, 2H, OCH_2), δ4.89 (s, 2H, CH_2CCl_3).

IR (Neat) ν_{max}/cm^{-1} : 2940 (C-H), 1775 (C=O)

MS (CI) : m/z = 221 (M^+)

Trichloroethyl methoxypropionate [113];

A solution of methoxypropionic acid (1.6g, 15.4mM) and thionyl chloride (1.8g, 15.4mM) in methylene chloride (30ml) was heated under reflux for 3h. 2,2,2-Trichloroethanol (2.3g, 15.4mM) and pyridine (1.2g, 15.4mM) were added and the mixture was refluxed for a further 3h. The cooled mixture was washed with brine (4 x 50ml), dried ($MgSO_4$) and the solvent removed under reduced pressure. The residue was distilled, (36°C at 0.03mmHg), to afford [113] as a colourless oil in 58% yield (2.1g, 9.01mM).

^1H NMR ($CDCl_3$, 250Hz) : δ2.72 (t, J=6.16Hz, 2H, CH_2), δ3.37 (s, 3H, CH_3), δ3.72 (t, J=6.17Hz, 2H, OCH_2), δ4.75 (s, 2H, CH_2CCl_3).

IR (Neat) ν_{max}/cm^{-1} : 2880 (C-H), 1750 (C=O).

MS (CI) : m/z = 235 (M^+)

Ethyl fluorobutyrate;

Silver (I) fluoride[201b] (2.0g, 15.8mM) was added to a stirred solution of ethyl 2-bromobutyrate (1.5g, 7.7mM) in acetonitrile (20ml) and water (1ml). The mixture was stirred at 18°C for 24h and then diethyl ether (50ml) was added. The mixture was filtered, dried ($MgSO_4$) and the solvent removed under reduced pressure. The residue was distilled, (61°C, 60mmHg), to afford the product as a colourless oil in 86% yield (890mg, 6.6mM).

^1H NMR ($CDCl_3$, 250MHz) : δ1.04 (t, J=7.39Hz, 3H, CH_3), δ1.32 (t, J=7.22Hz, 3H, CH_3), δ1.84-2.02 (m, 2H, CH_2), δ4.26 (q, J=7.20Hz, 2H, CH_2), δ4.86 (dm, $^2J_{PH}$=49.05Hz, $^3F_{PH}$=24.41Hz, 1H, CH)

^{19}F NMR ($CDCL_3$, 400MHz) : -δ193.98 (dt, $^2J_{PH}$=49.10Hz, ^3J=24.43Hz, 1F, CHF), lit[201].

o-Pyridylethan-1-ol [133];

$NaBH_4$ (1.5g, 39.6mM) was added in small portions to a stirred solution of o-acetylpyridine (3.0g, 24.6mM) in 50:50 diethyl ether : ethanol (30ml) and the mixture stirred for 2h. Acetone (2ml) and diethyl ether (50ml) were added and the mixture washed with water (2 x 5ml). The solvent removed was under reduced pressure and the residue was taken up in diethyl ether (50ml), dried ($MgSO_4$) and the solvent was removed under reduced pressure to afford [133] as a colourless oil in 68% yield (2g, 16.6mM).

^1H NMR ($CDCl_3$, 250MHz) : δ1.49 (d, J=6.53Hz, 3H, CH_3), δ4.31 (s, 1H, OH), δ4.88 (q, J=6.42Hz, 1H, CH), δ7.10-7.30 (m, 2H, CH=CH), δ7.60-7.73 (m, 1H, CH), δ8.52 (d, J=4.25Hz, 1H, CH), lit[202].

IR (Neat) ν_{max}/cm^{-1} : 3280 (v broad OH), 2970, 1595, 900, 785, 750.

m-Pyridylethan-1-ol [134];

$NaBH_4$ (1.5g, 39.6mM) was added in small portions to a stirred solution of m-acetylpyridine (3.0g, 24.6mM) in 50:50 diethyl ether : ethanol (30ml) and the mixture was stirred for 2h. Acetone (2ml) and diethyl ether (50ml) were added and the mixture was washed with water (2 x 5ml). The solvent was removed under reduced pressure. The residue was taken up in diethyl ether (50ml), dried ($MgSO_4$) and the solvent removed under reduced pressure to afford [134] as a colourless clear oil in 64% Yield (1.9g, 15.9mM)

^1H NMR ($CDCl_3$, 200MHz) : δ1.47 (d, J=6.48Hz, 3H, CH_3), δ4.87 (q, J=6.46Hz, 1H, CH), δ7.22-7.27 (m, 1H, CH), δ7.65-7.73 (m, 1H, CH), δ8.30-8.43 (m, 2H, CH=CH) lit[202].

p-Pyridylethan-1-ol [135];

$NaBH_4$ (1.5g, 39.6mM) was added in small portions to a stirred solution of p-acetylpyridine (3.0g, 24.6mM) in 50:50 diethyl ether : ethanol (30ml) and the mixture was stirred for 2h. Acetone (2ml) and diethyl ether (50ml) were added and the mixture was washed with water (2 x 5ml). The solvent was removed under reduced pressure and the residue was taken up in diethyl ether (50ml), dried ($MgSO_4$) and the solvent removed under reduced pressure to afford [135] as a colourless oil in 71% Yield (2.2g, 17.5mM)

^1H NMR ($CDCl_3$, 200MHz) : δ1.44 (d, J=6.56Hz, 3H, CH_3), δ4.89 (q, J=6.60Hz, 1H, CH), δ7.38 (d, J=6.02Hz, 2H, m-Pyr), δ8.49 (d, J=4.98, 2H, o-Pyr), lit[202].

2-Furanethan-1-ol [136];

$NaBH_4$ (1g, 24.6mM) was added in small portions to a stirred solution of 2-acetylfuran (2.0g, 18mM) in methanol (20ml) and the mixture stirred for 2 hours. Acetone (2ml) was added and the reaction was stirred for 15min. Diethyl ether (100ml) was then added and the mixture was washed with water (2 x 5ml) and the solvent removed under reduced pressure. The residue was taken up in diethyl ether (50ml), dried ($MgSO_4$) and the solvent removed under reduced pressure. The title alcohol[136] was recovered as a colourless oil in 73% yield (1.5g, 13.3mM)

^1H NMR ($CDCl_3$, 250MHz) : δ1.50 (d, J=6.58Hz, 3H, CH_3), δ2.74 (s, 1H, OH), δ4.83 (q, J=6.54Hz, 1H, CH), δ6.10-6.32 (m, 3H, furan), lit[202].

2-Thiopheneethan-1-ol [137];

$NaBH_4$ (1g, 24.6mM) was added in small portions to a stirred solution of 2-acetylthiophene (2.0g, 15.75mM) in methanol (20ml) and the mixture stirred for 2h. Acetone (2ml) was added and the reaction mixture was stirred for a further 15min. Diethyl ether (100ml) was added and then the reaction was then washed with water (2 x 5ml) and the solvent removed under reduced pressure. The residue was taken up in diethyl ether (50ml), dried ($MgSO_4$) and the solvent removed under reduced pressure. The title alcohol [137] was recovered as a colourless oil in 64% yield (1.3g, 10.1mM).

^1H NMR ($CDCl_3$, 200MHz) : δ1.58 (d, J=6.42Hz, 3H, CH_3), δ2.69 (s, 1H, OH), δ5.09 (q, J=6.46Hz, 1H, CH), δ6.90-6.97 (m, 2H, CH-CH), δ7.20-7.25 (m, 1H, CH) lit[202].

2-Pyrroleethan-1-ol [138];

$NaBH_4$ (1g, 24.6mM) was added in small portions to a stirred solution of 2-acetylpyrrole (2.0g, 18.35mM) in methanol (20ml) and the mixture was stirred for 2h. Acetone (2ml) was added and the reaction was stirred for a further 15min. Diethyl ether (100ml) was added and the mixture washed with water (2 x 5ml) and the solvent removed under reduced pressure. The residue was taken up in diethyl ether (50ml), dried ($MgSO_4$) and the solvent was removed under reduced pressure. The title alcohol [138] was recovered as a colourless oil in 59% yield (1.2g, 10.8mM)

^1H NMR ($CDCl_3$, 200MHz) : δ1.51 (d, J=6.60Hz, 3H, CH_3), δ2.91 (s, 1H, OH), δ4.70-4.95 (m, 1H, CH), δ6.00-6.18 (m, 2H, CH-CH), δ6.67-6.72 (m, 1H, CH), δ8.90 (s, 1H, NH), lit[202].

3-Hydroxy-4-methylpentyne [141];

Ethynylmagnesium bromide (30ml of 0.5M, 15mM) was added to a stirred solution of iso-butyraldehyde (1.0g, 13.9mM) in THF (25ml) and the mixture was heated under reflux for 1h. The cooled mixture was poured into saturated $NaHCO_3$ solution (50ml) and then filtered. The precipitate was washed with diethyl ether and the filtrate extracted into diethyl ether (4 x 25ml). The ethereal extracts were combined, dried ($MgSO_4$) and the solvent removed under reduced pressure. The residue was distilled, (21°C at 0.5mmHg), to afford [141] as a colourless oil in 60% yield (0.87g, 8.34mM).

^1H NMR ($CDCl_3$, 250MHz) : δ1.02 (dd, J=5.11Hz J=6.62, 6H, $2CH_3$) δ1.89 (m, 1H, CH), δ1.99 (s, 1H, OH), δ2.46 (d, J=1.94Hz, 1H, CH), δ4.18 (dd, J=1.99Hz J=5.64Hz, 1H, CH), lit[203].

IR (Neat) ν_{max}/cm^{-1} : 3380 (broad OH), 3295, 2960, 2140.

3-Hydroxy-4-methylpentene [142];

Vinylmagnesium bromide (42ml of 1M, 42mM) was added to a stirred solution of iso-butyraldehyde (3.0g, 41.7mM) in THF (25ml) and the mixture was heated under reflux for 2h. The cooled mixture was poured into saturated $NaHCO_3$ solution (50ml) and filtered. The precipitate was washed with diethyl ether and the filtrate extracted into diethyl ether (4 x 20ml). The ethereal washings were combined, dried ($MgSO_4$) and the solvent was removed under reduced pressure. The residue was distilled, (18°C at 0.01mmHg), to afford [142] as a colourless oil in 56% yield (2.3g, 23.3mM).

^1H NMR ($CDCl_3$, 250MHz) : δ0.91 (dd, J=4.25Hz J=8.25Hz, 6H $2CH_3$), δ1.72 (m, 1H, CH) δ2.52 (s, 1H, OH), δ3.83 (dd, J=4.0Hz J=3.88Hz, 1H, CH), δ5.16 (m, 2H, CH_2), δ5.85 (m, 1H, CH) lit[204]. ^1H COSY was consistent with this structure.

IR (Neat) ν_{max}/cm^{-1} : 3480 (Broad OH), 3090, 2960, 1850, 1710, 1645.

3-Hydroxy-3-phenylpropyne [147];

Ethynylmagnesium bromide (38ml of 0.5M, 19mM) was added to a stirred solution of benzaldehyde (2.0g, 18.9mM) in THF (20ml) and the mixture was heated under reflux for 1h. The cooled solution was poured into saturated $NaHCO_3$ solution (50ml), filtered and the precipitate was washed with ether. The filtrate was extracted into diethyl ether (4 x 25ml) and the ethereal washings were combined, dried ($MgSO_4$) and the solvent removed under reduced pressure. The residue was distilled, (36°C at 0.01mmHg), to afford [147] as a colourless oil in 56% yield (1.4g, 10.6mM).

