Polymerisations of hydroxyacids performed by Candida rugosa lipase

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POLYMERISATIONS OF HYDROXYACIDS PERFORMED BY CANDIDA RUGOSA LIPASE.

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DECLARATION

The work contained in this thesis was carried out in the Chemistry Department at the University of Durham, between October 1994 and July 1997. All of the work was performed by the author unless otherwise indicated. It has not previously been submitted for a degree at this or any other university.

This thesis is dedicated to

my mother and father,

Ann and Derek,

and to my brother, Dominic.

You can find inspiration in

everything.

If you can't, then

you're not looking properly.

Giorgio Armani

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The final thanks, but by no means the least of the people to whom my gratitude extends, are for my parents, Ann and Derek, and brother, Dominic, without whose patience and encouragement these three years of toil would have been real hardship.

ABSTRACT

This thesis is describes the polymerisation of hydroxyacids catalysed by the *Candida rugosa* lipase (CRL). Chapter 1 involves a background introduction to enzymes in general and specifically lipases. Several commercial applications of lipases are discussed, followed by the use of lipases in the synthesis of polymeric materials.

Chapter 2 covers the experimental work carried out on our system to determine the optimal conditions for the enzymatic reaction to take place. For CRL these are using hexane at 55 °C. Several ω-hydroxyacids are investigated for the relative rates of reaction, using ^{1}H NMR and GPC analytical techniques. The use of molecular sieves is evaluated, as is reprecipitation from hexane, to establish the optimal method for obtaining high molecular weight polymers.

Chapter 3 includes a comparison of the six ω-hydroxyacids which were used, and expresses the observation that CRL has a preference for the hydroxyacids with 8 and 9 carbons. There is an investigation into the sequence of the assembly of the polymer using deuterium labelled monomers, and several functionalised monomers are also polymerised. A comparison is made between the enzymatic and a chemically ($Ti(OBu)_4$) catalysed process.

Chapter 4 introduces molecular modelling studies, and docks 9-hydroxy-nonanoic acid into the cavity beyond the enzyme active site. Various potential affinity labels are designed to covalently modify the active site cavity, and are thus synthesised to determine if the polymerisation process can be inhibited. Finally in this Chapter an investigation into the stereospecificity of CRL is undertaken, using 10-hydroxy-undecanoic acid, and a binding mechanism between the enzyme and the substrate is proposed.

Chapter 5 outlines the synthetic pathways to all of the materials which are synthesised in this thesis, and Chapter 6 describes the experimental routes to each of these materials.

LIST OF ABBREVIATIONS

APEE	N-Acetyl phenylalanine ethyl ester
BAEP (69)	N-Bromoacetylethanolamine phosphate
1-BrU (70)	1-Bromoundecane
11-BrUA (71)	11-Bromoundecanoic acid
CRL	*Candida rugosa* lipase
DP	Degree of polymerisation
DSC	Differential scanning calorimetry
Et 3-HB (66)	Ethyl 3-hydroxybutyrate
Et 6-HH (63)	Ethyl 6-hydroxyhexanoate
GC	Gas Chromatography
GCL	*Geotrichum candidum* lipase
GPC	Gel permeation chromatography
HB (76)	Hexyl butyrate
10-HDA (42)	10-Hydroxydecanoic acid
12-HDDA (54)	12-Hydroxyhexadecanoic acid
HDSC (68)	Hexadecanesulphonyl chloride
16-HHDA (55)	16-Hydroxyhexadecanoic acid
9-HNA (53)	9-Hydroxynonanoic acid
8-HOA (52)	8-Hydroxyoctanoic acid
HPL	Human pancreatic lipase
11-HUA (43)	11-Hydroxyundecanoic acid
IR	Infra red
LAPH	Liver acetone powder horse
LAPP	Liver acetone powder pig
MALDITOF MS	Matrix assisted laser desorption ionisation time of flight mass spectrometry
Me 8-BrAcO (73)	Methyl 8-bromoacetyloctanoate

Me 8-HO (59)	Methyl 8-hydroxyoctanocate
Me 11-BrAcU (72)	Methyl 11-bromoacetylundecanoate
Me 11-HU (64)	Methyl 11-hydroxyundecanoate
Me 12-HS (65)	Methyl 12-hydroxystearate
M_n	Number average molecular weight
MPC (67)	*O*-Menthyl hexylphosphonochloridate
M_W	Weight average molecular weight
NMR	Nuclear magnetic resonance
PCL	*Pseudomonas cepacia* lipase
PHA	Polyhydroxy acid
PHB	Poly (*R*)-3-hydroxybutyrate
PPL	Porcine pancreatic lipase
PS-30	*Pseudomonas* lipase
SDS	Sodium dodecylsulphate
SG	*Streptomyces griseus* lipase
TCEB (74)	Trichloroethyl butyrate
TLC	Thin layer chromatography

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CHAPTER ONE

INTRODUCTION

1.0: INTRODUCTION.

1.1: The early history of enzymes.

Enzymes are biological catalysts[1], which operate in cells, and can increase rates of biochemical reactions by up to a factor of 10^{12}. Enzymes have evolved and each catalyses a single reaction on a particular substrate.

1997 was the 100th anniversary of Eduard Büchner's observation that yeast extracts which contained no living cells were responsible for the fermentation of sugar to alcohol and carbon dioxide[2]. The species causing the fermentation he named "zymase", which he claimed was present in the yeast cells. Although the fermentation process had been used since ancient times for brewing and bread-making in Egypt, this was the first time scientists had faced the concept of the fermentation process being mediated by a non-living species. This fermentation process was the first biochemical pathway to be elucidated by Embden and Meyerhof[3]. In 1926 Sumner isolated a single protein from a crude extract, and demonstrated that the protein had catalytic activity. The enzyme was urease, which hydrolysed urea to carbon dioxide and ammonia[2]. Sumner crystallised urease in 1928, the first enzyme to be crystallised, and shortly after pepsin, trypsin and chymotrypsin were all crystallised.

1.2: Hydrolase enzymes.

The hydrolase group of enzymes catalyses the hydrolytic cleavage of C-O, C-N, C-C and various other bonds (such as P-O)[4], found in biological macromolecules: nucleic acids are hydrolysed by nucleases, polysaccharides by glycosidases, and polypeptides by peptidases or proteases. Peptidases hydrolyse amide and ester bonds, and are classified by the group which carries out the catalytic cleavage. These enzymes are: 1) the serine proteases; 2) the cysteine proteases; 3) the aspartyl proteases; and 4) the metalloproteases[2].

The active site residues of the serine proteases consists of a catalytic serine,

which acts as the initial nucleophile during the hydrolytic process, assisted by a histidine and an aspartate residue[5,6,7], operating together as a 'catalytic triad', although in some cases aspartic acid is replaced by glutamic acid[8].

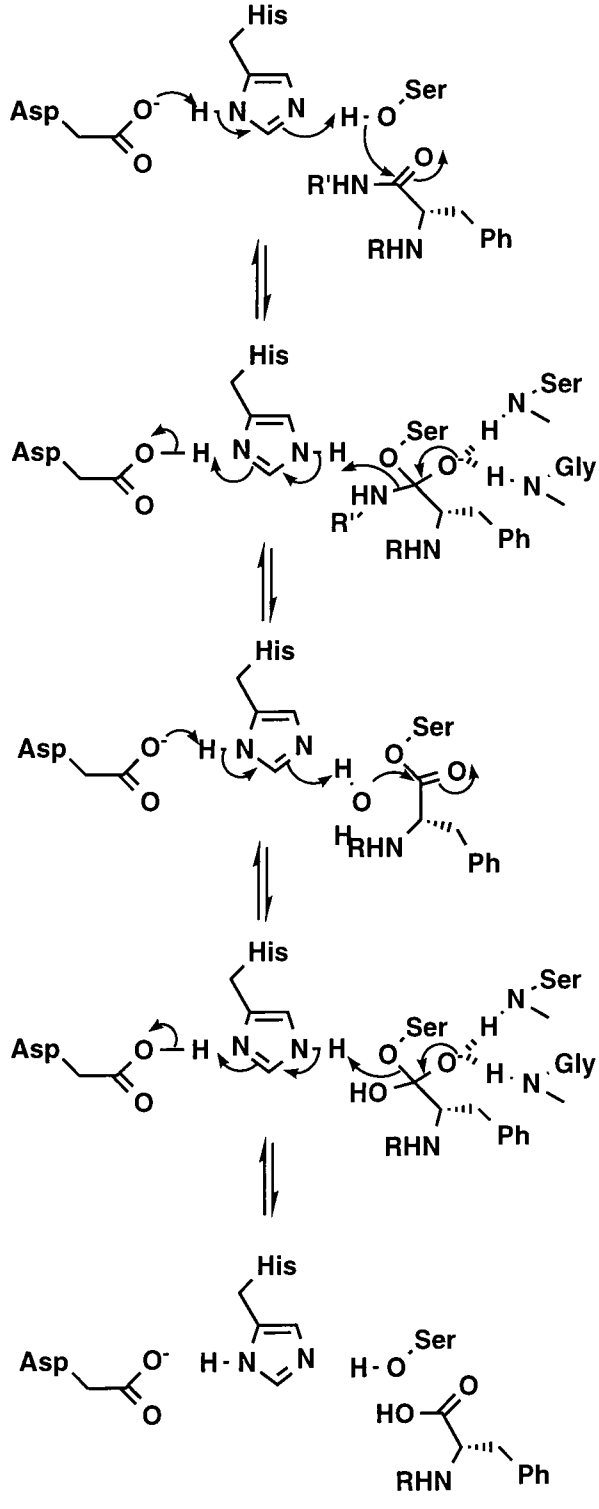


Figure 1.1. *Mechanism of amide hydrolysis by α–chymotrypsin[2].*

The serine residue lies within hydrogen bonding distance of the N-1 ring nitrogen of the histidine residue, while the N-2 ring nitrogen is within hydrogen bonding distance of the carboxylate group of the aspartate or glutamate residue[5]. A proton-relay system operates to transfer the charge on aspartate to generate a serine alkoxide nucleophile. The alkoxide attacks the amide carbonyl, to generate a tetrahedral intermediate. This intermediate breaks down to an acyl-enzyme complex with the elimination of RNH_2. The acyl-enzyme complex is then hydrolysed by a molecule of water, which is activated by the same type of process. The enzyme returns to its original state, releasing the carboxylic acid into the medium. In this way serine proteases hydrolyse amides to amines and acids.

α-Chymotrypsin is one of the best studied serine proteases, with 241 amino acids, cleaving polypeptide chains after aromatic amino acid residues (phenylalanine, tyrosine or tryptophan). This specificity for aromatic residues is attributed to a hydrophobic binding pocket adjacent to the catalytic triad, compatible with the aromatic side chains of these amino acids, which stabilises the acyl-enzyme complex[2].

Certain organophosphorus and chloromethylketones compounds have been designed as inhibitors of serine proteases. The organophosphorus inhibitors, such as diisopropylphosphofluoridate, form a phospate-enzyme ester at the active site serine. The tetrahedral geometry of the phosphorus closely resembles the tetrahedral geometry of the transition state for hydrolysis of the enzyme-substrate complex, and is thus tightly bound by the enzyme[2].

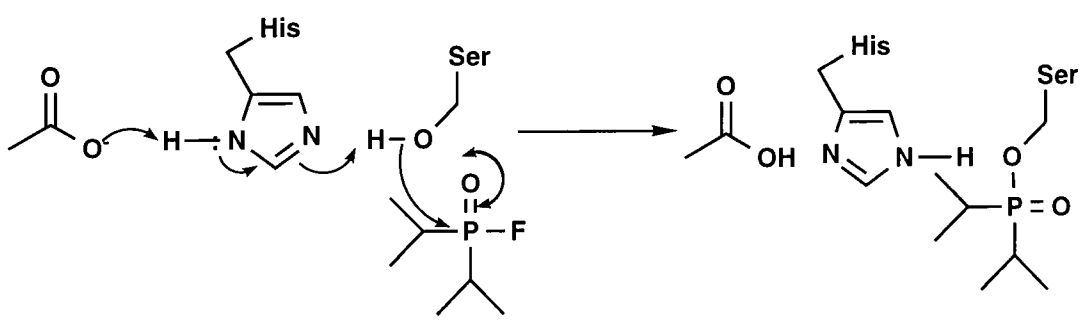


Figure 1.2. *The organophosphorus inhibitor, diisopropylphosphofluoridate.*

Chloromethylketone analogues covalently modify the histidine residue, by the sequence of events shown in Figure 1.3, rendering it inoperable.