^1H NMR ($CDCl_3$, 250MHz) : δ2.67 (d, J=2.67Hz, 1H, CH), δ4.46 (d, J=2.77, 1H, CH), δ7.35-7.59 (m, 5H, Ph) lit[205].

IR (Neat) ν_{max}/cm^{-1} : 3300cm^{-1} (v broad, OH), 3040, 2880, 2100.

3-Hydroxy-3-phenylpropene [148];

Vinylmagnesium bromide (24ml of 1M, 24mM) was added to a stirred solution of benzaldehyde (2.5g, 23.6mM) in THF (25ml) and the mixture heated under reflux for 2h. The cooled solution was poured into saturated $NaHCO_3$ solution (50ml), filtered and the precipitate was washed with diethyl ether. The filtrate was extracted into diethyl ether (4 x 25ml) the ethereal washings were combined, dried ($MgSO_4$) and the solvent removed under reduced pressure. The residue was distilled, (37°C at 0.05mmHg), to afford [148] as a colourless oil in 54% yield (0.68g, 12.6mM).

^1H NMR ($CDCl_3$, 250MHz) : δ2.57 (m, 1H, CH), δ5.09-5.32 (m, 2H, CH_2), δ5.92-6.06 (m, 1H, CH), δ7.22-7.32 (m, 5H, Ph), lit[209].

IR (Neat) ν_{max}/cm^{-1} : 3360 (broad, OH), 3060, 2360, 1635, 1600, 925, 830, 710, 700.

MS (CI) : m/z = 135 (M^+)

3-Hydroxynonyne [144];

Ethynylmagnesium bromide (28.6ml of 0.5M, 14.3mM) was added to a stirred solution of heptaldehyde (1.6g, 14mM) in THF (25ml) and the mixture heated under reflux for 1h. The cooled solution was poured into saturated $NaHCO_3$ solution (50ml), filtered and the precipitate was washed with diethyl ether. The filtrate was extracted into diethyl ether (4 x 25ml) and the ethereal extracts were combined, dried ($MgSO_4$) and the solvent removed under reduced pressure. The residue was distilled, (42.5°C at 0.55mmHg), to afford [144] as a colourless oil in 56% yield (1.1g, 7.85mM).

^1H NMR ($CDCl_3$, 250MHz) : δ0.89 (t, J=7.4Hz, 3H, CH_3), δ1.29 (m, 8H, $4CH_2$), δ1.43 (m, 2H, CH_2), δ2.08 (s, 1H, OH), δ2.46 (d, J=3.25Hz, 1H, CH), δ4.36 (dt, J=7.53Hz J=2.35Hz, 1H, CH) lit[206].

IR (Neat) ν_{max}/cm^{-1} : 3300 (broad, OH), 2920, 2090 (sharp), 1680.

2-Hydroxy-2-phenylpropane methoxyacetate [151a];

Methoxyacetic acid (1.3g, 14.6mM) was added to a stirred solution of DCC (3g, 14.5mM) in diethyl ether (30ml) and the mixture was stirred for 15min. 2-Hydroxy-2-phenylpropane (2g, 14.7mM) and DMAP (10mg, 0.08mM) were then added. The mixture was stirred for 24h at 18°C. the reaction was filtered and the solvent removed under reduced pressure. The residue was chromatographed over silica gel and eluted with methylene chloride to afford [151a] as a yellow oil in 64% yield (1.9g, 9.27mM)

^1H NMR ($CDCl_3$, 250MHz) : δ1.81 (s, 6H, $2CH_3$), δ3.41 (s, 3H, OCH_3), δ3.99 (s, 2H, OCH_2), δ7.20-7.40 (m, 5H, Ph)

IR (Neat) ν_{max}/cm^{-1} : 2980 (v broad), 1755 (C=O), 1600 (Ar c=c), 765 (Ar C-H), 700 (Ar C-H)

MS (CI) : m/z = 223 (M^+ + H)

2-Hydroxy-2-phenylpropane butyrate [151b];

Butyllithium (2ml, 3.6mM) was added to a stirred solution of 2-hydroxy-2-phenylbutane (500mg, 3.6mM) in diethyl ether (30ml) at -78°C. The solution was stirred for 10min and then butyryl chloride (383mg, 3.6mM) was added and the solution allowed to warm to ambient temperature with stirring. The solution was filtered and the solvent removed under reduced pressure. The residue was chromatographed over silica gel and eluted with petrol : diethyl ether 80:20 to afford [151b] as a yellow oil in 74% yield (545mg, 2.64mM).

^{1}H NMR ($CDCl_3$, 250MHz) : δ0.93 (t, J=7.42Hz, 3H, CH_3), δ1.65 (m, 2H, CH_2), δ1.76 (s, 6H, $2CH_3$), δ7.23-7.35 (m, 5H, Ph).

IR (Neat) ν_{max}/cm^{-1} : 2970, 1735 (C=O), 1605 (Ar C=C), 765 (Ar C-H), 700 (Ar C-H).

MS (CI) : m/z = 221 (M^+ + H)

2-Hydroxy-2-phenylbutane methoxyacetate [152a];

Methoxyacetic acid (1.3g, 14.6mM) was added to a stirred solution of DCC (3g, 14.5mM) in diethyl ether (30ml) and the mixture was stirred for 15minutes. 2-Hydroxy-2-phenylbutane (2.2g, 14.7mM) and DMAP (10mg, 0.08mM) were then added and the reaction stirred at 18°C for 24h. The reaction was filtered and the solvent removed under reduced pressure. The residue was chromatographed over silica gel and eluted with methylenechloride. The title compound [152a] was recovered as a yellow oil in 63% yield (2.0g, 9.2mM).

^{1}H NMR ($CDCl_3$, 200MHz) : δ0.79 (t, J=7.40Hz, 3H, CH_3), δ1.86 (s, 3H, CH_3), δ2.08 (m, 2H, CH_2), δ3.44 (s, 3H, OCH_3), δ4.04 (s, 2H, OCH_2), δ7.20-7.35 (m, 5H, Ph).

IR (Neat) ν_{max}/cm^{-1} : 2960 (C-H), 1750 (C=O), 1600 (Ar C=C), 760 (Ar C-H), 700 (Ar C-H).

MS (CI) : m/z = 223 (M^+ + H)

Analysis : C = 70.1%, H = 8.1% (required for $C_{13}H_{18}O_3$ C = 70.35%, H = 8.25%)

2-Hydroxy-2-phenylbutane butyrate [152b];

Butyllithium (1.8ml of 1.8M, 3.24mM) was added to a stirred solution of 2-hydroxy-2-phenylbutane (500mg, 3.33mM) in diethyl ether (30ml) at -78°C. The solution was stirred for 10min and then butyryl chloride (355mg, 3.7mM) was added and the solution allowed to warm to ambient temperature with stirring. The reaction was worked up as for [151b] to afford [152b] as a colourless oil in 78% yield (571mg, 2.6mM).

^{1}H NMR ($CDCl_3$, 200MHz) : δ0.78 (t, J=7.50Hz, 3H, CH_3), δ0.96 (t, J=7.34Hz, 3H, CH_3), δ1.65 (m, 2H, CH_2), δ1.82 (s, 3H, CH_3), δ2.03 (dq, J=1.74Hz J=7.46Hz, 2H, CH_2) δ2.31 (t, J=7.14Hz, 2H, CH_2), δ7.20-7.40 (m, 5H, Ph).

IR (Neat) ν_{max}/cm^{-1} : 2960 (C-H), 1735 (C=O), 1600 (Ar C=C), 760 (Ar C-H), 730 (Ar C-H), 700 (Ar C-H).

MS (CI) : m/z = 221 (M^+ + H)

3-Hydroxy-3-phenylbutyne [153];

Ethynylmagnesium bromide (84ml of 0.5M, 42mM) was added to a stirred solution of acetophenone (5.0g, 41.7mM) in THF (50ml) and the mixture heated under reflux for 2h. The cooled solution was poured into saturated $NaHCO_3$ solution (50ml) and filtered. The precipitate was washed with diethyl ether and the filtrate extracted into diethyl ether (4 x 30ml). The ethereal washings were combined, dried ($MgSO_4$) and the solvent removed under reduced pressure. The residue was distilled, (45°C at 0.02mmHg), to afford [153] as a colourless oil in 48% yield (2.9g, 19.8mM).

^{1}H NMR ($CDCl_3$, 200MHz) : δ1.80 (s, 3H, CH_3), δ2.68 (s, 1H, CH), δ7.28-7.68 (m, 5H, Ph), lit[209].

IR (Neat) n_{max}/cm^{-1} : 3400 (broad OH), 3000 (C-H), 2130 (alkyne), 1670, 1600 (Ar C=C), 750 (Ar C-H), 700 (Ar C-H).

MS (CI) : m/z = 147 (M^+ + H)

3-Hydroxy-3-phenylbutyne methoxyacetate [153a];

Methoxyacetic acid (1.2g, 13.7mM) was added to a stirred solution of DCC (2.8g, 13.7mM) in diethyl ether (30ml) and the solution stirred for 15min. 3-Hydroxy-3-phenylbutyne (2.0g, 13.7mM) and DMAP (10mg, 0.08mM) were then added and the mixture stirred at 18°C for 24h. The reaction was worked up as for [149a] to afford [153a] as a colourless oil in 85% yield (2.5g, 11.65mM).

^{1}H NMR ($CDCl_3$, 200MHz) : δ1.95 (s, 3H, CH_3), δ2.85 (s, 1H, CH), δ3.43 (s, 3H, OCH_3), δ4.04 (s, 2H, OCH_2), δ7.25-7.65 (m, 5H, Ph).

IR (Neat) ν_{max}/cm^{-1} : 3280 (alkyne C-H), 2980 (alkane C-H), 1770 (C=O), 765 (Ar C-H), 735 (Ar C-H), 700 (Ar C-H).

MS (CI) : m/z = 220 (M^+ + H)

3-Hydroxy-3-phenylbutyne butyrate [153b];

Butyllithium (1.9ml of 1.8M, 3.42mM) was added to a stirred solution of 3-hydroxy-3-phenylbutyne (500mg, 3.4mM) in diethyl ether at -78°C. The reaction was stirred for 10mins and then butyryl chloride (362mg, 3.4mM) was added and the reaction allowed to warm to ambient temperature with stirring. The reaction was worked up as for [151b] to afford [153b] as a colourless oil in 86% yield (636mg, 2.9mM).

^{1}H NMR ($CDCl_3$, 250MHz) : δ0.99 (t, J=7.39Hz, 3H; CH_3), δ1.55-1.77 (m, 2H, CH_2), δ1.89 (s, 3H, CH_3), d2.43 (t, J=7.29Hz, 2H, CH_2), d2.80 (s, 1H, CH), δ7.24-7.64 (m, 5H, Ph).

IR (Neat) n_{max}/cm^{-1} : 3300 (alkyne C-H), 2970 (alkane C-H), 1760 (C=O), 770 (Ar C-H), 735 (Ar C-H), 700 (Ar C-H).

MS (CI) : m/z = 221 (M^+ + H).

3-Hydroxy-3-phenylpentyne [154];

Ethynylmagnesium bromide (15ml of 0.5M, 7.5mM) was added to a stirred solution of propiophenone (1.0g, 7.5mM) in THF (25ml) and the mixture was heated under reflux for 2h. The cooled mixture was poured into saturated $NaHCO_3$ solution (50ml) and filtered. The precipitate was washed with diethyl ether and the filtrate extracted into diethyl ether (4 x 25ml). The ethereal washings were combined, dried ($MgSO_4$) and the solvent removed under reduced pressure. The residue was chromatographed over silica gel and eluted with petroleum ether : diethyl ether (90:10) to afford [154] as a colourless oil in 75% yield (0.9g, 5.62mM).