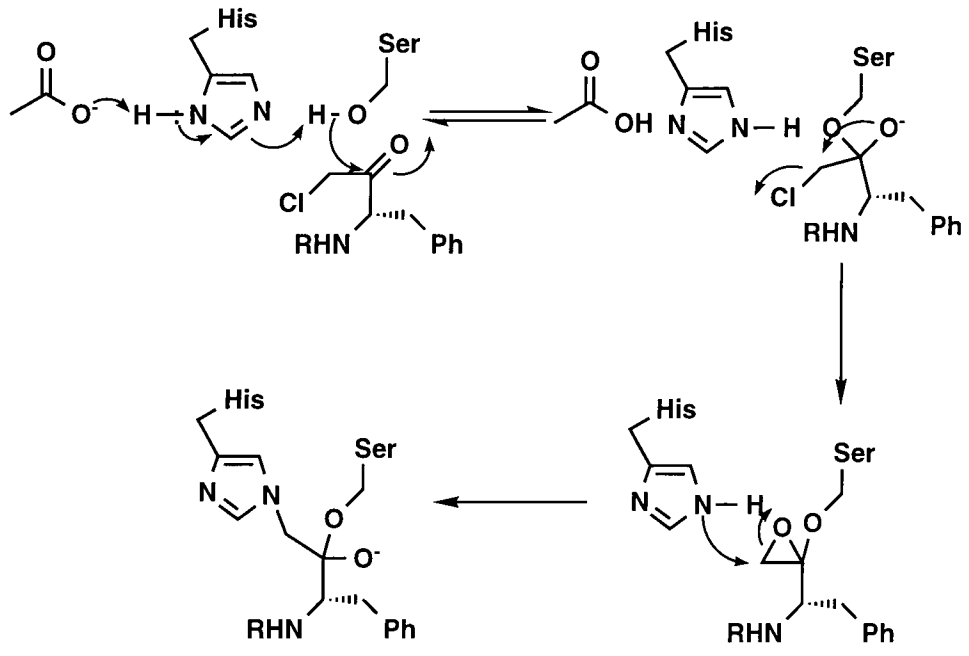


Figure 1.3. *Chloromethylketone inhibitors.*

Lipases belong to the group of enzymes which are inhibited by chloromethylketone inhibitors, and they catalyse the hydrolytic cleavage of triacyl glycerides to long chain fatty acids (water insoluble) and glycerol[9]. The hydrolytic action of lipases contrasts with the esterases, which catalyse the cleavage of short chain triacyl glycerides to short chain fatty acids (water soluble) and glycerol[10]. There are several well known lipase enzymes whose structures have been solved, such as *Geotrichum candidum* lipase (GCL) and *Human pancreatic* lipase (HPL)[7,10].

1.3: Structures of the lipase family.

The yeast *Candida rugosa* produces several lipase enzymes, of which five have had their amino acid sequences characterised[9]. They are proteins of 57 kDa molecular weight, with 534 amino acids. These five enzymes have significant homology with

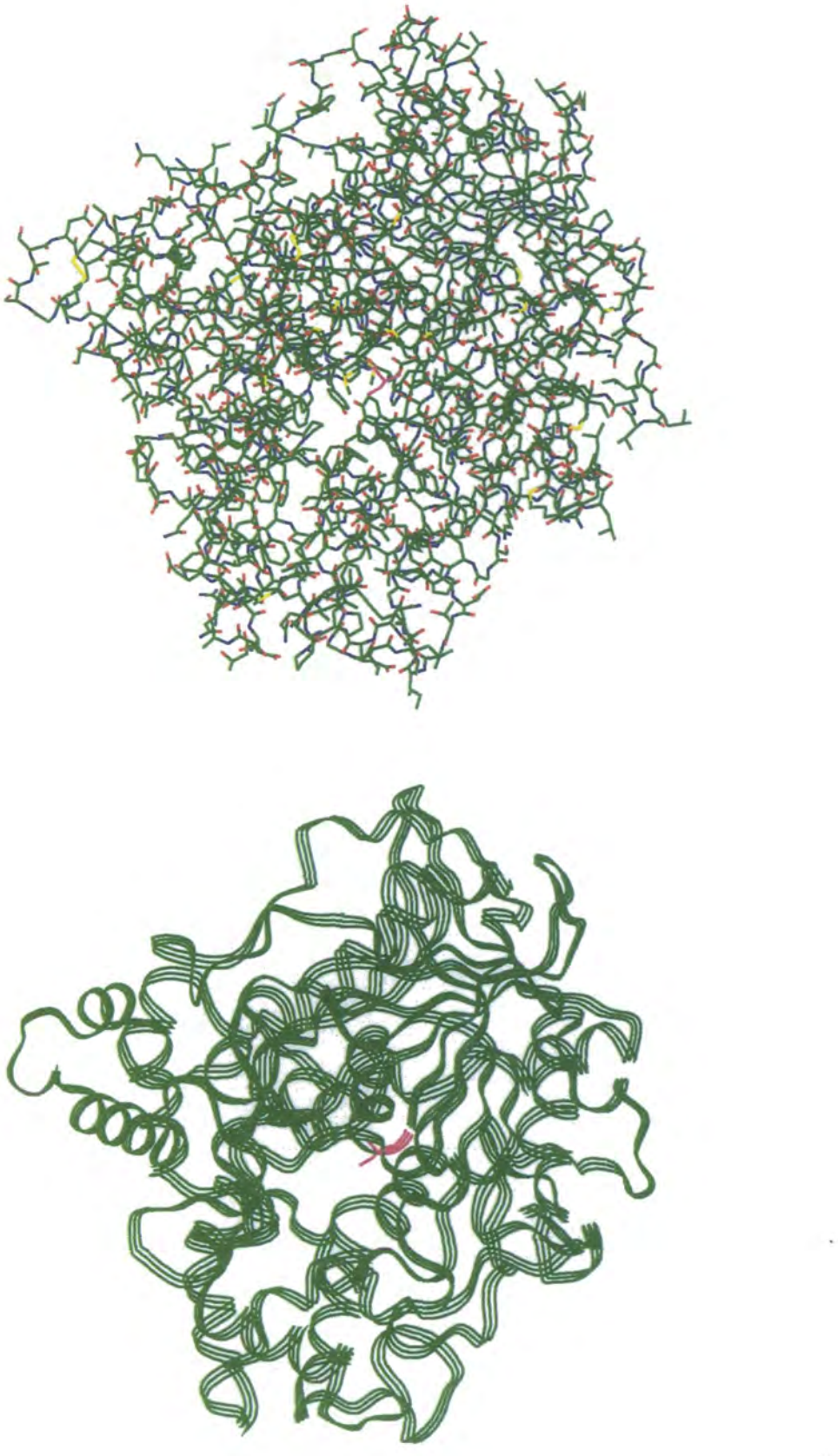
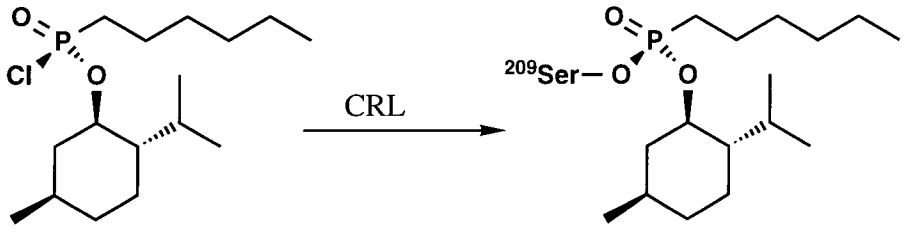


Figure 1.4. Candida rugosa *lipase, in world view and with the ribbon structure, portraying the α-helices and the β-sheets.*

over 66 % of their amino acid residues common.

The lipases from *Candida rugosa* have a similar structural organisation to lipases from other fungi, such as GCL[9]. All have had their three dimensional structures solved by X-ray analysis. GCL is a single domain molecule, with dimensions of approximately 65 x 60 x 45 Å[8]. It consists of α helices and a β sheet, with a central 11-strand β sheet, with most of the strands running parallel.[9]. The *Candida rugosa* lipase[9] is a single domain protein, belonging to the α/β hydrolase family of proteins. It consists of parallel β strands and α helices. Many of the C_α residues (84 %) can be superimposed upon the same residues from GCL, with only minor differences (<1 Å root mean square deviation)[11]. The structure of the *Candida rugosa* lipase is shown in Figure 1.4.

Cygler *et al.* [12] located the active site of this lipase by solving the structure with a phosphonate inhibitor covalently bound to the catalytically functional serine 209 residue. This was a major advance in the understanding of the action of lipase enzymes more generally, and showed unambiguously how the substrate and the enzyme interact. Figures 1.5 and 1.6 illustrate model structures of CRL with the inhibitor, menthyl hexylphosphate (1), bound.



(1)

Figure 1.5. *Cygler's phosphate inhibitor, and the inhibitor binding to CRL[12].*

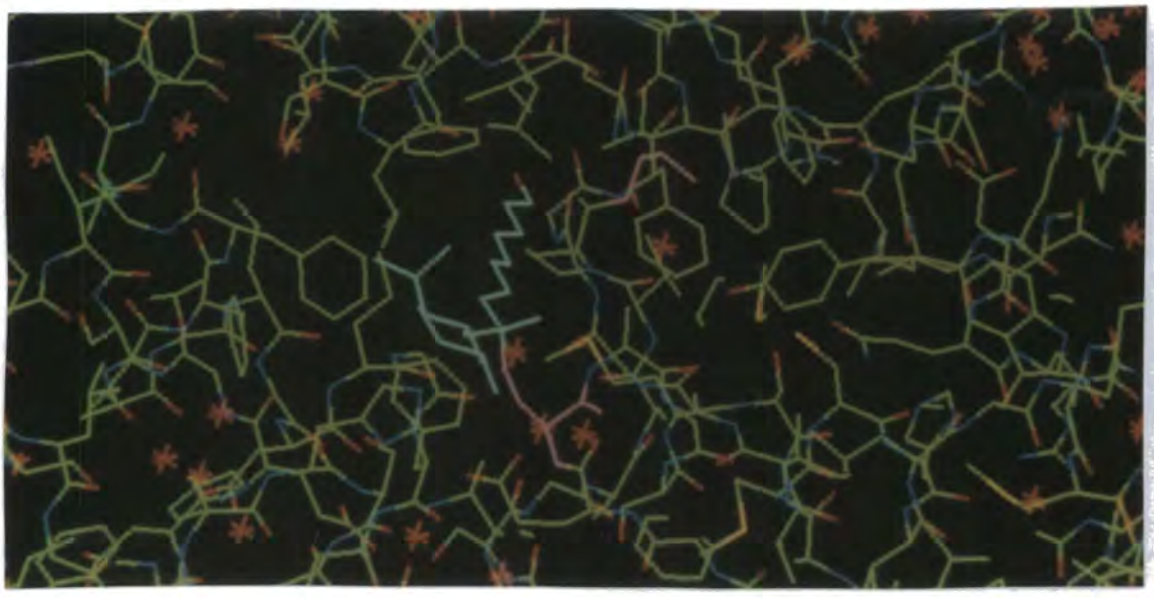


Figure 1.6. Candida rugosa *lipase, with a bound phosphonate inhibitor.*

The mammalian lipase, human pancreatic lipase, is also a single chain protein, consisting of 449 amino acids, with an α/β sheet, and a central parallel β sheet[7]. Thus the mammalian and fungal lipases have common structural features.

1.4: Interfacial activation.

Many lipases have a "lid" or "flap" covering the active site, making it inaccessible to aqueous solvents. This flap consists of a polypeptide chain, which can be as long as 40 amino acid residues as in the case of *Pseudomonas cepacia* lipase (PCL)[13], in a helical formation. The helix can pivot to alter the conformation and allow the substrate to enter the active site[14,15]. Major conformational differences occur between PCL, GCL and CRL, caused by residue deletions in this flap. A difference of upto 25 Å between the tips of the flap has been reported[14,16]. It is strange that in a family of enzymes which has such heavily conserved regions and such a high level of homology, that the structural similarities break down in regard to these flaps. Sections of PCL, GCL and CRL sequences can not be aligned satisfactorily, even when gaps are introduced to the amino acid sequences. This suggests a possible difference in structural organisation, or that this divergence could be due to the different

substrate specificity of the two enzymes[6,8].

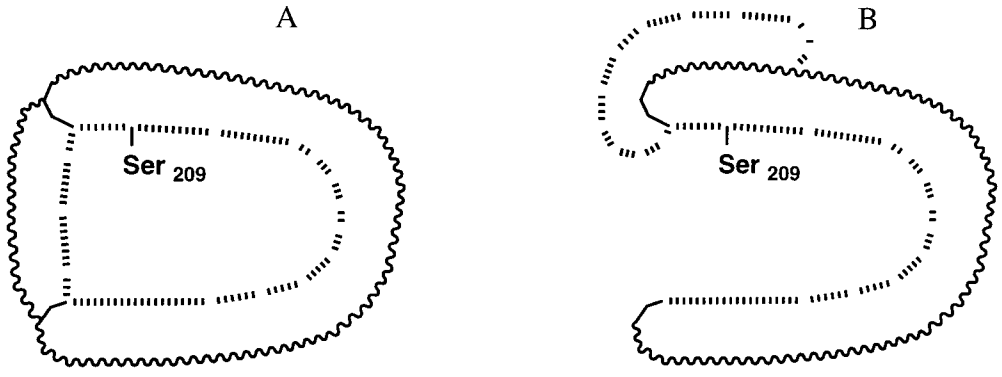


Figure 1.7. *Schematic models A and B represent the enzyme with the flap in the closed and open conformations respectively.*

The driving force for the conformational change of the flap is related to the environment of the enzyme. The hydrolysis of the triglycerides is facilitated by the enzyme operating at a lipid-water interface within the cell[17]. As the enzyme adsorbs onto the interface the flap opens to increase the hydrophobic contact, and to allow the triglyceride to enter the active site[18]. As the flap is opened there is a distinct change in the nature of the surface of the protein (see Figure 1.7).

In the closed form the surface is hydrophilic, being exposed to the aqueous solvent of the cell. When the helical flap is turned back, the hydrophilic surface becomes buried in a polar cavity, and the hydrophobic surface of the enzyme surrounding the active site is revealed, greatly increasing the non-polar character of the surface surrounding the catalytic triad[6,11]. The lipid environment at the interface helps to stabilise the enzyme in this open conformation. Increased substrate concentration at the interface also contributes to the increased rate of ester hydrolysis displayed by the enzyme at the interface. This has been termed interfacial activation[6].

The flap is hinged, in the case of CRL, at glutamic acid 66 and at proline 92[15].

A conformational change comes about through a *cis-trans* isomerisation of the proline residue, allowing differentiation between the open-active and the closed-inactive forms of the enzyme. Movement of the flap into the open conformation exposes both the active site (which is believed to be the lipid-binding site[13]), and the surrounding hydrophobic residues. The lipid-binding site, also termed the interfacial lipid recognition site, has a conserved sequence of amino acid residues throughout the hydrolase family: this common sequence is Gly - X - Ser - X - Gly/Ala, where X is usually Tyr or His[10,17] and lies between residues 207 to 211, with serine 209 involved in the hydrolysis of the ester bond.

1.5: The active site cavity.

Molecular modelling (in particular covalently modified organophosphorous molecules) has shown that in lipases such as *Candida rugosa* there is a cavity or tunnel beyond the catalytic triad[19,20,21]. Covalent modification of the enzyme with the derivatised phosphate ligand carrying a hydrocarbon chain allowed the hydrophobic cavity to be identified after X-ray analysis.

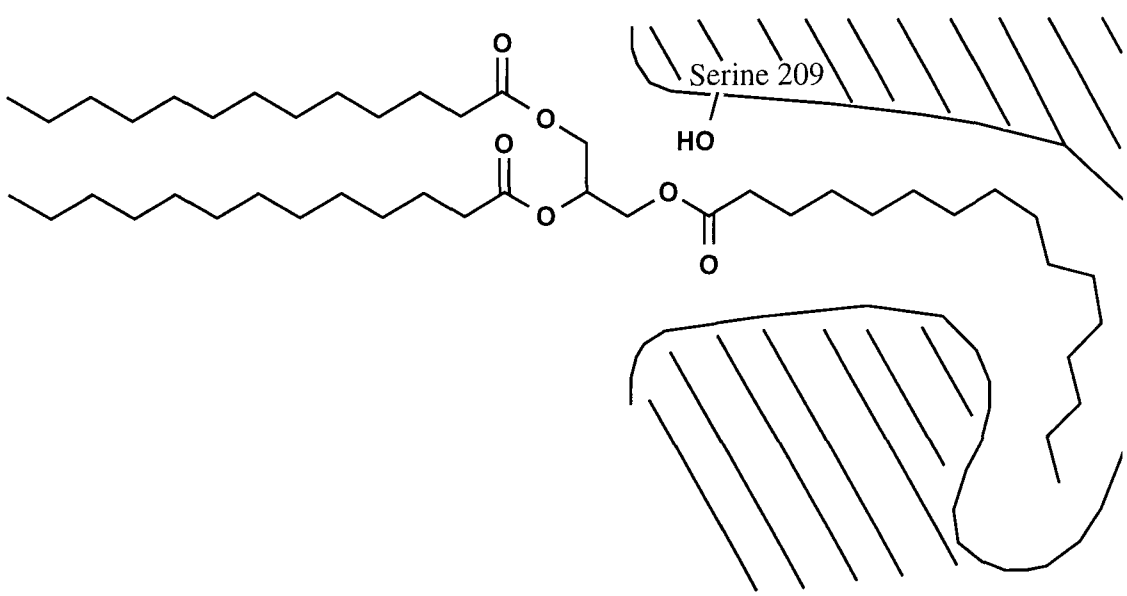


Figure 1.8. *Trihexadecanoyl glycerol with the* sn-*1 acyl chain bound in the active site tunnel. The* sn-*2 and* sn-*3 chains are pointing towards the solvent*[20].

This revealed that the triglyceride hydrocarbon chain enters the cavity and forms the acyl ester intermediate. The cavity assumes an L shape[20], heading initially towards the centre of the enzyme, and then bending back towards the protein surface, long enough to accommodate fatty acid chains of 18 carbons in length. The triglyceride adopts a "tuning fork" like conformation (see Figure 1.8), with one alkyl chain within the enzyme cavity, and the other two chains extending into the lipid layer of the interface, thus orientating an ester bond towards serine 209 for hydrolysis[20].

1.6: Enzymes in organic solvents.

In 1977 Klibanov *et al.* revolutionised the way that organic chemists thought about synthetic enzymatic reactions[22]. It was widely known that enzymes were catalysts for many important biological reactions, such as the hydrolysis of triacyl glycerides, occurring at the cell membrane. However, such hydrolytic enzymes were shown to work in reverse in dry organic solvents. When this had been attempted previously the catalytic activity of the enzyme decreased substantially, and the substrate specificity of the enzyme often disappeared[22]. In order to solve this problem reactions were attempted in organic solvents, or in aqueous-organic mixtures.

Water is required as the nucleophile in many of the enzyme catalysed reactions, and water participates in all the non-covalent interactions which maintain the natural state of the enzyme in its catalytically active conformation[23]. But, enzymes were able to act as catalysts in solvents which were 99.98 % anhydrous. This begged the question, how much water is required for lipase catalysis? The enzyme maintains a layer of essential water around the surface which plays an active part in maintaining the 3-dimensional conformation of the protein. If the enzyme is suspended in an organic solvent containing a small amount of water (< 0.02 %) then it becomes more thermally stable. A lipase from *Candida cylindracea* was active for several hours at 100 °C in dry tributyrin-heptanol, whereas an enzyme in a solution with a greater quantity of water (3 %) had a half-life of minutes[24]. Porcine pancreatic lipase (PPL) is still active at 100 °C in the transesterification reaction between tributyrin and heptanol, and its

activity is five times greater than the same system at 20 °C. The enzyme remains catalytically functional, maintaining its conformational integrity. The stability towards denaturation of the "dry" enzyme at this temperature appears to be due to water playing a role in lubricating the protein. Water is important in denaturation and the unfolding of the protein, such as the formation of incorrect scrambled structures, the destruction of S-S bonds *via* β-elimination, deamidation of asparagine and glutamine residues, and the hydrolysis of peptide bonds at aspartic acid residues[25].

Non-aqueous solvents such as acetone, acetonitrile, dioxane, dimethylsulphoxide, dimethylformamide, ethanol and methanol[22], all solvents miscible with water, have been widely studied in such reactions. A hydrophilic solvent partitions the essential water layer, deactivating the enzyme, and causing denaturation[25,26]. One technique used to increase the stability of the enzymes towards denaturation is immobilisation onto a solid support[22,27]. This technique has inadequacies, however, particularly deactivation of the enzyme when the concentration of the organic phase becomes higher than 90%. The enzyme often undergoes a conformational change on immobilisation, resulting in a subsequent loss of catalysis[22].

If a powdered enzyme is suspended in a biphasic mixture, containing a hydrophobic organic solvent, such as hexane or toluene, the water layer does not partition into the solvent, and thus it is not stripped from the enzyme[23]. Klibanov proposed that the non-aqueous solvent should be water-immiscible, such as choroform, diethyl ether, long chain alcohols and hydrocarbons. Such systems have many advantages: the enzyme does not need to be stabilised against the denaturing effect of the non-aqueous media, since they are not soluble in organic solvents. The percentage of the organic component is increased almost to unity, and thus the equlibrium of the system is in the non-hydrolytic direction. Substrates, dissolved in the organic phase, can diffuse into the aqueous phase, undergo a reaction, and diffuse back into the organic phase, which can be separated easily from the aqueous solvent, and thus the recovery of the product is simplified[22].

This system was developed further such that the water-insoluble substrate

became the organic phase in a biphasic aqueous-organic solvent mixture[27]. Optically active alcohols, such as 2-butanol, 2-octanol, 1-chloro-2-propanol and 2,3-dichloro-1-propanol and their butyric esters were prepared on a gram scale using CRL to perform the transesterification. In this system the fraction of water was extremely low, but the system remained biphasic[27].

A natural extension of this work was to explore reactions of enzymes in 100% organic solvents[28]. For example, PPL and CRL, in the powdered form, were shown to catalyse the esterification of n-butanol with racemic acids, such as 2-bromopropionic acid, 2-chloropropionic acid and 2-bromohexanoic acid to form the optically active esters, using hexane as the solvent. An investigation into the activity of enzymes in organic solvents found that catalytic activity was dependant on the pH of the aqueous solution from which the enzyme was initially recovered[23]. An enzyme is most active if it originates from an aqueous solution in which the pH is optimal for catalytic activity. For example, the activity of PPL is optimal when the enzyme is precipitated from an aqueous solution at pH 8.4, which is the optimal pH for activity in water. Activity decreases significantly when the enzyme is precipitated from an aqueous solution which is at non-optimal pH. Thus the enzymic activity depends on its pH history, and the enzyme "remembers" the pH of the last aqueous solution to which it was exposed, retaining that protonation state in the solid state in organic solvents[23].

Therefore it was thus established that lipases function in organic solvents, but several conditions had to be met: 1) hydrophobic organic solvents are better than hydrophilic organic solvents. The more polar hydrophilic solvents strip the essential layer of water from the enzyme; 2) the enzyme should be a fine powder, and the suspension should be continually agitated to decrease diffusional limitations of the organic substrate, rather than immobilising the enzyme onto a solid support; 3) The enzyme should be recovered from an aqueous solution at the optimal pH for activity, before deposition in the organic solution[23,29].

1.7: Enzymatic resolutions.

The use of enzymatic enantioselectivity has become increasingly important for the preparation of chiral synthons in the fine chemicals, agrochemicals and pharmaceutical industries[30,31]. There have been many investigations into the hydrolysis of chiral esters by enzymes to mediate kinetic resolutions. For example, Bornscheuer used a lipase from *Pseudomonas cepacia* to resolve racemic hydroxy esters, such as methyl 3-hydroxy-4-phenoxybutanoate (2), by either acylating the hydroxyl group (3), or by hydrolysis of the methyl ester (4). This gave both enantiomers of the racemate for the preparation of the β-blocker propanolol[32].

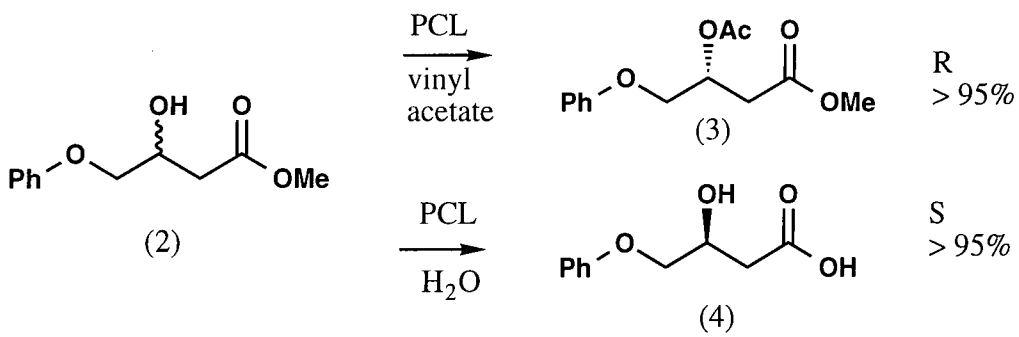


Figure 1.9. *Enzymatic resolution by hydrolysis or esterification[32].*

This type of resolution has also been carried out by Högberg[33]. 2-Methylalkanoic acids were resolved by esterification in cyclohexane with primary alcohols, catalysed by *Candida rugosa* lipase. The enzyme resolved the racemic 2-methyldecanoic acid (5) by esterification of the S-acid with *n*-tetradecanol (7) to give the enantiomerically pure S-ester (8), leaving the R-acid (6) untouched, as shown in Figure 1.10. This enzymatic resolution was used as the first step for the preparation of 2-methyldecanoic acid in > 99 % ee, and 2-methyldecanol in > 96 % ee, which have been used as intermediates in the preparation of insect pheromones[33].

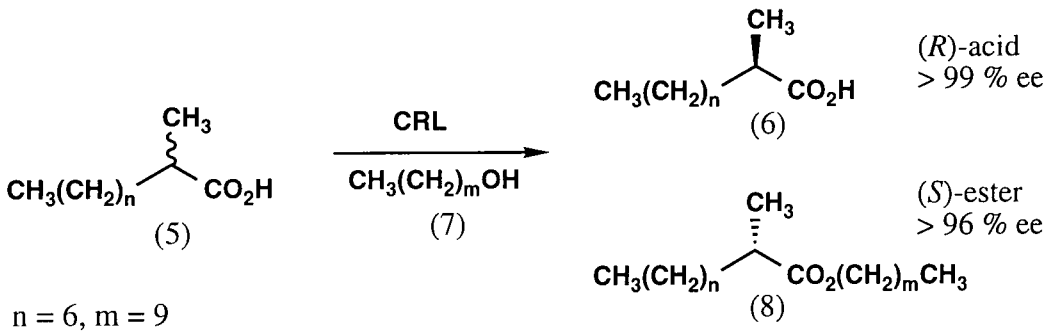


Figure 1.10. *Resolution of 2-methyldecanoic acid (5) using CRL*[33].

1.8: Organic reactions performed by enzymes.

Enzymes can also be used to undertake biotransformation reactions which would be difficult by the conventional tools of synthesis. Nitrile hydrolases have been used on a large scale to hydrolyse acrylonitrile (9) in the preparation of acrylamide (10), which is a monomer for the polymer industry (see Figure 1.11). The synthesis of penicillin derivatives also required the use of a hydrolase (penicillinase) to hydrolyse the amide (11) to an amine (12), without destroying the β-lactam ring system rendering the molecule active for side chain modifications (see Figure 1.12)[34].