^1H NMR ($CDCl_3$, 250MHz) : δ0.95 (dt J=2.25Hz J=9.27Hz, 3H, CH_3), δ1.96 (dq J=2.45Hz J=9.23Hz, 2H, CH_2), δ2.52 (s, 1H, OH), δ2.68 (s, 1H, CH), δ7.20-7.70 (m, 5H, Ph), lit[199a].

IR (Neat) ν_{max}/cm^{-1} : 3400 (broad OH), 2990 (C-H), 1675, 1600 (Ar C=C), 750 (Ar C-H), 700 (Ar C-H).

MS (CI) : m/z = 161 (M^+ + H).

3-hydroxy-3-phenylpentyne methoxyacetate [154a];

Methoxyacetic acid (563mg, 6.25mM) was added to a stirred solution of DCC (1.3g, 6.25mM) in diethyl ether (30ml). 3-Hydroxy-3-phenylpentyne (1.0g, 6.25mM) and DMAP (10mg) were then added and the mixture stirred at 18°C for 24h. The reaction was worked up as for [149a] to afford [152a] as a colourless oil in 79% yield (1.14g, 4.93mM).

^1H NMR ($CDCl_3$, 200MHz) : δ0.94 (t, J=7.38Hz, 3H, CH_3), δ1.95-2.35 (m, 2H, CH_2), δ2.85 (s, 1H, CH), δ3.43 (s, 3H, OCH_3), δ4.04 (s, 2H, OCH_2), δ7.26-7.60 (m, 5H, Ph).

IR (Neat) ν_{max}/cm^{-1} : 2940 (C-H), 1725 (C=O), 1590 (Ar C=C), 720 (Ar C-H).

MS (CI) : m/z = 233 (M^+ + H).

3-Hydroxy-3-phenylpentyne butyrate [154b];

Butyllithium (1.7ml of 1.8M, 3.06mM) was added to a stirred solution of 3-hydroxy-3-phenylpentyne (500mg, 3.12mM) in diethyl ether (30ml) at -78°C. The solution was stirred for 10min and then butyryl chloride (335.5mg, 3.12mM) was added and the solution allowed to warm to ambient temperature with stirring. The reaction was worked up as for [149b] to afford [152b] as a colourless oil in 83% yield (595mg, 2.59mM).

^{1}H NMR ($CDCl_3$, 200MHz) : δ0.94 (2t, 6H, $2CH_3$), δ1.55-1.75 (m, 2H, CH_2), δ1.90-2.25 (m, 2H, CH_2), δ2.27-2.36 (m, 2H, CH_2), δ2.81 (s, 1H, CH), δ7.25-7.58 (m, 5H, Ph).

IR (Neat) ν_{max}/cm^{-1} : 3280 (alkyne C-H), 2970 (alkane C-H), 1745 (C=O), 1600 (Ar C=C), 760 (Ar C-H), 700 (Ar C-H).

MS (CI) : m/z = 231 (M^+ + H).

3-Hydroxy-3-phenylhexyne [155];

Ethynylmagnesium bromide (68ml of 0.5M, 34mM) was added to a stirred solution of phenylbutan-1-one (5.0g, 34mM) in THF (25ml) and the mixture heated under reflux for 2h. The cooled solution was poured into saturated $NaHCO_3$ solution (50ml) and filtered. The precipitate was washed with diethyl ether and the filtrate was extracted into diethyl ether (4 x 25ml). The ethereal washings were combined, dried ($MgSO_4$) and the solvent removed under reduced pressure. The residue was distilled, (55°C at 0.02mmHg), to afford [153] as a colourless oil in 61% yield (3.6g, 20.7mM).

^{1}H NMR ($CDCl_3$, 200MHz) : δ0.85 (t, J=7.34Hz, 3H, CH_3), δ1.15-1.35 (m, 2H, CH_2), δ1.85-2.25 (m, 2H, CH_2), δ2.63 (s, 1H, CH), δ7.25-7.60 (m, 5H, Ph), lit[199a].

IR (Neat) ν_{max}/cm^{-1} : 3400 (broad OH), 2990 (C-H), 1600 (Ar C=C), 760 (Ar C-H), 700 (Ar OH).

MS (CI) : m/z = 175 (M^+ + H).

3-Hydroxy-3-phenylhexyne methoxyacetate [155a];

Methoxyacetic acid (517mg, 5.75mM) was added to a stirred solution of DCC (1.3g, 5.75mM) in diethyl ether (30ml). The reaction was stirred for 15min and then 3-hydroxy-3-phenylhexyne (1g, 5.75mM) and DMAP (10mg, 0.08mM) were added and the reaction stirred at 18°C for 24h. The reaction was worked up as for [151a] to afford [155a] as a colourless oil in 59% yield (834mg, 3.39mM).

^{1}H NMR ($CDCl_3$, 200MHz) : δ0.88 (t, J=7.38Hz, 3H, CH_3), δ1.15-1.35 (m, 2H, CH_2), δ1.85-2.25 (m, 2H, CH_2), δ2.85 (s, 1H, CH), δ3.42 (s, 3H, OCH_3), δ4.03 (s, 2H, OCH_2), δ7.20-7.60 (m, 5H, Ph).

IR (Neat) ν_{max}/cm^{-1} : 3280 (alkyne C-H), 2940 (alkane C-H), 2120 (alkyne C-C), 1765 (C=O), 730 (Ar C-H), 700 (Ar C-H).

MS (CI) : m/z = 247 (M^+ + H).

3-Hydroxy-3-phenylhexyne butyrate [155b];

Butyllithium (1.6ml of 1.8M, 2.88mM) was added to a stirred solution of 3-hydroxy-3-phenylhexyne (500mg, 2.87mM) in diethyl ether (30ml) at -78°C. The solution was stirred for 10min and then butyryl chloride (305.7mg, 2.87mM) was added and the reaction allowed to warm to ambient temperature with stirring. The reaction was worked up as for [151b] to afford [155b] as a colourless oil in 92% yield (644mg, 2.64mM).

^{1}H NMR ($CDCl_3$, 200MHz) : δ0.85-0.98 (2t, 6H, $2CH_3$), δ1.50-1.70 (m, 4H, $2CH_2$), δ1.85-2.20 (m, 2H, CH_2), δ2.31 (dt, J=1.34Hz J=7.52Hz, 2H, CH_2), δ2.81 (s, 1H, CH), δ7.25-7.56 (m, 5H, Ph).

IR (Neat) ν_{max}/cm^{-1} : 3270 (alkyne C-H), 2940 (alkane C-H), 2120 (alkyne C-C), 1740 (C=O), 1600 (Ar C=C), 760 (Ar C-H), 700 (Ar C-H).

MS (CI) : m/z = 245 (M^+ + H).

Analysis : C = 78.5%, H = 8.1% (required for $C_{16}H_{20}O_2$ C = 77.67%, H = 8.54%)

3-Hydroxy-3-phenylbutene [156];

Vinylmagnesium bromide (42ml of 1M, 42mM) was added to a stirred solution of acetophenone (5.0g, 42mM) in THF (25ml) and the mixture heated under reflux for 2h. The cooled solution was poured into saturated $NaHCO_3$ solution (50ml) and filtered. The precipitate was washed with diethyl ether and the filtrate was extracted into diethyl ether (4 x 25ml). The ethereal washings were combined, dried ($MgSO_4$) and the solvent removed under reduced pressure. The residue was distilled, (43°C at 0.05mmHg), to afford [156] as a colourless oil in 59% yield (3.7g, 24.78mM).

^1H NMR ($CDCl_3$, 250MHz) : δ1.66 (s, 3H, CH_3), δ5.12-5.33 (m, 2H, CH_2), δ6.12-6.23 (m, 1H, CH), δ7.25-7.50 (m, 5H, Ph), lit[207].

IR (Neat) ν_{max}/cm^{-1} : 3300 (broad OH), 2970 (C-H), 2250, 1600 (Ar C=C), 915 (Vinyl C-H), 730 (Ar C-H), 700 (Ar C-H).

MS (CI) : m/z = 149 (M^+ + H).

3-Hydroxy-3-phenylbutene methoxyacetate [156a];

Methoxyacetic acid (607.5mg, 6.75mM) was added to a stirred solution of DCC (1.4g, 6.75mM) in diethyl ether (30ml). The solution was stirred for 15min and then 3-hydroxy-3-phenylbutene (1.0g, 6.75mM) and DMAP (10mg) were added and the reaction stirred at 18°C for 24h. The reaction was worked up as for [151a] to afford [156a] as a colourless oil in 56% yield (832mg, 3.78mM).

^1H NMR ($CDCl_3$, 200MHz) : δ1.93 (s, 3H, CH_3), δ3.44 (s, 3H, OCH_3), δ4.04 (s, 2H, OCH_2), δ5.20-5.33 (m, 2H, CH_2), δ6.25-6.35 (m, 1H, CH), δ7.20-7.38 (m, 5H, Ph).

IR (Neat) ν_{max}/cm^{-1} : 2940 (C-H), 2250, 1750 (C=O), 1600 (Ar C=C), 920 (vinyl C-H), 760 (Ar C-H), 730 (Ar C-H), 700 (Ar C-H).

MS (CI) : m/z = 221 (M^+ + H).

3-Hydroxy-3-phenylbutene butyrate [156b];

Butyllithium (1.9ml of 1.8M, 3.42mM) was added to a stirred solution of 3-hydroxy-3-phenylbutene (500mg, 3.38mM) in diethyl ether at -78°C. The solution was stirred for 10min and then butyryl chloride (360mg, 3.38mM) was added and the reaction allowed to warm to ambient temperature with stirring. The reaction was worked up as for [151b] to afford [156b] as a colourless oil in 94% yield (693mg, 3.31mM).

^{1}H NMR ($CDCl_3$, 200MHz) : δ0.96 (t, J=8.12Hz, 3H, CH_3), δ1.55-1.80 (m, 2H, CH_2), δ1.90 (s, 3H, CH_3), δ2.33 (t, J=7.24, 2H, CH_2), δ5.21-5.33 (m, 2H, CH_2), δ6.19-6.34 (m, 1H, CH), δ7.20-7.45 (m, 5H, Ph).

IR (Neat) ν_{max}/cm^{-1} : 2980 (C-H), 2250, 1730 (C=O), 1600 (Ar C=C), 910 (vinyl C-H), 730 (Ar C-H), 700 (Ar C-H).

MS (CI) : m/z = 219 (M^+ + H).

References.

1. L. Stryer, *Biochemistry*, W. H. Freeman and Co, 2 Ed, 1981.

2. J. Retey, *Angew. Chem. Int. Ed. Engl.*, 1990, **29**, 355.

3. L. Pauling, *Chem. Eng. News.*, 1946, **24**, 1375.

4. L. Michaelis, M. Menten, *Biochem. Z*, 1913, **49**, 333.

5. IUPAC and IUB, *Enzyme Nomenclature*, Elsevier, 1972.

6. M. I. Page, A. Williams, *Enzyme Mechanisms*, Royal Society of Chemistry, 1987.

7. G. M. Whitesides and C-H. Wong, *Angew. Chem. Int. Ed. Engl.*, 1985, **24**, 617.

8. G. H. Davies, R. H Green, D.R. Kelly and S. M. Roberts, *Bittransformations in Preparative Organic Chemistry.The Use of Isolated and Whole Cell Systems in Synthesis*, Academic Press, London, 1989.