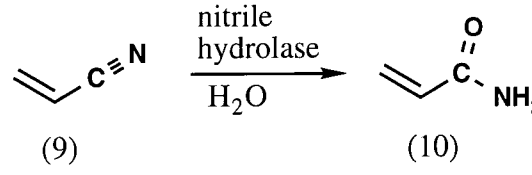
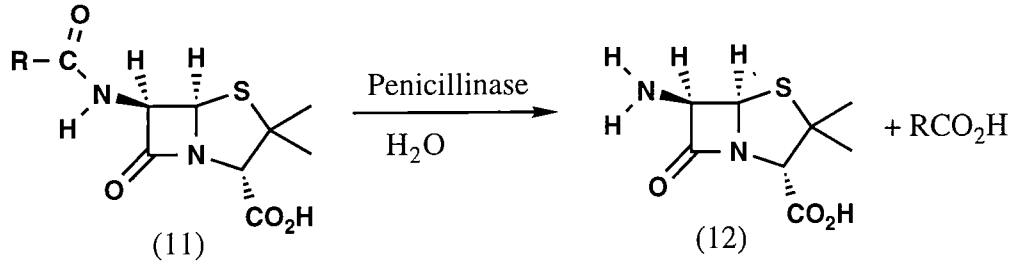


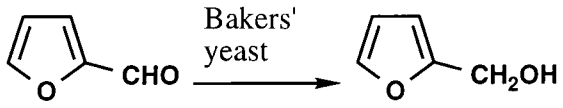
Figure 1.11. *Preparation of acrylamide (10) from acrylonitrile (9)*[34].



R = alkyl or aryl

Figure 1.12. *Enzymatic amide hydrolysis to prepare penicillin derivatives*[34].

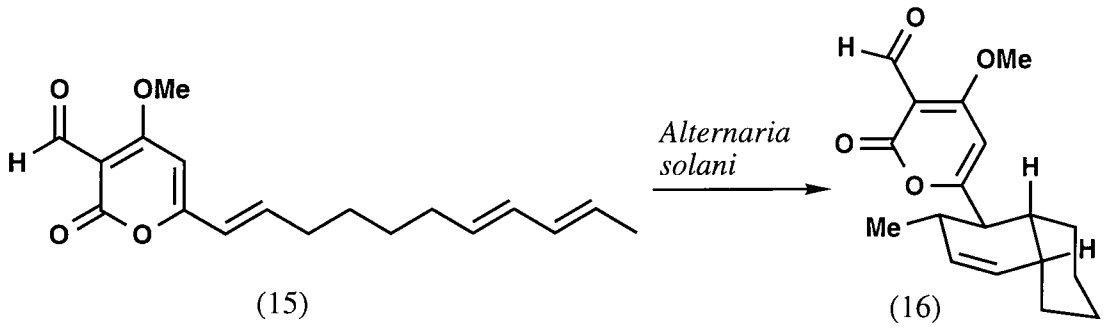
Enzymes have also been shown to catalyse the reduction or dehydrogenation of substrates, such as Bakers' yeast mediated reduction of furaldehyde (13) to furfuryl alcohol (14)[34].



(13) (14)

Figure 1.13. *Aldehyde reduction performed by Bakers' yeast*[34].

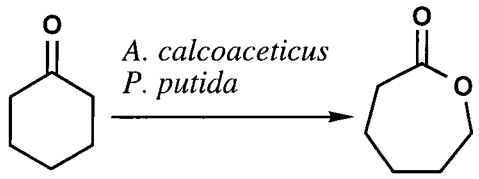
Interestingly, an enzyme from *Alternaria solani* has been shown to catalyse the Diels Alder cycloadditions of prosolanapyrone (15) as the last step in solanapyrone (16) biosynthesis in a 92 % ee[35] (see Figure 1.14).



(15) (16)

Figure 1.14. *Formation of solanapyrone (16) from prosolanopyrone (15) by a Diels Alderase from* Alternaria solani *(92 % ee)*[35].

In a further example of the diversity of enzymes in biotransformations the biological Baeyer-Villiger reaction has been widely explored[36,37,38]. *Acinetobacter calcoaceticus* when incubated with cyclohexanone (17)[36,37,38] produces caprolactone (18), as does a monooxygenase from *Pseudomonas putida*[38].



(17) (18)

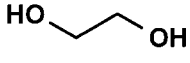
Figure 1.15. *An enzymatic Baeyer-Villiger reaction*[36,37,38].

1.9: Lipase catalysed polymerisations performed in organic media.

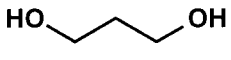
The formation of polymers, polyesters and macrocyclic lactones has been explored using lipases in organic media, and this aspect of lipase biotransformations forms the basis of the thesis. Developments in this field impact on environmental issues to produce clean, biodegradable polymers, prepared from renewable raw materials. Two types of systems have arisen for the lipase catalysed polymerisation: the AA-BB system, in which AA represents a diacid or a diester, and BB represents a diol; and the AB system, in which AB represents a hydroxy acid or hydroxy ester.

1.9.1: [AA-BB] Systems.

Early work on enzymatic polymerisations was undertaken by Okumura *et al.* who used a lipase from *Aspergillus niger* to polymerise dicarboxylic acids (with between six and fourteen carbons) with 1,2-ethanediol (19) and 1,3-propanediol (20)[39].



1,2-ethanediol
(19)



1,3-propanediol
(20)

Oligomers were obtained between 1,13-tridecanedioic acid (21) and 1,3-propanediol (20), when stirred at 30 °C. The oligomer consisted mainly of a heptamer with three molecules of the diacid and four molecules of the diol, as shown in Figure 1.16[39].

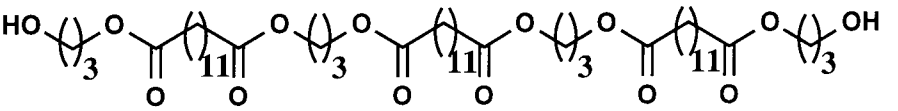
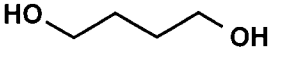


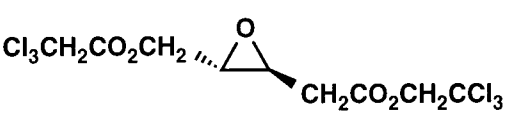
Figure 1.16. *The oligomer of Okumura, produced from the monomers 1,13-tridecanoic acid (21) and 1,3-propanediol (20)*[39].

Many of the early investigations involved the use of 1,4-butanediol (22) as the BB component in the system. Wallace and Morrow[40,41] investigated lipase mediated polymerisation with bis (2,2,2-trichloroethyl) epoxyadipate (23), using PPL in anhydrous diethyl ether, THF or dichloromethane. Enantiomerically enriched polymers (up to 95 % ee) of high mass were generated after five days at ambient temperature with this system. To date this system shows the only polymer formed by enzymatic resolution, and as such the technology has been patented[40,41].



1,4-butanediol

(22)

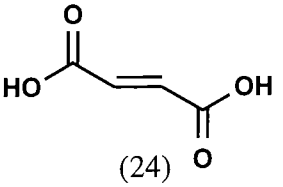


bis (2,2,2-trichloroethyl) epoxyadipate

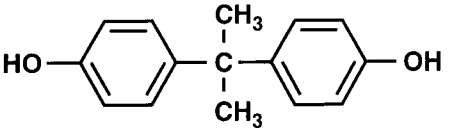
(23)

An Israeli group[42,43] have also investigated 1,4-butanediol (22)[42] in the synthesis of alkyds (complex unsaturated polyesters) using diesters of fumaric acid (24). Aromatic diols[43], such as bisphenol A (4,4'-isopropylidene diphenol) (25) were also employed in these studies, although with no success. The reactions with fumaric acid (24) were carried out in acetonitrile or THF at 37 °C. There was no isomerism of the "all trans" double bond in the fumaric acid (24), rendering the enzymatic synthesis more controlled than the chemical synthesis of alkyds, in which the double bond is readily isomerised. Roberts[44] investigated the polycondensation of 1,4-butanediol (22) with adipic acid (26) by lipozyme M-20 (*Mucor miehei*). The highest degrees of polymerisation were achieved in diisopropyl ether at a temperature of 40-44 °C. This

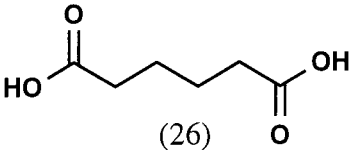
enzymatic polymerisation was the first system in which molecular sieves were employed to remove the water which was generated during the condensation reaction. The sieves served to suppress the competitive hydrolytic reaction, and thus increased molecular weights were achieved.



(24) fumaric acid

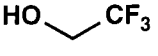


(25) bisphenol A, or 4,4'-isopropylidene diphenol



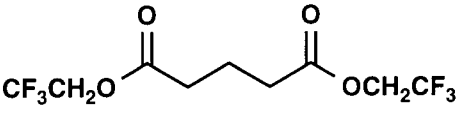
(26) adipic acid

Several of the investigations into AA-BB systems involve the condensation of esters with 1,4-butanediol (22), to generate 2,2,2-trifluoroethanol (27). Morrow *et.al.*[45] employed PPL to polymerise bis (2,2,2-trifluoroethyl) glutarate (28) with 1,4-butanediol (22). Either 1,2-dimethoxybenzene (29) or 1,3-dimethoxybenzene (30) was used as the solvent at 40 °C, and high masses (M_W = 40 000 by GPC) were achieved in 7 days.



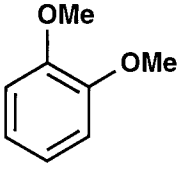
2,2,2-trifluoroethanol

(27)



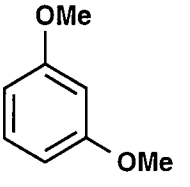
bis (2,2,2-trifluoroethyl) glutarate

(28)



1,2-dimethoxybenzene

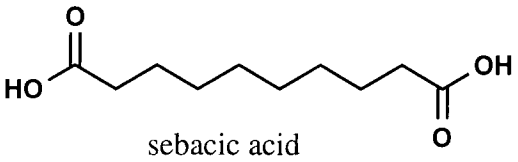
(29)



1,3-dimethoxybenzene

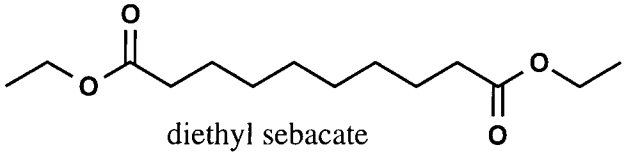
(30)

Linko *et al.*[46,47,48,49] polymerised sebacic acid (31), diethyl sebacate (32) or bis (2,2,2-trifluoroethyl) sebacate (33) with 1,4-butanediol (22) in 1,2-dimethoxybenzene (29) and generated high molecular weights (up to M_W = 46 000), also after 7 days. These reactions were performed at 37 °C with the *Mucor miehei* lipase.



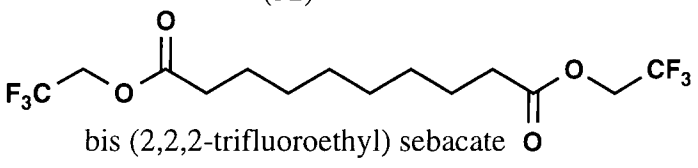
sebacic acid

(31)



diethyl sebacate

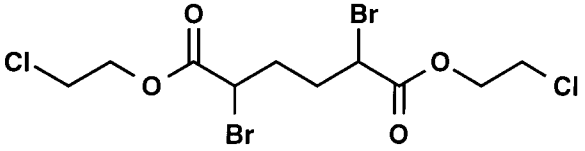
(32)



bis (2,2,2-trifluoroethyl) sebacate

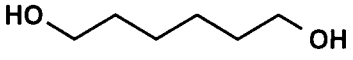
(33)

Investigations on these $[AA\text{-}BB]_x$ systems were extended to polymerisations between bis (2-chloroethyl) (±)-2,5-dibromoadipate (34) and 1,6-hexanediol (35)[50]. The reaction was stirred at 45 °C using lipases from *Aspergillus niger* and *Chromobacterium* species for seven days. Optically active trimers and pentamers were isolated with $[\alpha]_D$ of +4.5 and +4.3 respectively. Both trimer and pentamer had the diol at the termini.



bis (2-chloroethyl)-2,5-dibromoadipate

(34)



1,6-hexanediol

(35)

The work of Kobayashi[51,52,53,54,55] in Japan has been very wide ranging in terms of the number of monomers investigated as substrates for enzymatic

polymerisation. Some of the early work involved polyester synthesis using a lipase from *Pseudomonas fluorescens* to hydrolyse a cyclic anhydride (succinic anhydride (36)). Subsequent treatment with a diol (1,8-octanediol (37)) in toluene generated a high molecular weight polyester[51] (see Figure 1.17).

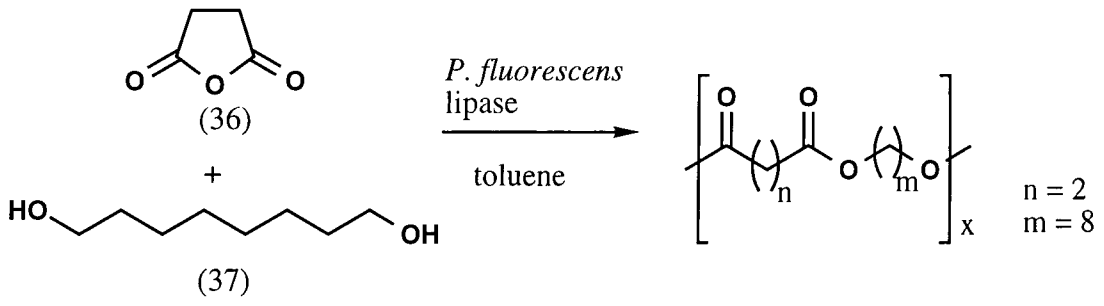


Figure 1.17. *Polymerisation of succinic acid (36) and 1,8-octanediol (37)[51].*

The formation of macrocyclic lactones by lipase catalysis was reported in several papers in the late 1980's[56,57,58]. Sih[56] used CRL, PPL and a lipase from the *Pseudomonas* species to condense diacids (with 2 to 12 carbons) and diols (with 5 to 16 carbons). The formation of the monolactone and dilactone at 65 °C was accompanied by some trilactone and oligomeric ester formation. The product profile was dependant on several factors, but particularly if the temperature was reduced to lower than 45 °C then no lactone formation was observed[56].

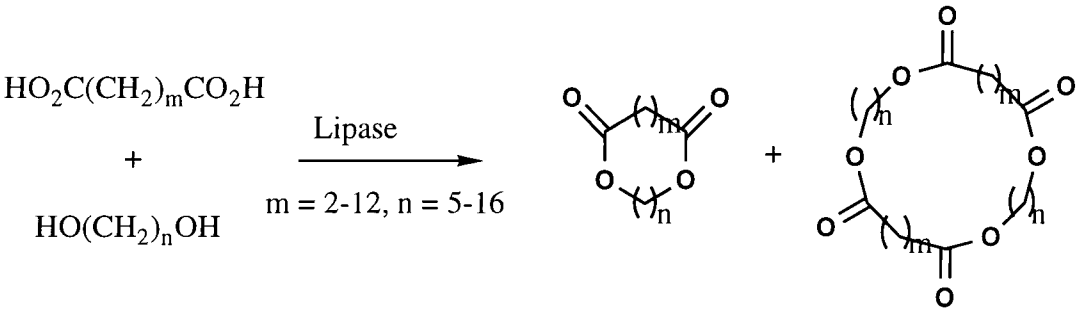


Figure 1.18. *Formation of macrocyclic lactones according to Sih[56].*

In many of the early investigations into enzyme catalysed AA-BB polymerisations the products formed were largely oligomeric[40,41,44]. Generally when a dimer formed the terminal acid was rapidly esterified, resulting in a trimer with diol termini. This dominance of diols is due to their higher solubility over any of the diacids which form. Thus despite using equimolar quantities of both the diol and diacid, diols dominate the product mixture.

Water is formed during the course of many of the polycondensation reactions, and if it is not removed it will start to hydrolyse the polyesters which have formed. This problem has been addressed by using 4 Å molecular sieves[44]. The sieves act to remove this excess water from the system, without removing the enzyme's essential aqueous layer. Figure 1.19 shows the apparatus which Roberts used when he first reported the use of molecular sieves for this purpose.

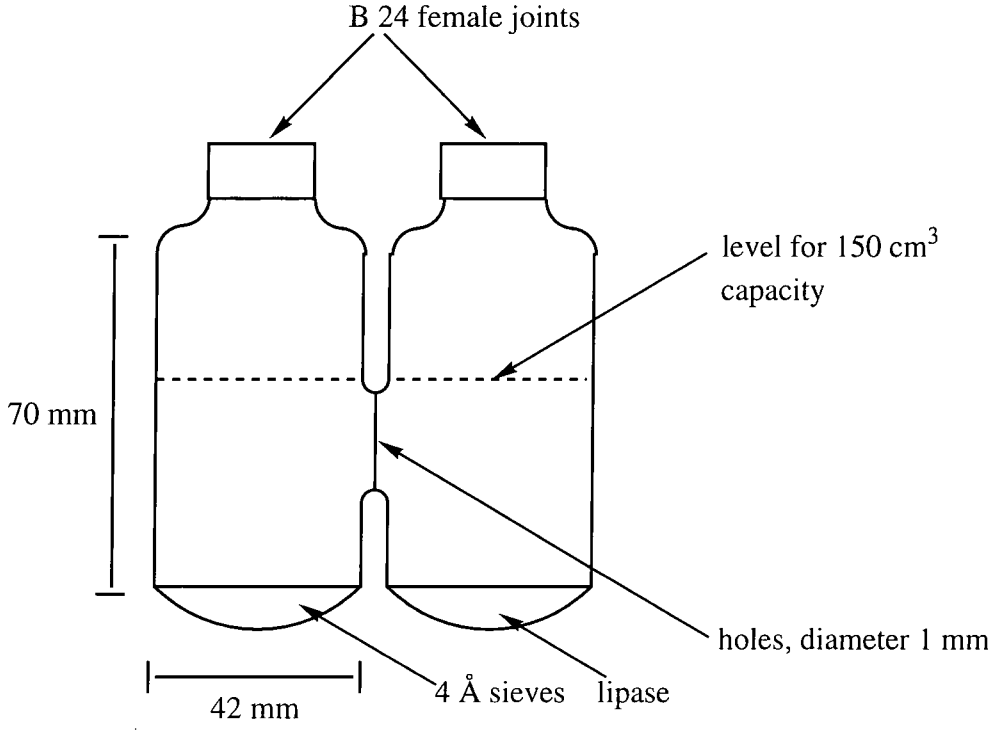
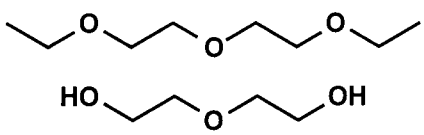


Figure 1.19. *The apparatus employed by Roberts, when using molecular sieves[44].*

The use of the two chambers allowed the sieves to be agitated throughout the

experiment, without the risk of damaging the enzyme through abrasion. Roberts observed that the molecular sieves increased the molecular weight (M_W) of the polymer by a factor of two. However, the effect of the sieves decreased with time, and was negligible if the sieves were not present at the start of the reaction[44].

2,2,2-Trifluoroethanol (27) was produced in the condensation of bis (2,2,2-trifluoroethyl) glutarate (28) with 1,4-butanediol (22)[45] and bis (2,2,2-trifluoroethyl) sebacate (33) with 1,4-butanediol (22)[46,47,48,49]. If allowed to accumulate this by-product of the condensation denatures the enzyme, and hinders attainment of higher mass material. If this alcohol is removed by the use of a vacuum then denaturation of the enzyme is prevented. Thus, by employing a high boiling solvent, such as 1,2 dimethoxybenzene (29), bis (2-ethoxyethyl) ether (38) or diethylene glycol (39), the trifluoroethanol (27) could be removed when the vacuum was applied periodically. Thus vacuum in these reactions can increase the higher mass threshold of the material[45].

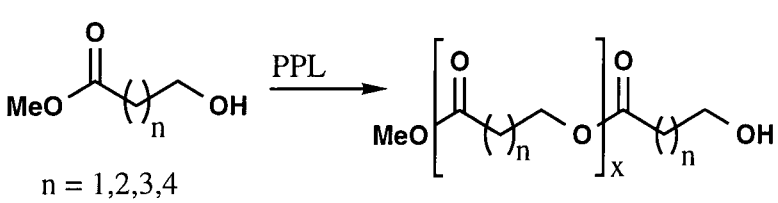


bis (2-ethoxyethyl) ether (38)

diethylene glycol (39)

1.9.2: [A-B] Systems.

The use of A-B type monomers has advantages over the AA-BB type as the stoichiometry of the functional groups is kept constant. At large dilution intramolecular condensation to form lactones could be performed on the primary alcohols, and using secondary alcohols lactonisation occurred even in concentrated solutions. However, transesterification reactions using ω-hydroxy methyl esters (with 3 to 6 carbons in the chain) with PPL in organic solvents formed straight chain oligomers[58].



n = 1,2,3,4

Figure 1.20. *Polymerisation of ω-hydroxy esters[58].*

Synthesis of a chiral, optically active polymer from a *meso* monomer has been reported. Dimethyl β-hydroxyglutarate (40) was employed with horse liver acetone powder (LAPH), pig liver acetone powder (LAPP) and the protease from *Streptomyces griseus* [59]. The reaction was slow, affording only oligomers after ten days, but in all cases observed the product was stereospecific and optically active after purification by thin layer chromatography (TLC).

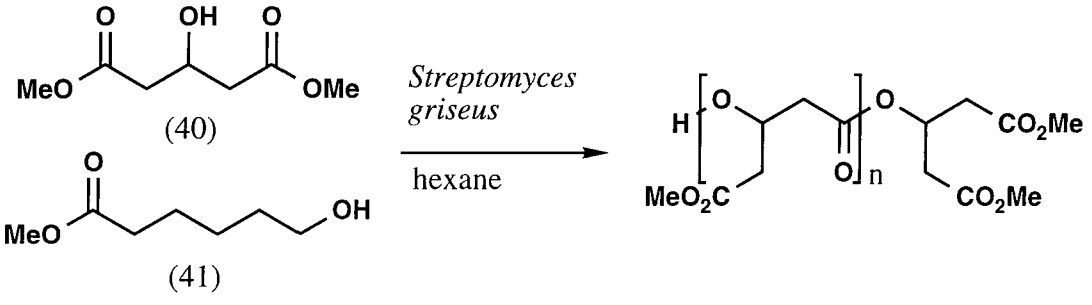
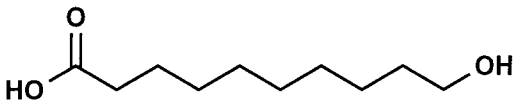


Figure 1.21. *Polymerisation of dimethyl β-hydroxyglutarate (40) and methyl 6-hydroxyhexanoate (41)*[59].

Following earlier work Gutman[60] investigated the polycondensation reaction with methyl 6-hydroxyhexanoate (41) using PPL. In *n*-hexane at reflux temperature (69 °C) a degree of polymerisation (DP) of about 100 was possible. However, in this study lactone formation was also detected. In agreement with earlier work[56], observations were made that the macrolactone content of the reaction was higher when the temperature of the reaction was higher. There was also a general increase of mass (M_W) with increase in the duration of the experiment.

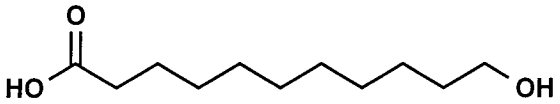
The early use of molecular sieves[44] prompted further investigations into their use as water-retaining agents[61,62], using *Candida rugosa* lipase at 55 °C, in hexane, with ω-hydroxy acids. When the molecular sieves were employed the number average molecular weight (M_n) of the polymer of 10-hydroxydecanoic acid (42) increased dramatically. This monomer contains approximately 1 % sebacic acid (31), and thus the polymer became end-capped with the diacid, rendering it unable to take further part in the polymerisation process[61]. A monomer such as 11-hydroxyundecanoic acid

(43), which did not contain any diacid, generated material of high molecular weight (35 000 by GPC) under the same conditions. This was an upper limit due to the insolubility of the polymer in the solvent[62].



10-hydroxydecanoic acid

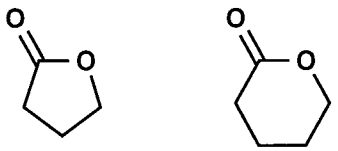
(42)



11-hydroxyundecanoic acid

(43)

AB systems can be divided into two categories: those using open chain ω-hydroxy acids and esters and those using lactone rings. γ-Butyrolactone (44), δ-valerolactone (45) and ε-caprolactone (18) have been studied in ring opening polymerisation reactions using *Pseudomonas fluorescens* lipase, both as homo- and co-polymers[52]. Generally the homopolymer generates materials of higher mass than the copolymers [the homopolymer of valerolactone (45) produced materials of higher mass (M_n = 7000) than the copolymer of valerolactone (45) and caprolactone (18) (M_n = 3700)]. An increase in the reaction temperature also had an influence on the molecular weight. All of the polymerisations in these studies were performed without solvent (neat). Other investigations[63,64] into the ring-opening polymerisation of, for example, ε-caprolactone (18) used PPL in heptane at 65 °C, achieving modest masses (M_n = 1600). This suppresion of molecular weight was rationalised by water being present in the reaction mixture, in excess of that bound to the enzyme[63].



γ–butyrolactone δ-valerolactone

(44) (45)

Kobayashi[53] has proposed a mechanism for these ring opening polymerisations. Firstly the lactone is hydrolysed by water, catalysed by the enzyme. Secondly, the serine 209 residue attacks the carbonyl bond of the hydroxy acid, forming an acyl enzyme intermediate. Thirdly, a second ω-hydroxy acid molecule acts as a nucleophile, attacking the acyl enzyme intermediate, and hydrolysing the ester bond. This generates an ester from the two hydroxy acid molecules, water to propagate the reaction, and returns the enzyme to its original state[53].