9. J. B. Jones, *Asymmetric Synthesis*, 1985, **5**, 309.

10. H. L. Herzog, M. A. Yevnik, P. L. Perlman, A. Nobile and E. B. Hershberg, *J. Am. Chem. Soc.*, 1953, **75**, 266.

11. P. Kevene and A. Walti, *Organic Synthesis collected volumes*, 1943, **2**, 545.

12. A. I. Meyers and R. A. Amos, *J. Am. Chem. Soc.*, 1980, **102**, 870.

13. K. Mori and K. Tanida, *Heterocycles*, 1981, **15**, 1171.

14. M. Hirama and M. Uei, *J. Am. Chem. Soc.*, 1982, **104**, 4251.

15. M. R. Uskokovic, R. L. Lewis, J. J. Partridge, C. W. Despreeaux and D. L. Pruess, *J. Am. Chem. Soc.*, 1979, **101**, 6742.

16. Y. Takaishi, Y-L. Yang, D. DiTullio and C. J. Sih, *Tetrahedron Lett.*, 1982, **23**, 5489.

17. W. H. Pirkle, D. L. Sikkenga and M. S. Pavlin, *J. Org. Chem.*, 1977, **42**, 384.

18. a) M. Bucciarelli, A. Forni, I. Moretti and G. Torre, *J. Chem. Soc., Chem. Commun.*, 1978, 456.
    b) A. Forni, I. Moretti and G. Torre, *J. Chem. Soc., Chem. Commun.*, 1977, 731.

19. D. R. Boyd and D. C. Neil, *J. Chem. Soc., Chem. Commun.*, 1977, 31.

20. a) M. Hirama, H. Shimizu and M. Iwashita, *J. Chem. Soc., Chem. Commun.*, 1983, 599.
    b) D. W. Brooks, M. Castro de Lee and R. Peevey, *Tetrahedron Lett*, 1984, **25**, 4623
    c) D. Seebach, M. F. Zuger, F. Giovannini, B.Sonnleiter, and A. Fiechter, *Angew. Chem. Int. Ed. Engl.*, 1984, **23**, 151.
    d) D. W. Brooks, R. P. Kellogg and C. S. Cooper, *J. Org. Chem.*, 1987, **52**, 192.

21. R. U. Lemieux and J. Giguere, *Can. J. Chem.*, 1951, **29**, 678.

22. a) D. W. Brooks, P. G. Grothaus and W. L. Irwin, *J. Org. Chem.*, 1982, **47**, 2820.
    b) D. W. Brooks and K. W. Woods, *J. Org. Chem.*, 1987, **52**, 2036.
    c) D. W. Brooks, H. Mazdiyasni and P. G. Grothaus, *J. Org. Chem.*, 1987, **52**, 3223.
    d) K. Mori and H. Mori, *Tetrahedron*, 1986, **42**, 5531.

e) Ito R. Masahara R. Tsukida K, *Tetrahedron Lett*, 1977, **29**, 2767

f) M. Nakazaki, H. Chikamatsu, K. Naemura, M. Nishiro, H. Murakami andM. Asao, *J. Chem. Soc., Chemm. Commun.*, 1978, 667.

23. L. Velluz, J. Valls and G. Nomine, *Angew. Chem. Int. Ed. Engl.*, 1965, **4**, 181.

24. C. J. Sih, J. B. Heather, G. P. Peruzzotti, P. Price and R. Sood, *J. Am. Chem. Soc.*, 1973, 95, 1676.

25. a) T. Fujisawa, T. Itoh and T. Sato, *Tetrahedron Lett.*, 1984, **25**, 5083.

b) G. Frater, U. Muller and W. Günther, *Tetrahedron*, 1984, **40**, 1269.

26. a) D. Seebach, S. Roggo, T. Maetzke, H. Braunschweiger, J. Cercus and M. Krieger, *Helv. Chim. Acta.*, 1987, **70**, 1605.

b) R. W. Hoffmann, W. Helbig and W. Ladner, *Tetrahedron Lett.*, 1982, **23**, 3479.

27. R. F. Newton, J. Paton, D. P. Reynolds and S. Young, *J. Chem. Soc., Chem. Commun.*, 1979, 908.

28. a) C. J. Sih, R. G. Salomon, P. Price, R. Sood and G. Peruzzotti, *Tetrahedron Lett.*, 1972, 24, 2435.

b) K. Mori and H. Akao, *Tetrahedron Lett.*, 1980, **36**, 91.

29. H. R. Levy, F. A. Loewus and B. Vennesland, *J. Am. Chem. Soc.*, 1957, **79**, 2949.

30. J. B. Jones, D. W. Sneddon, W. Higgins and A. J. Lewis, *J. Chem. Soc., Chem. Commun.*, 1972, 856.

31. H. Simon, J. Bader, H. Gunther, S. Neumann and J. Thomas, *Angew. Chem. Int. Ed. Engl.*, 1985, **24**, 539.

32. J. B. Jones and K. E. Taylor, *J. Chem. Soc., Chem. Commun.*, 1973, 205.

33. P. Blattmann and J. Retey, *J. Chem. Soc., Chem. Commun.*, 1970, 1394.

34. J. B. Jones and J. F. Beck, *Tech. Chem. NY*, 1976, **10**, 107.

35. D. R. Dodds and J. B. Jones, *J. Am. Chem. Soc.*, 1988 ,**110**, 577.

36. D. R. Dodds and J. B. Jones, *J. Chem. Soc., Chem. Commun.*, 1982 1080-

37. M. Nakazaki, H. Chikamatsu and M. Taniguchi, *Chem. Lett.*, 1982, 1761.

38. L. K. Lam, I. A. Gair and J. B. Jones, *J. Org. Chem.*, 1988, **53**, 1611.

39. V. Prelog, *Pure Appl. Chem.*, 1964, **9**, 119.

40. J. B. Jones and I. J. Jakovac, *Can. J. Chem.*, 1982, **60**, 19.

41. H. Simon, H. Gunther, J. Bader and W. Tischer, *Angew. Chem. Int. Ed. Engl.*, 1981, **20**, 861.

42. H. G. W. Leuenberger, W. Boguth, E. Widmer and R. Zell, *Helv. Chim. Acta.*, 1976, **59**, 1832.

43. H. G. W. Leuenberger, W. Boguth, R. Barner, M. Schmid and R. Zell, *Helv. Chim. Acta.*, 1979, **62**, 455.

44. A. J. Irwin and J. B. Jones, *J. Am. Chem. Soc.*, 1977, **99**, 1625.

45. J. Fried and J. C. Sih, *Tetrahedron Lett.*, 1973, 3899.

46. M. E. Jung and L. M. Speltz, *J. Am. Chem. Soc.*, 1976, **98**, 7882.

47. R. V. Stevens, K. T. Chapman, C. A. Stubbs, W. W. Tamm and K. F. Albizati *Tetrahedron Lett.*, 1982, **23**, 4647.

48. A. J. Irwin and J. B. Jones, *J. Am. Chem. Soc.*, 1977, **99**, 556.

49. J. B. Jones and K. P. Lok, *Can. J. Chem.*, 1979, **57**, 1025.

50. C. Bally and F. Leuthardt, *Helv. Chim. Acta.*, 1970, **53**, 732.

51. P. Herold, P. Mohr and G. Tamm, *Helv. Chim. Acta.* 1983, **66**, 744.

52. C. S. Chen, Y. Fujimoto, G, Girdaukas and C. J. Sih, *J. Am. Chem. Soc.*, 1982, **104**, 7294.

53. J. B. Jones, A. W. Finch and I. J. Jakovac, *Can. J. Chem.* 1982, **60**, 2007.

54. E. Abushanab, D. Reed, F. Suzuki and C. J. Sih, *Tetrahedron Lett.*, 1978, **37**, 3415.

55. G. Pelsy and A. M. Klibanov, *Biochim. Biophys. Acta.*, 1983, **742**, 352.

56. J. Tramper, A. Nagel, H. C. van der Plas and F. Muller, *J. R. Neth. Chem. Soc.*, 1979, **98**, 224.

57. K. N. Murray and S. Chaykin, *J. Biol. Chem.*, 1966, **241**, 3468

58. G. Stohrer and G. B. Brown, *J. Biol. Chem.*, 1969, **244**, 2498.

59. D. M. Valerino and J. J. McCormack, *Biochim. Biophys. Acta.*, 1969 **184** 154-

60. L. Greenlee and P. Handler, *J. Biol. Chem.* 1964, **239**, 1090.

61. C. J. Suckling, *Enzyme Chemistry. Impact and Applications.*, Chapman and Hall, London, 2nd edn., 1990.

62. J. M. Hill and P. J. G. Mann, *Biochem. J.*, 1964, **91**, 171.

63. C. M. McEwan Jr. and A. J. Sober, *J. Biol. Chem.*, 1967, **242**, 3068.

64. a) J. B. Jones, *Tetrahedron*, 1986, **42**, 3351.
b) J. B. Jones, *Tetrahedron*, 1986, **42**, 3403.

65. A. Moradian and S. A. Benner, *J. Am. Chem. Soc.*, 1992, **114**, 6980.

66. N. Cohen, W. F. Eichel, R. J. Lopresti, C. Neukom and G. Saucy, *J. Org. Chem.*, 1976, **41**, 3505.

67. Q. Branca and A. Fischli, *Helv. Chim. Acta.*, 1977, **60**, 925.

68. R. A. Johnson, M. E. Herr, H. C. Murray and G. S. Fenken, *J. Org. Chem.*, 1970, **35**, 622.

69. D. G. H. Ballard, A. Courtis, I. M. Shirley and S. C. Taylor, *J. Chem. Soc., Chem. Commun.*, 1983, 954.

70. A. M. Klibanov, Z. Berman and B. N. Alberti, *J. Am. Chem. Soc.*, 1981, **103**, 6263.

71. S. G. Cohen and A. Milovanovic, *J. Am. Chem. Soc.*, 1968, **90**, 3495.

72. B. Cambou and A. M. Klibanov, *Apl. Biochem. Biotechnol.*, 1984, **9**, 255.

73. W. K. Wilson, S. B. Baca, Y. J. Barber, T. L. Scallen, C. J. Morrow, *J. Org. Chem.*, 1983, **48**, 3960.

74. a) S. Ramaswamy, R. A. H. F. Hui and J. B. Jones, *J. Chem. Soc., Chem. Commun.*, 1986, 1545.
b) G. Gil, E. Ferre, A. Meou, J. Le Petit and C. Triantaphylides, *Tetrahedron Lett.*, 1987, **28**, 1647.

75. S. Iriuchijima and N. Kojima, *Agric. Biol. Chem.*, 1984, **48**, 2055.

76. E. J. Toone and J. B. Jones, *Can. J. Chem.*, 1987, **65**, 2722.

77. T. Katsuki and K. B. Sharpless, *J. Am. Chem. Soc.*, 1980, **102**, 5974.

78. W. E. Lander and G. M. Whitesides, *J. Am. Chem. Soc.*, 1984, **106**, 7250.

79. U. T. Bhalerao, L. Dasaradhi, P. Neelahanlan and N. W. Fadnarsis, *J. Chem. Soc., Chem. Commun.*, 1991, 1197.

80. a) N. A. Porter, J. D. Byers, K. M. Holden and D. B. Menzel, *J. Am. Chem. Soc.*, 1979, **101**, 4319.
b) C-H. Lin, D. L. Alexander, C. G. Chidester, R. R. Gorman and R. A. Johnson, *J. Am. Chem. Soc.*, 1982, **104**, 1621.

81. C-S. Chen, Y. Fujimoto and C. J. Sih, *J. Am. Chem. Soc.*, 1981, **103**, 3580.

82. Y-F. Wang, C-S. Chen, G. Girdaukas and C. J. Sih, *J. Am. Chem. Soc.*, 1984, **106**, 3695.

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

84. a) F-C. Huang, L. F. Hsulee, R. S. D. Mittal, P. R. Ravikamur, J. A. Chan, C. J. Sih, E. Caspi and C. R. Eck, *J. Am. Chem. Soc.*, 1975, **97**, 4144.
b) Y-F. Wang, T. Izawa, S. Kobayashi ans M. Ohno, *J. Am. Chem. Soc.*, 1982, **104**, 6465.
c) P. Mohr, M. Tori, P. Grussen, P. Herold and C. Tamm, *Helv. Chim. Acta.*, 1982, **65**, 1412.
P. Herold, P. Mohr and C. Tamm, *Helv. Chim. Acta.*, 1983, **66**, 744.
C-S. Chen, Y. Fujimoto, G. Girdaukas and C. J. Sih, *J. Am. Chem. Soc.*, 1982, **104**, 7294.
D. Gani and D. W. Young, *J. Chem. Soc., Chem. Commun.*, 1983, 576.

85. M. Ohno, S. Kobayashi, T. Limori, Y-F. Wang and T. Izawa, *J. Am. Chem. Soc.*, 1981, **103**, 2405.

86. F. Bjorkling, J. Boutelje, S. Galenbeck, K. Hult, T. Norin and P. Szmulik, *Tetrahedron*, 1985, **41**, 1347.

87. a) K. Laumen and M. Schneider, *Tetrahedron Lett.*, 1984, **25**, 5875.
b) T. Fujisama, T. Kobori and H. Ohno, *J. Chem. Soc., Chem. Commun.*, 1976, 189.
c) T. Tanaka, S. Kurozumi, T. Toru, S. Miura, M. Kobayashi and S. Ishimoto, *Tetrahedron*, 1976, **32**, 1713.
S. Miura, S. Kurozumi, T. Toru, T. Tanaka, M.
d) Kobayashi, S. Matsubara and S. Ishimoto, *Tetrahedron*, 1976, **32**, 1895.

88. a) M. Schneider, N. Engel, P. Honicke, G. Heinemann and H. Gorisch, *Angew. Chem. Int. Ed. Engl.*, 1984, **23**, 67.
H-J. Gais and K. L. Lukas, *Angew. Chem. Int. Ed. Engl.*, 1984, **23**, 142.
b) S. Kobayashi, K. Kamiyama, T. Limori and M. Ohno, *Tetrahedron Lett.*, 1984, **25**, 2557.

89. G. Sabbioni and J. B. Jones, *J. Org. Chem.*, 1987, **52**, 4565.

90. G. Sabbioni, M. L. Shea and J. B. Jones, *J. Chem. Soc., Chem. Commun.*, 1984, 236.

91. J. B. Jones, R. S. Hinks and P. G. Hulkin, *Can. J. Chem.*, 1985, **63**, 452.

92. J. P. Greenstein, *Adv. Protein Chem.*, 1954, **9**, 121.

93. G. M. Anatharamaiah and R. W. Roeske, *Tetrahedron Lett*, 1982, **23**, 3335.

94. a) J. E. Baldwin, M. E. Christie, S. B. Haber and L. I. Kruse, *J. Am. Chem. Soc.*, 1976, **98**, 3045.
b) K. Mori and H. Iwasawa, *Tetrahedron Lett.*, 1980, **36**, 2209.

95. F. Cecere, G. Galli and F. Morisi, *F.E.B.S. Lett.*, 1975, **57**, 192.

96. S. L. Regen, A. Singh, G. Oehme and M. Singh, *J. Am. Chem. Soc.*, 1982, **104**, 791.

97. Y. Y. Lin and J. B. Jones, *J. Org. Chem.*, 1973, **38**, 3575.

98. A. Taunton-Rigby, *J. Org. Chem.*, 1973, **38**, 977.

99. a) H. T. Huang and C. Niemann, *J. Am. Chem. Soc.*, 1951, 73, 475.
b) J. L. Abernethy, D. Srulevitch and M. J. Ordway Jr, *J. Org. Chem.*, 1975, **40**, 3445.

100. K. Oyama, S. Nishimura, Y. Nonaka, K-I. Kihama and T. Hashimoto, *J. Org. Chem.*, 1981, **46**, 5241.

101. a) W. Kullmann, *J. Biol. Chem.*, 1980, **255**, 8234.
b) W. Kullmann, *Biochem. J.*, 1984, **220**, 405.

102. G. Pelsey and A. M. Klibanov, *Biotechnol. Bioeng.*, 1983, **25**, 919.

103. a) G. Bellucci, G. Berti, G. Catelani and E. Mastrolli, *J. Org. Chem.*, 1981, **46**, 5148.
b) G.Bellucci, G. Berti, R. Bianchini, P. Cetera and E. Mastrolli, *J. Org. Chem.*, 1982, **47**, 3105.

104. D. M. Jerina, H. Ziffer and J. W. Daly, *J. Am. Chem. Soc.*, 1970, **92**, 1056.

105. a) G. M. Whitesides, M. Siegel and P. Garrett, *J. Org. Chem.*, 1975, **40**, 2516.
b) J. M. Lewis, S. L. Haynie and G. M. Whitesides, *J. Org. Chem.*, 1979, **44**, 864.

106. D. C.Crans and G. M. Whitesides, *J. Org. Chem.*, 1983, **48**, 3130.

107. B. L. Hirschbein, F. P. Mazenod and G. M. Whitesides, *J. Org. Chem.*, 1982, **47**, 3765.

108. a) C-H. Wong and G. M. Whitesides, *J.Am. Chem. Soc.*, 1981, **103**, 4890.
b) C-H. Wong, J. Gordan, C. L. Cooney and G. M. Whitesides, *J. Org. Chem.*, 1981, **46**, 4676.

109. a) C-H. Wong and G. M. Whitesides, *J.Org. Chem.*, 1983, **48**, 3199.
b) C-H. Wong, F. P. Mazenod and G. M. Whitesides, *J. Org. Chem.*, 1983, **48**, 3493.

110. O. Abril, D. C. Crans and G. M. Whitesides, J. Org. Chem., 1984, 49, 1360.

111. J. R. Moran and G. M. Whitesides, *J. Org. Chem.*, 1984, **49**, 704.

112. a) D. R. Walt, V. M. Rios-Mercadillo, J. Auge and G. M. Whitesides, *J. Am. Chem. Soc.*, 1980, **102**, 7805.
b) D. R. Walt, M. A. Findeis, V. M. Rios-Mercadillo and G. M. Whitesides, *J. Am. Chem. Soc.*, 1984, **106**, 234.

113. a) V. M. Rios-Mercadillo and G. M. Whitesides, *J. Am. Chem. Soc.*, 1979, **101**, 5828.
b) D. C. Crans and G. M. Whitesides, *J. Am. Chem. Soc.*, 1985, **107**, 7008.
D. C. Crans and G. M. Whitesides, *J. Am. Chem. Soc.*, 1985, **107**, 7019.

114. J. R. Barrio, J. E. Egbert, E. Henze, H. R. Schelbert and F. J. Baumgartner, *J. Med. Chem.*, 1982, **25**, 93.

115. G. A. Kochetov, *Biochem. SSR.*, 1986, **51**, 1721.

116. a) J. Bolte, C. Demuynek and H. Samaki, *Tetrahedron Lett.*, 1987, 28, 5525.
b) T. Ziegler, A. Straub and F. Effenberger, *Angew. Chem. Int. Ed. Engl.*, 1988, **27**, 716.

117. M. Kapuscinski, F. P. Franke, I. Flanigan, J. K. MacLeod and J. F. Williams, *Carbohydrate Res.*, 1985, **140**, 69.

118. A. Macali, D. Aldinucci and F. Pasletti, *Carbohydrate Res.*, 1985, **143**, 288.

119. D. C. Myles, P. J. Andrulis and G. M. Whitesides, *Tetrahedron Lett.*, 1991, **32**, 4835.

120. a) M. D. Bednarski, E. S. Simon, N. Bischofberger, W-D. Fessner, M-J. Kimm, W. Lees, T. Saito, H. Waldmann and G. M. Whitesides, *J. Am. Chem. Soc.*, 1989, **111**, 627.
b) I. A. Rose, *J. Am. Chem. Soc.*, 1958, **80**, 5835.

121. a) M. H. Ghalambar and E. C. Heath, *J. Biol. Chem.*, 1962, **237**, 2427.
b) T-H. Chiu and D. S. Feingold, *Biochemistry*, 1969, **8**, 98.

122. a) E. Racker, *J. Biol. Chem.*, 1952, **196**, 347.
b) W. E. Price and B. L. Horecker, *J. Biol. Chem.*, 1960, **235**, 1292.

123. G. M. Whitesides and C-H. Wong, *Angew. Chem. Int. Ed. Engl.*, 1985, **24**, 617,

124. A. R. Battersby, *Chem. in Britain*, 1984, **20**, 611.

125. C. Auge, S. David and C. Gautheron, *Tetrahedron Lett.*, 1984, **25**, 4663.

126. a) W. Becker and E. Pfeil, *J. Am. Chem. Soc.*, 1966, **88**, 4299.
b) L. Jaenicke and J. Preun, *Eur. J. Biochem.*, 1984, **138**, 319,
c) E. Hochuli, *Helv. Chim. Acta.*, 1983, **66**, 489.
d) W. Becker, H. Freund and E. Pfeil, *Angew. Chem. Int. Ed. Engl.*, 1964, **4**, 1079.

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

128. C. Fugati, P. Grasseli, S. Servi, F. Spreafico, C. Zirotti and P. Casati, *J. Org. Chem.*, 1984, **49**, 4089.

129. a) R. Benardi, C. Fuganti, P. Grasseli and G. Marinoni, *Synthesis*, 1980, 50.
b) C. Fuganti, P. Grasseli and S. Servi, *J. Chem. Soc., Chem. Commun.*, 1982, 1285.

130. C. Fuganti, P. Grasseli, F. Spreafico and C. Zirotti, *J. Org. Chem.*, 1984, **49**, 543.

131. C. Fuganti and P. Grasseli, *J. Chem. Soc., Chem. Commun.*, 1982, 205.

132. R. D. Libby, J. A. Thomas, L. W. Kaiser and L. P. Hager, *J. Biol. Chem.* 1982, **257**, 5030.

133. S. L. Niedlemann and S. D. Levine, *Tetrahedron Lett.*, 1968, **27**, 4057.

134. S. G. Sogo, T. S. Widlanski, J. H. Hoare, C. E. Grimshaw, G. A. Berchtold and J. R. Knowles, *J. Am. Chem. Soc.*, 1984, **106**, 2701.

135. P. J. Card and W. D. Hitz, *J. Am. Chem. Soc.*, 1984, **106**, 5348.

136. Y. Ooi, T. Hashimoto, N. Mitsuo and T. Sotoh, *Tetrahedron Lett.*, 1984, **25**, 2241.

137. A. Gross, S. Geresh and G. M. Whitesides, *Appl. Biochem. Biotechnol.*, 1982, **8**, 415.

138. H. G. Khorana, K. L. Agarwal, P. Besner, H. Buchi, M. H. Caruthers, P. J. Cashion, M. Fridkin, E. Jay, K. Kleppe, A. Kumar, P. C. Loewen, R. C. Miller, K. Minamoto, A. Panet, U. L. RajBhandary, B, Ramamoorthy, T. Seikiya and T. Takeya, *J. Biol. Chem.*, 1976, **251**, 565.