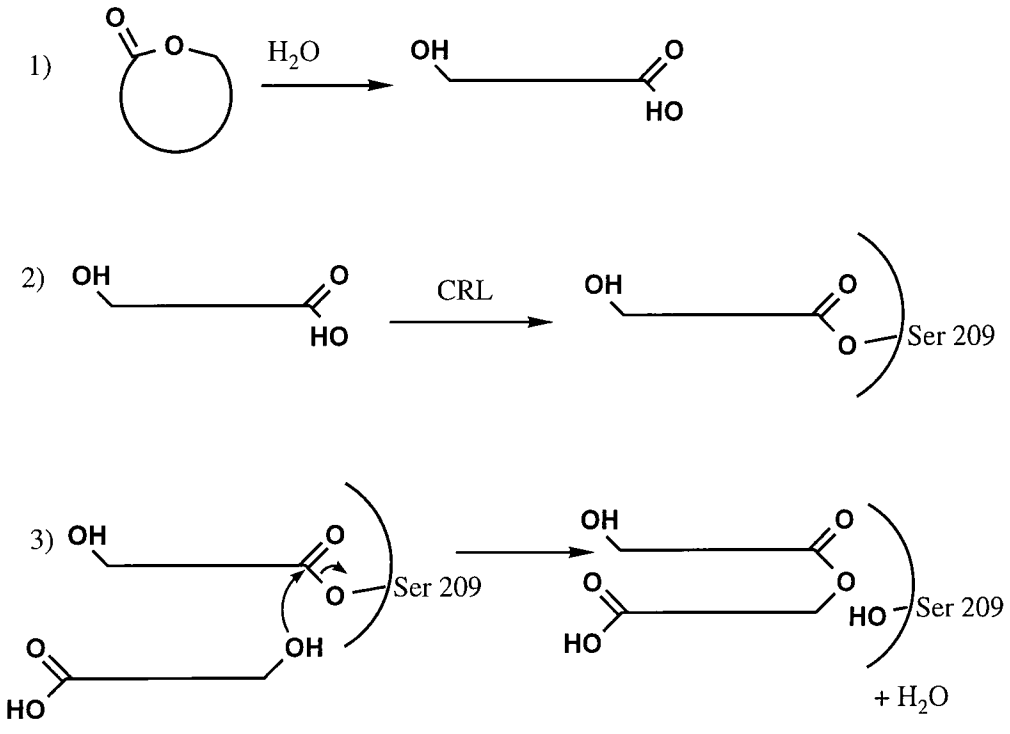
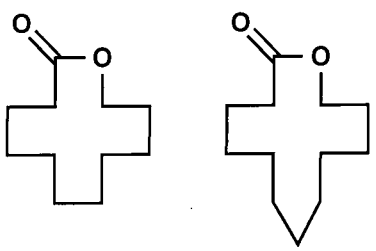


Figure 1.22. *Kobayashi's mechanism for ring opening polymerisation of lactones[53].*

An investigation[54,55] was undertaken to compare the reactivity of caprolactone, 11-undecanolide (46) and 12-dodecanolide (47) with the lipases from *Pseudomonas fluorescens,* porcine pancreas and *Candida cylindracea.* These reactions were performed neat, at 75 °C. The rate of polymerisation of (46) and (47) was faster than that of caprolactone. The larger macrolides clearly have a lower ring strain than

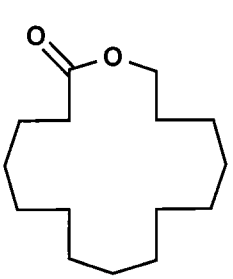
caprolactone, and thus the increased rate of polymerisation appears to be contra-intuitive[54]. In view of these observations the rate-determining step of the reaction was suggested to be the formation of the acyl-enzyme intermediate, with the serine alkoxide of the lipase attacking the lactone initially. The enzyme's preference for the larger lactone is most likely due to binding affinity, since the macrolides adopt a conformation which more closely mimics the conformation of a triacyl glyceride, the natural substrate[54].



11-undecanolide (46)

12-dodecanolide (47)

When lipase PS-30 was immobilised onto Celite a high mass polyester was generated[64] with neat ω-pentadecalactone (48) (70 °C for 24 hours). The author speculated in the paper[64] that the increased lipase activity when immobilised could be due to a conformational alteration, resulting in the enhanced accessibilty of the active site to the substrate molecules.



pentadecalactone (48)

Non-polar solvents such as isooctane, diethyl ether and *n*-hexane proved to be the best for polymerisation. In contrast diisopropyl ether, toluene and THF generated only low mass oligomers. Low mass oligomers only were found in solvents in which

the oligomers were soluble, *ie*, once the oligomers had formed, they were removed from the surface of the enzyme into the bulk solvent by diffusion. When the solvent employed was more hydrophobic, and the oligomers were not soluble in the solvent, the growing ester chain remained associated with the enzyme, and was able to undergo further polymerisation. Thus the reaction in bulk, at optimal concentration of substrate, afforded the highest DP.

Solvent	Temperature °C	Yield %	Mw
No solvent	70	95	1900
Isooctane	70	62	1130
Diisopropyl ether	70	59	1035
n-Hexane	70	61	850
Tetrachloromethane	70	69	395
Benzene	70	73	295
Acetonitrile	70	90	200

Table 1.1. *Enzymatic polymerisation of methyl 6- hydroxyhexanoate*[60].

1.10: Profile of the assembly of high mass polymers .

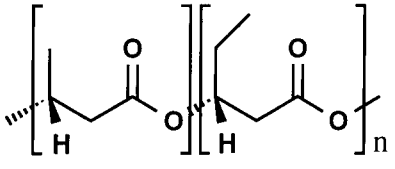
A very rapid inital build-up of polymer over the first five hours was observed in systems employing ω-hydroxy acids[61,62]. This was followed by a slower, gradual build up of the higher mass polyesters in the latter stages of the reaction (over several days). In the late stages of these reactions (10 days) lower mass oligomers re-emerged. The conclusion drawn from this was that the hydrated molecular sieves offered a surface on which the polymer can deposit. Water, previously adsorbed by the sieves, desorbed, and catalysed the hydrolysis of the polymers to the lower mass oligomers which were observed.

1.11: Natural and synthetic polymerisations.

Polyhydroxy acids (PHAs), such as poly (*R*)-3-hydroxybutyrate (PHB), are

found widely in microorganisms[66] and used as storage materials due to their low solubility and high molecular weight. They were first described in 1925 by Lemoigne who found the PHAs in *Bacillus megaterium.*

In the late seventies and early eighties ICI developed a poly (β-hydroxybutyrate) synthesised by the bacteria *Alcaligenes eutrophus*, which they have marketed as Biopol[67]. The copolymer consisted of (*D*)-3-hydroxybutyrate (49) and (*D*)-3-hydroxypentanoate (50) (or (*D*)-3-hydroxyvalerate). Biopol is used to imitate the natural polymeric material PHB, and has commercial advantages over the natural material in terms of its physical properties, which are similar to polypropylene.



(49) (50)

(*D*)-3-hydroxybutyrate (49)

(*D*)-3-hydroxyvalerate (50)

Figure 1.23. *The structure of Biopol, and its constituent monomers*[67].

1.12: Synthetic routes to polyesters.

Many chemical catalysts have been used to synthesise polymeric materials similar to PHB. Catalysts using metals such as zinc[68], aluminium[68] and titanium[69] have all been reported recently. Diethylzinc and triethylaluminium catalysts were used with water to ring open (*S*)-β-butyrolactone (44) in a stereochemically controlled manner, and then polymerise the monomeric units formed from the ring opening reaction. These two catalysts gave PHB of varying mass and polydispersities, depending on the system employed and the catalyst used.

The titanium catalyst, $Ti(OBu)_4$, has been used to polymerise each of the enantiomers of methyl β-hydroxyisobutyrate (51)[69]. The materials used in this study were isomeric with 3-hydroxybutyrate (44), and demonstrated a straightforward synthetic method for obtaining homochiral polymers isomeric to natural PHB. The optical purity of the monomeric material has a significant influence on the properties of

the polymer. Using either the *R* or *S* enantiomer of methyl β-hydroxyisobutyrate (51) as the starting monomer, the recovered polymer in each case was crystalline[69]. However, the racemate generated an amorphous material[70]. Thus, optical purity can dramatically improve physical properties.

1.13: Molecular weight analysis of polymers.

The analysis of the mass of the polymeric materials generated from lipase catalysed reactions has been evaluated using various methods. In their studies Wallace and Morrow[40,41] used end group analysis by ^{1}H NMR, which allows the mass of the polymer to be determined by comparing the integrals of the terminal groups in the monomer to the same groups in the polymer. Assuming that all the end groups are from polymeric materials and are not monomers, an estimate of the average molecular weight (M_W) can be evaluated. This method has subsequently been used widely.

Gel permeation chromatography (GPC) measures the molecular mass by eluting the polymer from a series of columns packed with 100 Å, 10^3 Å and 10^5 Å beads. This is a size exclusion chromatographic technique, *ie*, separation according to the size of the solute molecules in solution. The polymeric material travels through the columns, with the smaller molecules being retained in the pores between the packing. The higher mass materials are too large to fit the pores, and remain in the interparticulate volume (*ie*, the solvent). The larger molecules (greater hydrodynamic volume) elute first from the column, and are monitored as they emerge using a light scattering technique, with the retention time of the material inversely proportional to its mass. Various different mass values for a polymeric material can be obtained using GPC, although the most commonly used values are M_n, the number average molecular weight (or the median), and the M_W, the weight average molecular weight (or the mean). Calibration is performed using standards, usually polystyrene, which have a molecular mass range from 60 to 30 000 000. With polystyrene as a standard caution must be used in expressing the absolute mass of polyesters by GPC.

Matrix assisted laser desorption ionisation time of flight mass spectrometry

(MALDITOF) is a more recent method for the analysis of polymers. The MALDITOF MS technique is a soft method of analysing materials using mass spectrometry and has become invaluable for analysing high mass biomolecules and polymer distributions. MALDITOF MS is a mass precise technique, which shows unique peaks for individual oligomers within the overall distribution of the polymer[71].

Using the same materials for analysis by both GPC and MALDITOF MS, it has been observed that the GPC technique using polystyrene molecular weight standards overestimated the mass of a polymer by approximately 25 %[71]. However, the MALDITOF MS technique has limitations. Although it is a soft ionisation technique, and as such does not cause fragmentation of the polymer, there are problems with skewing of the distribution towards the lower mass end. There is a preferential desorption of oligomers over polymers, if there is inadequate acceleration of the higher mass polymers, once ionisation has occurred[71].

CHAPTER TWO

ENZYMATIC REACTION CONDITIONS AND OPTIMISATION

2.0: ENZYMATIC REACTION CONDITIONS AND OPTIMISATION.

2.1: Aims and objectives.

The objectives of this chapter are to probe the following areas related to the *Candida rugosa* lipase catalysed polymerisation of ω-hydroxy acids:

1) Establish the optimal conditions for polymerisation.

2) Investigate the purity and amount of enzyme required for catalytic activity.

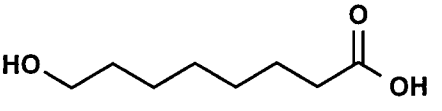
3) Study the length specificity of the enzyme for ω-hydroxy acid monomers.

4) Evaluate parameters affecting the molecular weights of the resultant polymers.

5) Control of the polydispersity of the polymeric material obtained.

2.2: Selecting the enzyme and the conditions for polymerisation.

Preliminary screening of several Sigma and Amano lipases was carried out in the laboratory prior to starting this project[72]. This led to the selection of the *Candida rugosa* lipase as a good catalyst for the polymerisation of the ω-hydroxy acids. It then became necessary to establish the optimal conditions for the *Candida rugosa* catalysed polymerisation process. A temperature profile was investigated using hexane as the solvent. As a control system 8-hydroxyoctanoic acid (8-HOA) (52) was used as the substrate for the polymerisation (for the synthesis of 8-HOA (52) see section 5.2.1). The initial phase of the reaction was rapid, and therefore the reactions were worked up after 30 mins. Table 2.1 and Figure 2.1 show the molecular weight (M_W) / temperature relationship determined by GPC analysis.



8-hydroxyoctanoic acid

(52)

From Table 2.1 and Figure 2.1 several conclusions can be drawn. The esterification is optimal at 55 °C. There was a significant, albeit slow, reaction at 0 °C,

extending the temperature range of the enzyme in hexane beyond that of the enzyme in water. The molecular weights then increase with increase in temperature, as expected, up to a maximum at 55 °C. The reaction slows dramatically in refluxing hexane.

Time/Temp	0 °C	20 °C	35 °C	45 °C	55 °C	60 °C	69 °C
0.5h	829	1587	1865	2423	2457	2392	1032
1.5h	1243	1713	2016	3137	3372	2769	1140
3h	1132	2065	2304	3417	3671	3465	1233
5h	1304	2144	2601	3519	4050	3858	1141

Table 2.1. *Molecular weights (M_W) from GPC analysis for 8-HOA (52) at various temperatures.*

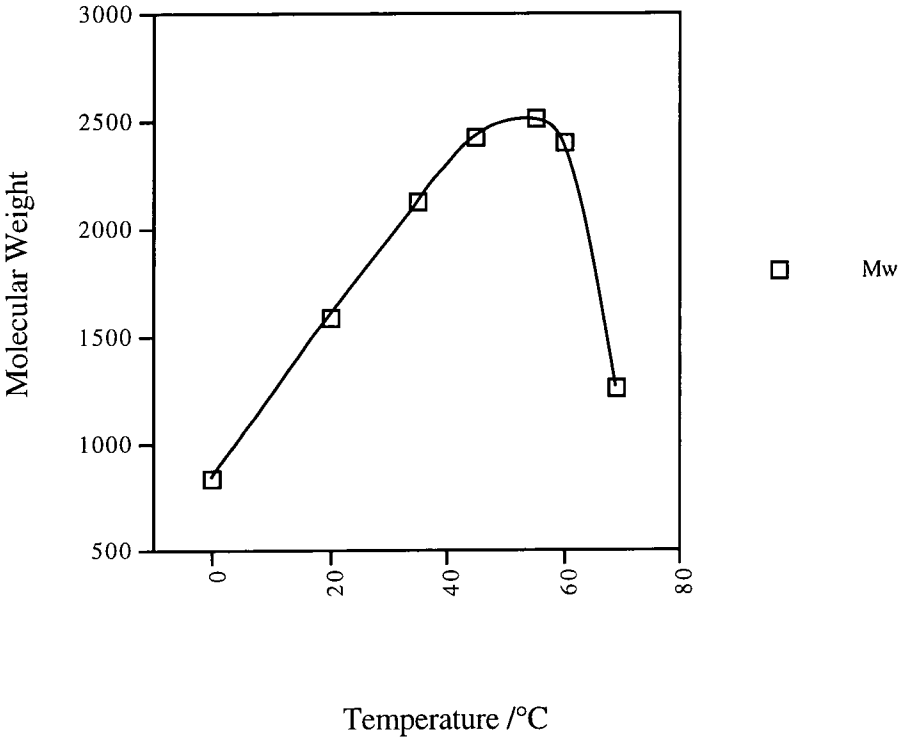


Figure 2.1. *Temperature versus mass profile for 8-HOA (52) polymerisation.*

The reactions carried out at both 0 °C and at 69 °C were not performed on a shaker in the standard way (see section 6.2.1), but under ice cooling or in refluxing solvent respectively, using a magnetic stirrer to mix the suspension. However, these variations in reaction conditions are not anticipated to significantly alter the polymerisation process. Figure 2.1 clearly shows that the lipase has optimal activity between 55-60 °C, and indicates a dramatic decline in activity above this temperature, which is most probably due to enzyme deactivation. Thus there is a steady increase in the rate of polymerisation until this point is reached.