139. a) T. Kayama, A. Saito, K. Ogura and S. Seto, *J. Am. Chem. Soc.*, 1980, **102**, 3614.
b) M. Kobayashi, T. Koyama, K. Ogura, S. Seto, F. J. Ritter and I. E. M. Bruggemann-Rotgans, *J. Am. Chem. Soc.*, 1980, **102**, 6602.

140. A. Gross, O. Abril, J. M. Lewis, S. Geresh and G. M. Whitesides, *J. Am. Chem. Soc.*, 1983, **105**, 7428.

141. G. D. Markham, E. W. Hafner, C. W. Tabor and H. Tabor, *J. Biol. Chem.*, 1980, **255**, 9082.

142. a) G. A. Bahadur, J. E. Baldwin and J. J. Usher, *J. Am. Chem. Soc.*, 1981, **103**, 7650.
b) J. E. Baldwin, R. M. Adlington, N. J. Turner, B. P. Domayne-Hayman, H-H. Ting, A. E. Derome and J. A. Murphy, *J. Chem. Soc., Chem. Commun.*, 1984, 1167.
J. E. Baldwin, R. M. Adlington, A. E. Derome, H-H. Ting and N. J. Turner, *J. Chem. Soc., Chem. Commun.*, 1984, 1211.

c) J. E. Baldwin, E. P. Abraham, R. M. Adlington, G. A. Bahadur, B. Chakravarli, B. P. Dormayne-Hayman, L. D. Field, S. L. Flitsch, G. S. Jayatilake, A. Spakovskis, H-H. Ting, N. J. Turner, R. L. White and J. J. Usher, *J. Chem. Soc., Chem. Commun.*, 1984, 1225.
d) I. J. Hollander, Y. Q. Shen, J. Heim and A. L. Demain, *Science*, 1984, **224**, 610.
e) S. Wolfe, A. L. Demain, S. E. Jensen and D. W. S. Westlake, *Science*, 1984, **226**, 1386.

143. a) S. Kobayashi, K. Kamiyama, T. Limori and M. Ohno, *Tetrahedron Lett.*, 1984, **25**, 2557.
b) Y-F. Wang and C-J. Sih, *Tetrahedron Lett.*, 1984, **25**, 4999.

144. J. B. Jones and M. M. Mehes, *Can. J. Chem.*, 1979, **57**, 2245.
145. G. Guanti, L. Banfi, E. Narisano, R. Riva and S. Thea, *Tetrahedron Lett.*, 1986, **27**, 4639.

146. J. B. Jones and H. M. Schwartz, *Can. J. Chem.*, 1982, **60**, 335.
J. B. Jones and H. M. Schwartz, *Can. J. Chem.*, 1982, **60**, 1030.

147. F. Bjorkling, T. Boutelje, S. Gatenbeck, K. Hult and T. Norin, *Tetrahedron Lett.*, 1985, **26**, 4957.

148. F. Bjorkling, T. Boutelje, M. Hjalmarsson, K. Hult and T. Norin, *J. Chem. Soc., Chem. Commun.* ,1987, 1041.

149. G. Langrand, J. Baratti, G. Buono, C. Triantaphylides, *Tetrahedron Lett.*, 1986, **27**, 29.

150. K. Martinek, A. V. Levashov, N. Klyachko, Y. L. Khemelnitski and I. V. Berezin, *Eur. J. Biochem.*, 1986, **155**, 453.

151. P. L. Luisi, *Angew. Chem. Int. Ed. Engl.*, 1985, **24**, 439.

152. F. M. Menger and K. Yamada, *J. Am. Chem. Soc.*, 1979, **101**, 6731.

153. S. Barbaric and P. L. Luisi, *J. Am. Chem. Soc.*, 1981, **103**, 4239.

154. P. L. Luisi, F. J. Bonner, A. Pellegrini, P. Wiget and R. Wolf, *Helv. Chim. Acta.*, 1979, **62**, 740.

155. C. Grandi, R. E. Smith and P. L. Luisi, *J. Biol. Chem.*, 1981, **256**, 837.

156. P. Luthi and P. L. Luisi, *J. Am. Chem. Soc.*, 1984, **106**, 7285.

157. R. Jost, E. Brambilla, J. C. Monti and P. L. Luisi, *Helv. Chim. Acta.*, 1980, **63**, 375.

158. R. Hillhorst, R. Spruijt, C. Laane and C. Veeger, *Eur. J. Biochem.*, 1984, **144**, 459.

159. K. Martinek and A. N. Semenov, *J. Appl. Biochem.*, 1981, **1**, 93.

160. a) A. Zaks and A. M. Klibanov, *J. Biol. Chem.*, 1988, **263**, 3194.
b) A. M. Klibanov, *Acc. Chem. Res.*, 1990, **23**, 114.
c) A. M. Klibanov, *Trends In Biochem. Sci.*, 1989, **14**, 141.
d) P. J. Halling, *Trends In Biochem. Tech.*, 1989, **7**, 50.
e) A. Zaks and A. J. Russell, *J. Biotechnol.*, 1988, **8**, 259.

161. a) P. A. Fitzpatrick and A. M. Klibanov, *J. Am. Chem. Soc.*, 1991, **113**, 3166.
b) S-H. Wu, F-Y. Chu and K-T. Wang, *Bioorg. Med. Chem. Lett.*, 1991, **1**, 339.
c) A. L. Gutman, K. Zuobi and A. Boltansky, *Tetrahedron Lett.*, 1987, **28**, 3861.

162. a) A. Zaks and A. M. Klibanov, *J. Biol. Chem.*, 1988, **263**, 8017.
b) S. H. M. van Erp, E. O. Kamenskaya and Y. L. Khmelnitsky, *Eur. J. Biochem.*, 1991, **202**, 379.
c) T. M. Stokes and A. C. Oehlshlager, *Tetrahedron Lett.*, 1987, **28**, 2091.
d) S. A. Khan, P. J. Halling and G. Bell, *Enzyme Microb. Technol.*, 1990, **12**, 453.
e) P. J. Halling, *Biocatalysis*, 1987, **1**, 109.
f) H. C. Goderis, C. Anpe, M. P. Feylen, B. L. Fouwe, W. M. Guffens, S M. Van-Cauwenberg and P. P. Tobback, *Biotechnol. Bioeng.*, 1987, **30**, 258.

163. a) U. Bornschever, S. Schapohler and K. Schugerl, *Tetrahedron Asymm.*, 1991, **2**, 1011.
b) L. K. P. Lam, R. A. H. F. Hui and J. B. Jones, *J. Org. Chem.*, 1986, **51**, 2047.
c) A. Zaks and A. M. Klibanov, *Science*, 1984, **224**, 1249.

164. L. Kuittingen, B. Sjursnes, P. Halling and T. Anthonsen, *Tetrahedron*, 1992, **48**, 5259.

165. G. M. Ramos-Tombo, H. P. Schar, X. Fernandez, I. Busquets and O. Ghisalba, *Tetrahedron Lett.*, 1986, **27**, 5707.

166. a) G. Kirchner, M. P. Scollar and A. M. Klibanov, *J. Am. Chem. Soc.*, 1985, **107**, 7072.
b) P. E. Sonnet, *J. Org. Chem.*, 1987, **52**, 3477.
c) G. Lagrand, M. Secchi, G. Buono, J. Baratti and C. Triantaphylides, *Tetrahedron Lett.*, 1985, **26**, 1857.
d) H. Hemmerle and H-J. Gais, *Tetrahedron Lett.*, 1987, **28**, 3471.
e) Y. Okahata, Y. Fujimoto and K. Ijiro, *Tetrahedron Lett.*, 1988, **29**, 5133.
f) D. Lutz, A. Guldner, R. Thums and P. Schreier, *Tetrahedron Asymm.*, 1990, **1**, 783.
g) C-S. Chen, G. Girdaukas and C. J. Sih, *J. Am. Chem. Soc.*, 1987 **109** 2812.

167. D. Bianchi, P. Cesti and E. Battistel, *J. Org. Chem.*, 1988, **53**, 5531.

168. a) K. Yamamoto, T. Nishioka and J. Oda, *Tetrahedron Lett.*, 1988, **29**, 1717.
b) Y. Yokahata and K. Ijiro, *J. Chem. Soc., Chem. Commun.*, 1988, 1394.

169. B. Cambou and A. M. Klibanov, *J. Am. Chem. Soc.*, 1984, **106**, 2687.

170. B. Morgan, A. C. Oehlshlager and T. M. Stokes, *Tetrahedron*, 1991, **47**, 1611.

171. M. Therisod and A. M. Klibanov, *J. Am. Chem. Soc.*, 1986, **108**, 5638.

172. Y-F. Wang, S-T. Chen, K. K. C. Liu and C-H. Wong, *Tetrahedron Lett.*, 1989, **30**, 1917.

173. a) K. Burgess and L. D. Jennings, *J. Am. Chem. Soc.*, 1991, **113**, 6129.
b) K. Burgess and L. D. Jennings, *J. Am. Chem. Soc.*, 1990, **112**, 7434.
c) C-S. Chen, Y-C. Liu and M. Marsella, *J. Chem. Soc., Perkin Trans. 1*, 1990, 2559.

174. a) C. R. Johnson and H. Sakaguchi, *Synlett*, 1992, 813.
b) K. J. Harris, Q-M. Gu, Y-E. Shih, G. Girdaukas and C. J. Sih, *Tetrahedron Lett*, 1991, **32**, 3941.
c) R. Seemayer and M. P. Schneider, *Tetrahedron Asymm.*, 1992, **2**, 827.

175. J. C. Carretero and E. Dominguez, *J. Org. Chem.*, 1992, **57**, 3867.

176. Y-F. Wang, J. J. Lalonde, M. Momongan, D. E. Bergbreiter and C-H. Wong, *J. Am. Chem. Soc.*, 1988, **110**, 7200.

177. B. Berger, C. G. Rabiller, K. Konigsberger, K. Faber and H. Griengl, *Tetrahedron Asymm.*, 1990, **1**, 541.

178. B. Berger and K. Faber, *J. Chem. Soc., Chem. Commun.*, 1991, 1198.

179. T. Miyazawa, S. Kurita, S. Ueji, T. Yamada and S. Kuwata, *J. Chem. Soc., Perkin Trans. 1*, 1992, .

180. a) A. L. Gutman, K. Zuobi and T. Bravdo, *J. Org. Chem.*, 1990, **55**, 3546.
b) A. Makita, T. Hihira and Y. Yamada, *Tetrahedron Lett.*, 1987, **28**, 805.
c) A. L. Gutman, K. Zuobi and A. Boltansky, *Tetrahedron Lett.*, 1987, **28**, 3861.

181. A. C. Margolin, J-Y. Crenne and A. M. Klibanov, *Tetrahedron Lett.*, 1987, **28**, 1607.

182. a) A. L. Margolin and A. M. Klibanov, *J. Am. Chem. Soc.*, 1987, **109**, 3802.
b) A. L. Margolin, D-F. Tai and A. M. Klibanov, *J. Am. Chem. Soc.*, 1987, **109**, 7885.