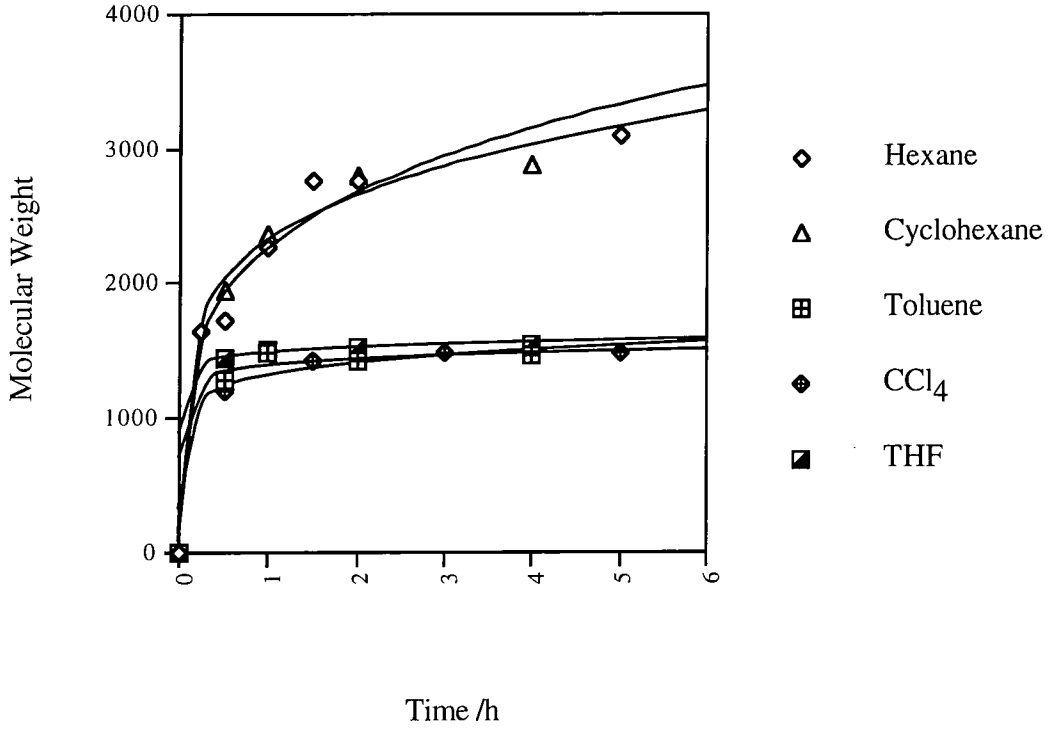


Figure 2.2. *The rate of polymerisation for 8-HOA (52) in various non-polar solvents.*

The literature studies into lipase catalysed polymerisations have used various dry hydrophobic organic solvents. For example, both Roberts[44] and Wallace and Morrow[40,41] employed anhydrous diethyl ether for the *Mucor miehei* and porcine pancreatic lipases respectively, whereas Gutman[59] used hexane in his polymerisations

with LAPH, LAPP and *Streptomyces griseus*. (SG). Having established the optimal temperature for the reaction with *Candida rugosa*, an investigation was initiated to establish the best solvent for the polymerisation. Again polymerisation of 8-HOA (52) was taken as a standard system, and the polymerisations were all studied at 55 °C . Figure 2.2 shows the resultant molecular weight (M_W) / time profile for the various solvents over a six hours period, from which several conclusions emerge. All of the rates were initially rapid, achieving molecular weights (M_W) of over 1000 within 30 minutes. This demonstrates again that these reactions are fast. The highest molecular weights were associated with the non-polar solvents, with hexane and cyclohexane behaving similarly. Toluene, CCl_4, and THF all gave similar molecular weights. However, it is clear that for these latter three solvents the polymerisation stopped after 30 minutes, and there was no further progression of the reaction.

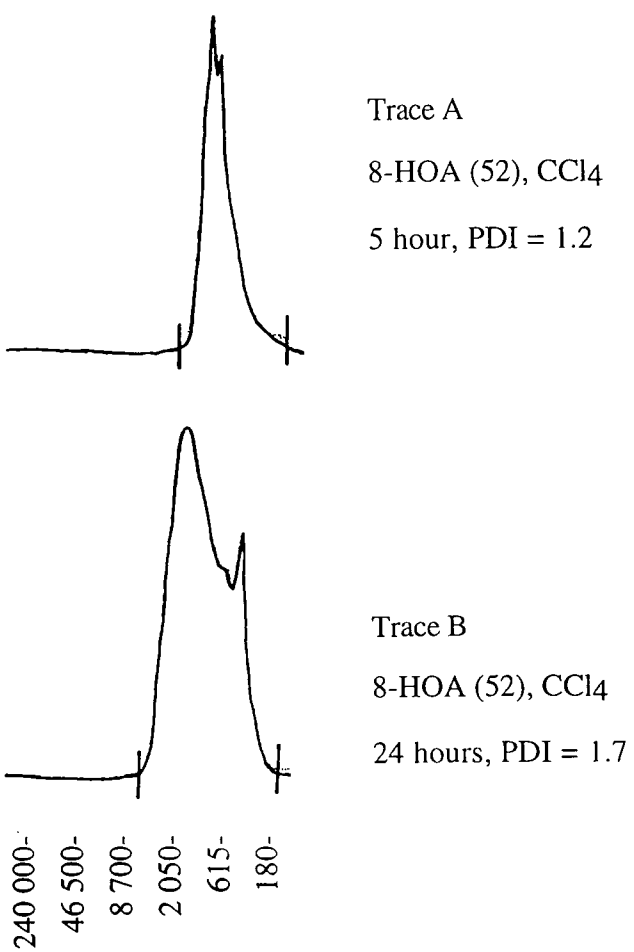


Figure 2.3. *GPC of 8-HOA (52) polymerisation product in CCl_4, after 5 hours (GPC Trace A) and 24 hours (GPC Trace B).*

Interestingly, the material recovered from the reaction in CCl_4 had a relatively low polydispersity (1.2). A reaction with CCl_4 was performed to explore this further, for a longer period of time (up to 24 hours). The resultant mass was higher (M_W = 1900), and the polydispersity had increased (1.7), similar to a typical polydispersity for material of this molecular weight when using hexane. It was therefore concluded that the tight polydispersity was a function of the low molecular weight of the polymer, and not a particular solvent effect (see Figure 2.3).

2.3.1: Electrophoresis and catalytic activity.

Electrophoresis is a technique for separating macromolecules, such as proteins, which have a net charge. The macromolecule is denatured using sodium dodecyl sulphate (SDS, a detergant) and mercaptoethanol. Denaturation occurs due to the apolar tails of the SDS molecules associating with the hydrophobic groups in the protein. The unfolded protein is then dissolved in a gel and placed in an electric field. The strength of the magnetic field and the net charge of the protein (due to the number of SDS groups attached to the protein) are the driving force for migration towards the electrodes and separation of the individual components. Comparison of a protein against given molecular weight standards allows the molecular mass of the protein to be determined[73].

The enzyme preparations obtained from the commercial suppliers Sigma and Amano are crude. Thus the homogeneity of protein from Sigma and Amano was assessed. Additionally, there is a significant amount of salt in each batch. Samples of lipase were subjected to electrophoresis. After the enzymes had been precipitated from solutions of chloroform and methanol (to remove the salts), they were placed on the gel with several standard proteins (M_W from approximately 14 000 - 60 000 Da). Figure 2.4 shows the image of an electrophoresis gel which was used to purify CRL. Column 1 is the molecular weight standards (1 µl). Column 2 contains 1 µl of a solution of the enzyme from Sigma. The quantity of enzyme increases across the gel, up to column 8, which contains 100 µl of the solution. The gels were stained to show the proteins more clearly, with the more concentrated samples showing a deeper stain.

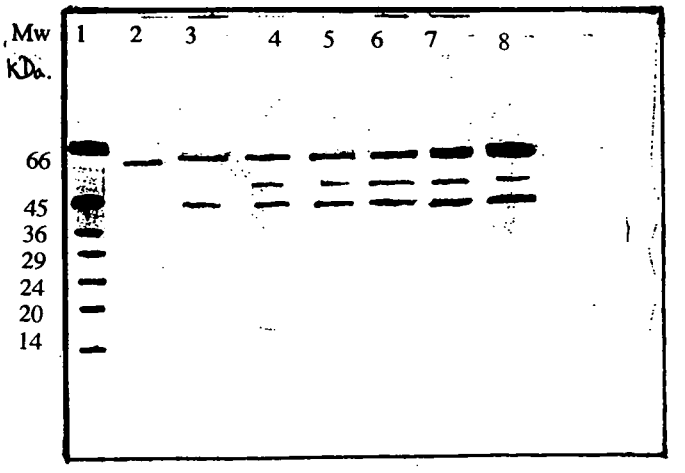


Figure 2.4. *Image of the electrophoresis gel. In column 1 are the molecular weight standards, with increasing concentrations of Sigma* Candida rugosa *lipase through columns 2-8.*

Three protein bands were present in each sample with molecular weights of approximately 57 000, 53 000 and 45 000 Da. This becomes progressively clearer with increasing concentration. The top band is in good agreement with the molecular mass of the *Candida rugosa* lipase purified by Cygler[8]. The middle band at 53 kDa, and the lower band at 45 kDa are most probably iso-enzymes from the Sigma and Amano preparations of their lipases[11].

A significant point to emerge from this analysis was the quantity of protein present in each sample. The molecular weight standard used was 2 μg of albumin (Mw = 60 kDa), whereas the largest lipase sample (column 8) contained 200 μg of material, yet the lipase stain at 57 kDa was significantly less intense than that of the standard. Therefore the CRL present in the Sigma batches appears to be less than 1 % of the total mass of the sample. The remainder in each sample is presumably precipitated salt. Thus when an enzyme reaction uses 2.5 g crude enzyme, less than 25 mg of the protein is actually present. Typically therefore a ratio of 1:10, catalyst to monomer, is used.

2.3.2: Studies using a reduced quantity of enzyme.

Literature preparations for enzyme catalysed polymerisations have used a ratio of substrate to crude enzyme of 1:1[59], 4:1[55] and 7:1[44]. However, none of these studies used pre-purified enzymes. The experiments performed in this lab prior to this study had used a ratio of monomer to enzyme of 1:10 (see section 6.1.1). The mass of the protein contained within the enzyme sample is such that the stoichiometry of the enzyme is significantly lower than the substrate, as discussed above (see section 2.3.1). An experiment was set up in which the concentration of the enzyme present in the reaction was decreased by an order of magnitude, such that the mass ratio of monomer to crude lipase was now 1:1. However, the ratio of substrate to lipase is estimated to be 100:1.

9-HNA (53) was used in this study as it emerged as the best substrate for this reaction (see section 2.5). For the synthesis of 9-HNA (53) see section 5.3. Analysis of the resultant polymer after 31 hours using 150 mg 9-HNA (53) and 150 mg crude enzyme (1.5 mg lipase) gave a material with an upper limit of 24 000. However, the majority of the material was oligomeric, as can be seen from Figure 2.5. This was a relatively slow reaction, and it took 31 hours (from ^{1}H NMR) before the monomer was completely consumed, thus although the enzyme was active this % catalyst was above a practical working threshold.

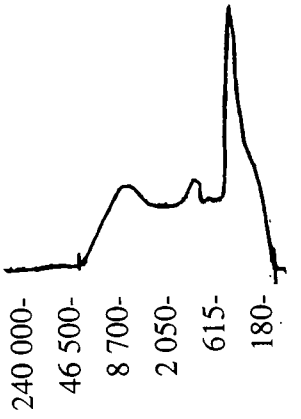
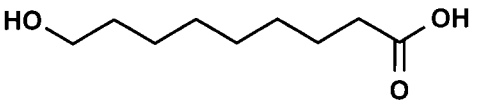


Figure 2.5. *GPC trace of 9-HNA (53) polyester with an enzyme to monomer ratio of 1:100.*



9-hydroxynonanoic acid

(53)

2.4.1: Polymerisations of 9–HNA (53).