183. a) T. Kitazume, K. Murata, Y. Kokusho and S. Iwasaki, *J. Fluorine Chem.*, 1988, **39**, 75.
b) T. Kitazume, T. Ikeya and K. Murata, *J. Chem. Soc., Chem. Commun.*, 1986, 1331.

184. a) N. W. Boaz, *Tetrahedron Lett.*, 1989, **30**, 2061.

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

186. J. Grunwald, B. Wirz, M. P. Scollar and A. M. Klibanov, *J. Am. Chem. Soc.*, 1986, **108**, 6732.

187. T. Sakurai, A. L. Margolin, A. J. Russell and A. M. Klibanov, *J. Am. Chem. Soc.*, 1988, **110**, 7236.

188. F. Effenberger, T. Ziegler and S. Forester, *Angew. Chem. Int. Ed. Engl.*, 1987, **26**, 458.

189. Y. Hata, Y. Matsuura, N. Tanaka, M. Kakudo, A. Sugihara, M. Iwai and Y. Tsujisaka, *J. Biochem.*, 1979, **86**, 1821.

190. J. D. Schrag, Y. Li, S. Wu and M. Cygler, *Nature*, 1991, **351**, 761.

191. C-S. Kaimal and M. Saroja, *Biochem. Biophys. Acta.*, 1989, **999**, 331.

192. C. Chapus and M. Semeriva, *Biochemistry*, 1976, **15**, 4988.

193. F. K. Winkler, A. D'Arcy and W. Hunziker, *Nature*, 1990, **343**, 770.

194. a) Y. Shimada, A. Sugiham, T. Lizumi and Y. Tominaga, *J. Biochem.*, 1990, **107**, 703.
b) P. Haduary, W. Sidler, W. Meister, W. Vetter and H. Wolfer, *J. Biol. Chem.*, 1991, **266**, 2021.
c) W. F. Ettinger, S. K. Thukral and P. E. Kolattukuddy, *Biochemistry*, 1987, **26**, 7883.

195. C-S. Chen and C. J. Sih, *Angew. Chem. Int. Ed. Engl.*, 1989, **28**, 695.

196. D. F. DeTar, *J. Org. Chem.*, 1980, **45**, 5166.

197. S-H. Wu, Z. W. Guo and C. J. Sih, *J. Am. Chem. Soc.*, 1990, **112**, 1990.

198. P. J. Halling, *Enzyme Microb. Technol.*, 1984, **6**, 513.

199. a) D. O'Hagan and N. A. Zaidi, *J. Chem. Soc., Chem. Commun.*, 1992, 947.
b) J. T. Lin, T. Yamazaki and T. Kitazumi, *J. Org. Chem.*, 1989, **57**, 3211.
c) T. H. Oberhauser, K. Faber and H. Griengl, *Tetrahedron*, 1989, **45**, 1679.
d) I. Brakenridge, R. McCague, S. M. Roberts and N. J. Turner, *J. Chem. Soc., Perkin Trans. 1.* 1993, 1093.

200. M. Takeshita, K. Terada, N. Akutsu, S. Yoshida and T. Sato, *Heterocycles*, 1987, **26**, 3051.

201. a) S. Hamman and C. G. Beguin, *Tetrahedron Lett.*, 1983, **24**, 57.
b) B. C. Saunders and G. J. Stacey, *J. Chem. Soc.*, 1948, 1773.

202. a) G. Gottarelli and B. Samori, *J. Chem. Soc., Perkin Trans.* 1., 1974, 1462.
b) M. Imuta and H. Ziffer, *J. Org. Chem.*, 1978, **43**, 3530.
c) J. Kopilov, E. Kariv and L. L. Miller, *J. Am. Chem. Soc.*, 1977, **99**, 3450.
d) M. Kasai, C. Froussions and H. Ziffer, *J. Org. Chem.*, 1983, **48**, 459.

203. A. B. Smith, P. A. Levenberg and J. Z. Sints, *Synthesis*, 1986, 184.

204. E. Montaudon, X. Lubeight and B. Maillard, *J. Chem. Soc., Perkin Trans. 1.*, 1991, 1531.

205. M. A. Henderson and C. H. Heathcock, *J. Org. Chem.*, 1988, **53**, 4736.

206. J. M. Schwab and D. C. J. Lin, *J. Am. Chem. Soc.*, 1985, **107**, 6046.

207. Y. Zhaomin, F. Yuzo, J. Tetsuro, M. Norioki, Y. Kazuhiro and T. Hiroshi, *J. Org. Chem.*, 1987, **52**, 3524.

208. B. S. Furniss, A. J. Hannaford, V. Rogers, P. W. G. Smith and A. R. Tatchell, Vogel's Textbook of Practical Organic Chemistry, Longmann London and New York, 4 Ed, 1989.

209. B. El Abi and H. Alper, *J. Org. Chem.*, 1991, **56**, 5357.

Appendix 1.

Publications 1989-1992.

V. F. Hogan, D. O'Hagan and J. Sanvoisin, *Indian J. Chem.*, 1992, **31**, 883. Rate Enhancement of the *Candida cylindracea* Lipase Catalysed Transesterifications in Organic Solvents: Enzymatic Reactions Below Zero.

Poster presentation at: IUPAC-NOST International Symposium on Enzymes in Organic Synthesis, University of Delhi, India, 1992 :- Rate Enhancement of the *Candida cylindracea* Lipase Catalysed Transesterifications in Organic Solvents: Enzymatic Reactions Below Zero.

Poster Presentation at: Lipases: Structure, Function and Applications in Biotransformations, University of Warwick, 1991 :- Rate Enhancement of the Transesterification Reaction Mediated by *Candida cylindracea* in Organic Solvents: Modification of the Acyl Moiety.

Appendix 2.

Colloquia, Lectures and Seminars given by invited speakers 1-October 1989 to 30-September 1992.
(* = Those attended)

F. Palmer (Nottingham University), Thunder and Lightning, 17- October- 1989. *

C. Floriani (University of Lausanne, Switzerland), Molecular Aggregates - A Bridge Between Homogeneous and Heterogeneous Systems, 25- October- 1989.

J. P. S. Badyal (Durham University), Breakthroughs in Heterogeneous Catalysis, 1- November- 1989.

N. N. Greenwood (University of Leeds), Novel Cluster Geometries in Metalloborane Chemistry, 9- November- 1989. *

J. E. Bercaw (California Institute of Technology), Synthetic Approaches to Ziegler-natta Polymerisation of Olefins, 10- November- 1989.

J. Becher (Odense University), Synthesis of New Macrocyclic Systems Using Heterocyclic Building Blocks, 13- November- 1989.

D. Parker (Durham University), Macrocycles, Drugs and Rock 'n' Roll, 16- November- 1989. *

D. J. Cole-Hamilton (St Andrews University), New Polymers from Homogeneous Catalysis, 29- November- 1989.

M. N. Huges (King's College, London), A Bug's Eye of the Periodic Table, 30- November- 1989. *

D. Graham (B. P. Research Centre), How Proteins Absorb to Interfaces, 4- December- 1989. *

R. L. Powell (ICI), The Development of CFC Replacements, 6- December- 1989. *

A. Butler (St Andrews University), The Discovery of Penicillin: Facts and Fancies, 7- December- 1989. *

J. Klinowski (Cambridge University), Solid State NMR Studies of Zeolite Catalysts, 13- December- 1989.

R. Huisgen (Universitat Munchen), Recent Mechanistic Studies of [2+2] Additions, 15- December- 1989.

R. N. Perutz (York University), Plotting the Course of C-H Activations with Organometallics, 24- January- 1990.

U. Dyer (Glaxo), Synthesis and Conformation of C-Glycosides, 31- January- 1990. *

J. H. Holloway (University of Leicester), Noble Gas Chemistry, 1- February- 1990.

D. P. Thompson (Newcastle University), The Role of Nitrogen in Extending Silicate Crystal Chemistry, 7- February- 1990.

Rev. R. Lancaster (Kimbolton Fireworks), Fireworks - Principles and Practice, 8- February- 1990. *

L. Lunazzi (University of Bologna), Application of Dynamic NMR to the Study of Conformational Enantiomerism, 12- February- 1990.

D. Sutton (Simon Fraser University, Vancouver B. C.), Synthesis and Applications of Dinitrogen and Diazo Compounds of Rhenium and Iridium. 14- February- 1990.

L. Crombie (Nottingham University), The Chemistry of Cannabis and Khat, 15- February- 1990. *

C. Bleasdale (Newcastle University), The Mode of Action of Some Anti-tumor Agents, 21- February- 1990. *

D. T. Clark (ICI Wilton), Spatially Resolved Chemistry (Using Natures Paradigm in the Advanced Materials Area), 22- February- 1990.

R. K. Thomas (Oxford University), Neutron Reflectometry from Surfaces, 28- February- 1990.

J. F. Stoddart (Sheffield University), Molecular Lego, 1- March- 1990. *

A. K. Cheetham (Oxford University), Chemistry of Zeolite Cages, 8- March- 1990.

I. Powis (Nottingham University), Spinning off in a Huff: Photodissociation of Methyl Iodide, 21- March- 1990.

J. M. Bowman (Emory University), Fitting Experiment with Theory in Ar-OH, 23- March- 1990

T. J. Simpson (University of Bristol), Polyketide Biosynthesis Past, Present and Future, 7- May- 1990. *

L. S. German (USSR Acadamy of Sciences Moscow), New Syntheses in Fluoroaliphatic Chemistry: Recent Advances in the Chemistry of Fluorinated Oxiranes, 9- July- 1990.

V. E. Platonov (USSR Acadamy of Sciences, Novosibirsk), Polyfluoindanes: Synthesis and Transformation 9- July 1990.

I. N. Rozhkof (USSR Acadamy of Sciences, Moscow), Reactivity of Perfluoroalkyl Bromides, 9- July- 1990.

W. A. MacDonald (ICI Wilton), Materials for the Space Age, 11- October- 1990.

M. Bochmann (University of East Anglia), Synthesis, Reactions and Catalytic Activity of Cationic Titanium Alkyls, 24- October- 1990. *

R. Soulen (South Western University, Texas), Preparation and Reactions of Bicycloalkanes, 26- October, 1990.

R. Jackson (Newcastle University), New Synthetic Methods: α-Amino Acids and Small Rings, 31- October- 1990. *

N. Logan (Nottingham University), Rocket Propellants), 1- November- 1990. *

D. Gerrard (British Petroleum), Raman Spectroscopy for Industrial Analysis, 7- November- 1990.

D. Jones (Newcastle University), Light and Airy Chemistry, 7- November- 1990. *

S. K. Scott (Leeds University), Clocks, Oscillations and Chaos, 8- November- 1990.

P. Kocovsky (Uppsala University), Stereo-Controlled Reactions Mediated by Transition and Non-Transition Metals, 9- November- 1990. *

T. Bell (SUNY, Stoney Brook, USA), Functional Molecular Architecture and Molecular Recognition, 14- November- 1990. *

J. Prichard (Queen Mary and Westfield College London), Copper Surfaces and Catalysts, 21- November- 1990.

B. J. Whitaker (Leeds University), Two-Dimensional Velocity Imaging of State-Selected Reaction Products, 28- November- 1990. *

D. Crout (Warwick University), Enzymes in Organic Synthesis, 29- November- 1990. *

P. G. Pringle (Bristol University), Metal Complexes with Functionalised Phosphines, 5- December- 1990. *

A. H. Cowley (University of Texas), New Organometallic Routes to Electronic Materials, 13- December- 1990.

B. J. Alder (Lawrence Livermore Labs., California), Hydrogen in all its Glory, 15- January- 1991.

P. Sarre (Nottingham University), Comet Chemistry, 17- January- 1991. *

P. J. Sadler (Birkbeck College London), Design of Inorganic Drugs: Precious Metals, Hypertension and HIV, 24- January- 1991. *

E. Sinn (Hull University), Coupling of Little Electrons with Big Molecules. Implications for the Active Site of Metalloproteins and othe Macromolecules, 30- January- 1991. *

D. Lacey (University of Hull), Liquid Crystals, 31- January- 1991. *

R. Bushby (Leeds University), Biradicals and Organic Magnets, 6- February- 1991. *

M. C. Petty (Durham University), Molecular Electronics, 14- February- 1991. *

B. L. Shaw (Leeds University), Syntheses with Coordinated, Unsaturated Phosphine Ligands, 20- February- 1991

J. Brown (Oxford University), Can Chemistry Provide Catalysts Superior to Enzymes, 28- February- 1991. *

C. M. Dobson (Oxford University), NMR Studies of Dynamics in Molecular Crystals, 6- March- 1991.

J. Markam (ICI Phamaceuticals), DNA Fingerprinting, 7- March- 1991. *

R. R. Schrock (Massachusettes Institute of Technology), Metal-Ligand Multiple Bonds and Metathesis Initiators, 24- April- 1991.

T. Hudlicky (Virginia Polytechnic Institute), Biocatalysis and Symmetry Based Approaches to the Efficient Synthesis of Complex Natural Products, 25- April- 1991. *

M. S. Brookhart (University of North Carolina), Olefin Polymerisations, Oligomerisations and Dimerisations Using Electophilic Late Transition Metal Catalysts, 20- June- 1991.

M. A. Brimble (Massey University, New Zealand), Synthetic Studies Towards the Antibiotic Griseusin-A, 29- July- 1991. *

J. A. Salthouse (University of Manchester), Son et Lumiere - a demonstration lecture, 17- October- 1991. *

R. Keeley (Metropolitan Police Forensic Science Lab.), Modern Forensic Science, 31- October- 1991.

B. F. G. Johnson (Edinburgh University), Cluster-Surface Analogies, 6- November- 1991.

A. R. Butler (St Andrews University), Traditional Chinese Herbal Brugs: A Different Way of Treating Disease, 7- November- 1991. *

D. Gani (St Andrews University), The Chemistry of PLP Dependent Enzymes, 13- November- 1991. *

I. Flemming, Use of Silicon in Stereochemistry, 19- November- 1991. *

R. More O'Ferrall (University College Dublin), Some Acid-Catalysed Rearrangements in Organic Chemistry, 20- November- 1991. *

I. M. Ward (IRC in Polymer Science, University of Leeds), The Science and Technology of Orientated Polymers, 28- November- 1991.

R. Grigg (Leeds University), Palladium Catalysed Cyclisations and Ion Capture Processes, 4- December- 1991. *

A. L. Smith (ex Unilever), Soap, Detergents and Blackpuddings, 5- December- 1991.

W. D. Cooper (Shell Research), Colloid Science, Theory and Practice, 11- December- 1991.

K. D. M. Harris (St Andrews University), Understanding the Properties of Solid Inclusion Compounds, 22- January- 1992.

A. Holmes (Cambridge University), Cycloaddition Reactions in the Service of the Synthesis of Pipridine and Indolizidine Natural Products, 29- January- 1992. *

D. E. Fenton (Sheffield University), Polynuclear Complexes of Molecular Clefts as Models for Copper Biosites, 12- February- 1992. *

E. J. Thomas (Manchester University), Applications of Organostannanes to Organic Synthesis, 19- February- 1992. *

J. F. Nixon (University of Sussex), Phosphoalkynes, New Building Blocks in Inorganic ond Organometallic Chemistry, 25- February- 1992.

M. L. Hitchman (Strathclyde University), Chemical Vapour Deposition, 26- February- 1992.

S. E. Thomas (Imperial Colege), Recent Advances in Organoiron Chemistry, 11- March- 1992. *

H. Maskill (Newcastle University), Concerted or Stepwise Fragmentation in a Deamination Type Reaction, 18- March- 1992. *

D. M. Knight (University of Durham (Philosophy Dept.)), Interpreting Experiments: The Beginnings of Electrochemistry, 7- April- 1992.

J-C. Gehret (Ciba-Geigy, Basel), Some Aspects of Industrial Agrochemical Research, 13- May- 1992. *

Conferences Attended Outside Durham.

St Andrews University: Biological Organic Chemistry 9- November- 1990.

T. J. Simpson, Biosynthesis of Polyketide Antibiotics: Problems in Molecular Recognition and Specificity.

D. Rees, Structure-Activity Relationships of Kappa Opioids

D. Robins, Biosynthesis of Alkaloids Derived from Ornithine and Lysine.

G. Wulff, Molecular Recognition in Synthetic Polymers.

Leeds RSC Regional Symposium 5- April- 1991.

A. P. Stanforth, Aspects of Heterocyclic Chemistry.

P. Bradly, Asymmetric Routes to Chiral Piperidines.

O. J. Tayler, Chemistry of Nucleotides Relating to Toxicology.

P. Steel, Approaches to the Taxol C-D Ring System.

D. Singh, Carbohydrate Chemistry in the Synthesis of Natural Products.

C. Rayner, Model Transformations 2,3-Epoxy Sulfides.

G. Stork, Regio- and Stereo-control Past and Present.

University of Warwick: Lipases: Structure, Function and Applications in Biotransformations 16 to 18- July- 1991.

H. L. Brockman, Towards Defining the Quality of the Interface: Lipid Organisation in the Regulation of Lipolysis.

R. Verger, Stereoselectivity and Inactivation of some Lipases.

B. Huge-Jensen, Structure and Applications of Fungal Lipases.

J. D. Schrag, The Structure of *Geotrichum candidum* Lipase.

F. K. Winkler, The Structure of Human Pancreatic Lipase Suggests a Locally Inverted Trypsin Like Mechanism.

E. Charlton, Purification and Properties of *Geotrichum candidum* Lipase.

P. J. Halling, Lipase Catalysis in Mainly Organic Media: Effects of Water Activity and pH.

Y. Inada, Ester Synthesis with Polyethylene Glycol Lipase Conjugate in Organic Solvents.

J. G. T. Klerkels, Lipase Kinetics in Two-Phase Emulsion System and the Application in Biotransformations.

K. Faber, Lipases in Organic Solvents: Advantages and Pitfalls.

P. E. Sonnet, Probes for Studying Lipase Selectivities: Synthesis, Analysis and Some Observations.

M. P. Schneider, Lipase Catalysed Preparation of Synthetically Useful Enantiomers.

"Stereochemistry at Sheffield" 18- December- 1991.

D. A. Evans, Studies in Asymmetric Synthesis.

S. V. Ley, Synthesis of Insect Antifeedants.

P. B. Dervan, A Chemists Approach to a General Solution for the Sequence Specific Recognition of Double Helical DNA.

D. Bellus, Some Stereochemical Challenges in Biologically Orientated Industrial Research.

D. Barton, The Invention of Chemical Reactions.

New Delhi (India): IUPAC-NOST International Symposium on Enzymes in Organic Synthesis 6 to 9- January- 1992.

J. B. Jones, Probing the Specificity of Synthetically Useful Enzymes.

C. Wandrey, Enzyme Reactiuon Engineering.

M. P. Schneider, Hydrolases in Organic Synthesis: Preparation of Enantiomerically Pure Compounds.

M. Bhupathy, Chemoenzymatic Synthesis of a Novel LTD_4 Antagonist.

K. Faber, Useful Hydrolytic Enzymes: Proteases, Lipases and Nitrilases.

T. Hudlicky, Enzymatic Hydroxylations of Arenes and Symmetry Conciderations in Efficient Synthetic Design of Oxygenated Natural Products.

C-H. Wong, Enzymes for Carbohydrate and Peptide Synthesis.

P. Balaram. The Design and Construction of Synthetic Protein Mimics.

D. Hilvert, Catalytic Antibodies: Perspectives and Prospects for the Future.

K. Rama Rao, New Trends in Biocatalysis in the Presence of Cyclodextrins.

H. S. Mosher, Preparative Synthesis of (R)-(+)-Ethanol-1-d by the Method of Simon; Synthesis of Derivatives; Application to More Complex Substrates.

H. Griengl, Enzyme Catalysed Transcyanohydrination.

M. Demuth, Light-Induced Selectivity Changes of Reductions with Yeast Enzymes.

S. Servi, The Formation of Minor Products from Reactive Unnatural Substrates and Baker's Yeast.

A. Kumar, A Novel Chemoenzymatic Enantioselective Synthesis of Some Clinically Effective CNS Drugs and Related Compounds.

D. H. G. Crout, Biotransformations in the Peptide and Carbohydrate Fields.

C. R. Johnson, Applications of Enzymes in the Synthesis of Bioactive Polyols.

H. E. Schoemaker, Chemo-Enzymatic Synthesis of Amino Acids and Derrivatives.

S. Kobayashi, Synthetic Study on an Antitumor Antibiotic Rhizoxin by Using an Enzymatic Process on Prochiral β-Substituted Glutarates.

D. Bianchi, Enzymatic Preparation of Optically Active Fungicides Intermediates in Aqueous and Organic Media.

A. M. Klibanov, Control of Enzymatic Selectivity by the Reaction Media.

A. J. Russell, Mechanistic Enzymology with Subtilisin in Non Aqueous Solvents.

A. V. Levashov, Microheterogeneous Surfactant-Based Systems as Media for Enzymatic Reactions.

B. K. Bachhawat, Enzyme Engineering and its Application in Model Lysosomal Storage Disease.

P. Friedrich, Proteolysis and Isopeptide Bond Formation in Nervous Tissue.

K. VS Rao, Synthetic Peptides as Inhibitors of Protein Tyrosine Kinases.

T. Norin, Enantio- and Regio-selectivity of Some Lipases: Control and Prediction.

V. S. Parmar, Potential Applications of Enzyme Mediated Transesterifiactions in the Synthesis of Bioactive Compounds.

C. Tamm, Pig Liver Esterase Catalysed Hydrolyses: Substrate Specificity and Stereoselectivity.

A. R. Fersht, Pathway and Stability of Protein Folding.

H. Simon, Properties and Mechanistic Aspects of Newly Found Redox Enzymes from Anaerobes Suitable for Bioconversions on Preparatory Scale.

D. Ranganathan, Enzyme Action: The Delineation of Novel Strategies Based on Reaction Mechanisms and Transition States.

K. Soda, D-Amino Acid Production with Thermostable Enzymes.

S. Chattopadhyay, Chemoenzymatic Synthesis of Some Bioactive Molecules.

P. Singh, Enzyme Mediated Dye Formation on a Solid Support and its Application in Medical Diagnostics.

K. Prasad, Synthesis of the Enantiomers of SDZ 62-834.

K. Ogura, Chain Length Distribution of the Products formed by Polyprenyl Diphosphate Synthase.

P. B. Terentiev, Regio- and Stereo-selective Hydroxylation of some Nitrogen Heterocycles by Microscopic Fungi.

S. M. Roberts, Exploitation of Microbiological Methods for the Synthesis of Biologically Active Natural Products and Analogues.

S. E. Godtfredson, Novel Uses of Biocatalysts in Enzymatic Synthesis and Organoc Chemical Processing.

D. Basavaiah, Enantioselective Synthesis Using Crude Enzymes.

C. Fuganti, Baker's Yeast in Organic Synthesis.