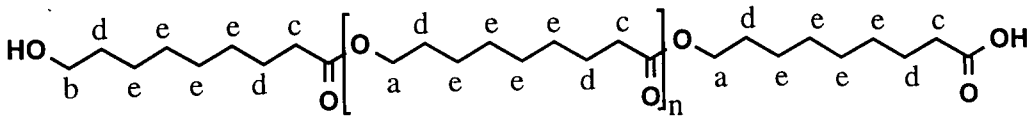
The polycondensation of ω-hydroxy acids generates water as a by product. A potential problem arises as the water can catalyse the hydrolysis of the polyesters, particularly in the latter stages of a given reaction. To obviate such competitive hydrolysis, molecular sieves have previously been added to the reaction[44]. A detailed study was undertaken to monitor the progress of *Candida rugosa* polymerisation with molecular sieves using 9-HNA (53) as the monomer. A comparison is made between polymerisations with and without molecular sieves in section 2.6.1.

2.4.2: Polymer assembly followed by ^{1}H NMR analysis.

Figure 2.6 shows the ^{1}H NMR spectrum of the 9-HNA (53) polymerisation with lipase and molecular sieves at an intermediate stage. Signals for the polymer / oligomer and monomer are assigned in the spectrum as follows. The signal for the hydroxymethyl end group of 9-HNA (53) occurs at 3.6 ppm, with the methylene group adjacent to the carboxylic acid at 2.3 ppm. The signals for the two methylene groups α to the hydroxyl group and β to the carboxylate group occur at 1.6 ppm. The four remaining methylene groups occur at 1.3 ppm.

In the polymeric material many of these peaks remain unaffected by the formation of the ester group. However, the change from a hydroxyl group to an ester group causes the $CH_2C\underline{H}_2OH$ signal to shift to lower frequency from 3.6 ppm (in the monomer) to 4.0 ppm in the ester ($CH_2C\underline{H}_2OCOCH_2$). Thus, by observing the loss of the peak at 3.6 ppm, and the development of the peak at 4.0 ppm, the consumption of the monomer can be monitored. Figure 2.5 shows a sample ^{1}H NMR spectrum of the 9-HNA (53)

polymer. The peak at 3.6 ppm is due to terminal hydroxyl groups (end groups) on the chains and monomeric material.



a = 4.0 ppm

b = 3.6 ppm

c = 2.3 ppm

d = 1.6 ppm

e = 1.3 ppm

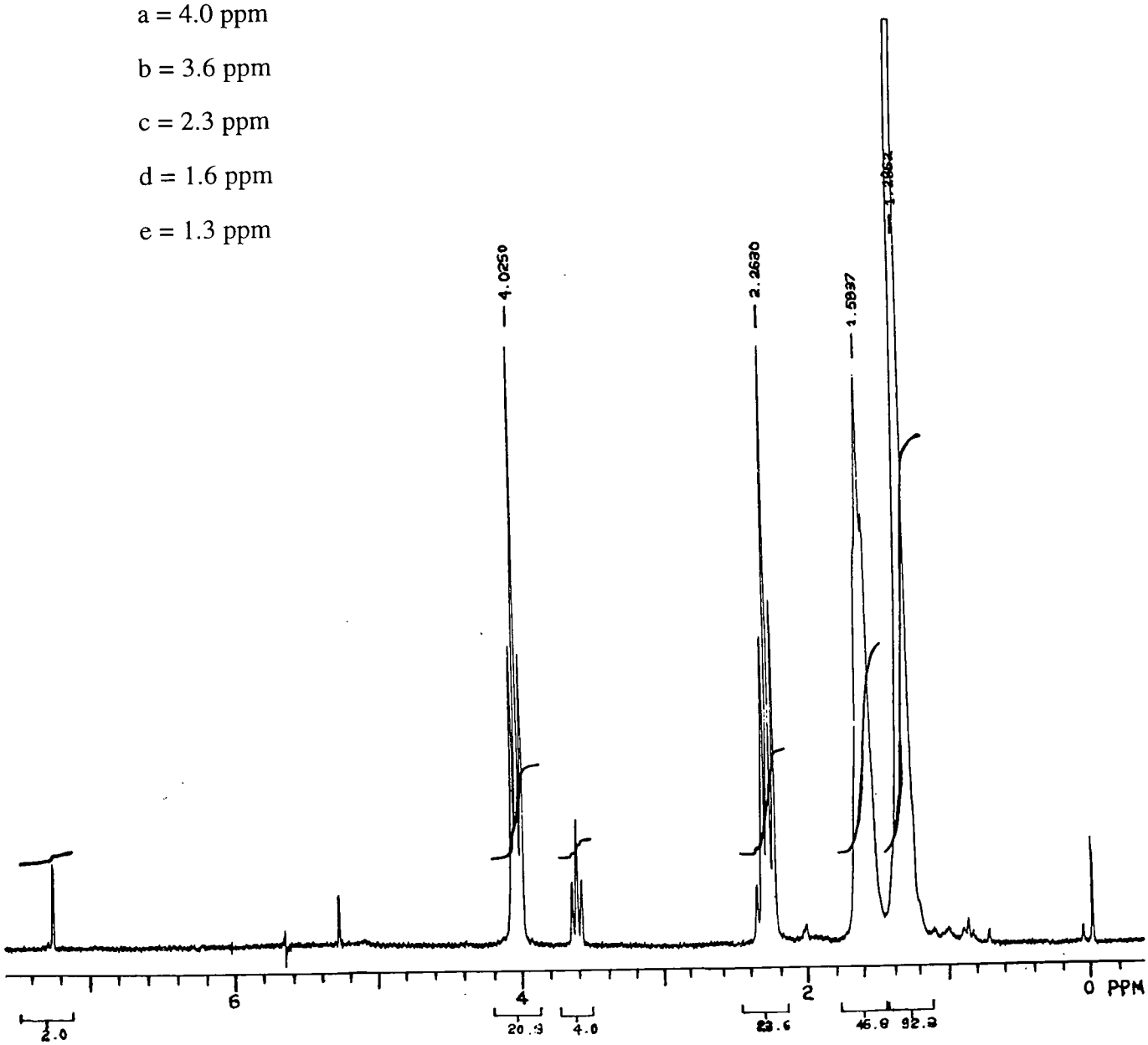


Figure 2.6. *^1H NMR spectrum of a polymer / oligomer of 9-HNA (53).*

2.5.1: Polymerisations of 8-HOA (52), 9-HNA (53), 10-HDA (42) and 11-HUA (43) by ^1H NMR.

Having established a relationship between the consumption of the monomer over

time (up to 12 hours) for 9-HNA (53), various other monomers were investigated. These were 8-HOA (52), 10-HDA (42) (commercially available), and 11-HUA (43) (for the synthesis of 11-HUA (43) see section 5.2.1). Using a standard set of conditions in each case (monomer 200 mg, molecular sieves 10.0 g, enzyme 2.0 g, hexane 30 ml) ^{1}H NMR analysis revealed that the 9-HNA (53) monomer was consumed over a period of 6 hours in an enzyme catalysed polymerisation. After 12 hours many of the oligomeric chains have been condensed, and the end group signal at 3.6 ppm had disappeared. 9-HNA (53) was the most rapidly consumed of the four monomers studied, and has been highlighted because of this. Figure 2.7 shows sections of three ^{1}H NMR spectra taken during polymerisation of 9-HNA (53), in which the formation of the ester signal is observed while the signal for the monomer decreases.

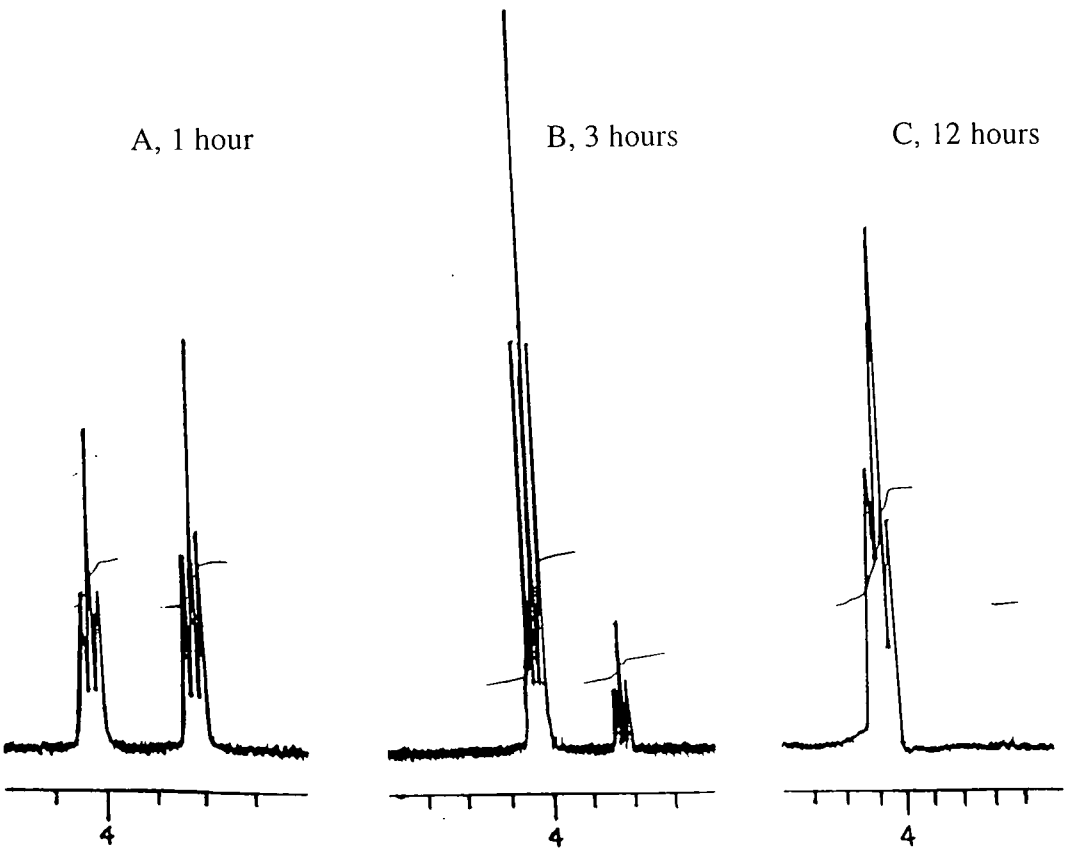


Figure 2.7. *Consumption of 9-HNA (53) in the early stages of the polymerisation process. ^{1}H NMR trace A is after 1 hour; ^{1}H NMR trace B after 3 hours; ^{1}H NMR trace C after 12 hours.*

Since the initial mass (200 mg) of the monomer was constant in all of the experiments a ratio of monomer:polymer was used to express the loss of the monomer and the build up of the polymer. From Figure 2.8, a graph of the change in the ratio of monomer to polymer, it can be seen that 9-HNA (53) was consumed within 6 hours, whereas the 8-HOA (52), 10-HDA (42) and 11-HUA (43) reactions still had significant residual monomer at that time. The 8-HOA (52) monomer was consumed more slowly relative to the 9-HNA (53) monomer, and the 10-HDA (42) and 11-HUA (43) were both consumed after a significantly longer time period (up to 24 hours), with 11-HUA (43) the slowest system studied.

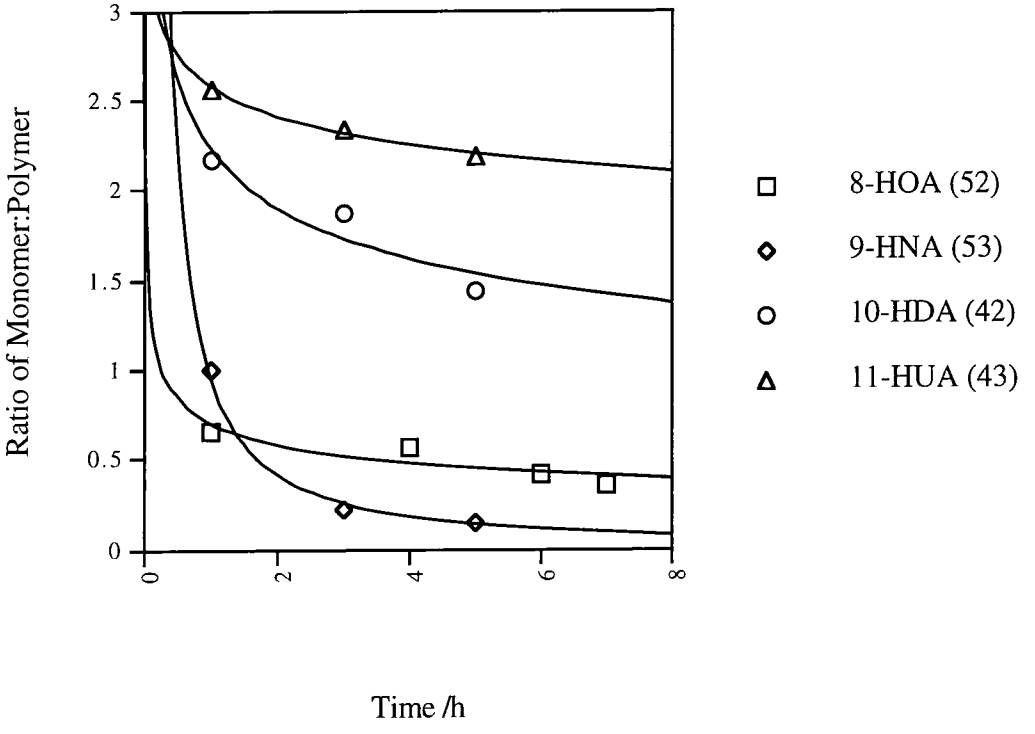


Figure 2.8. *Consumption of all four monomers with time, as measured by ^1H NMR.*

Clearly the reaction rates are not equal for each of the four systems. This suggests a length specificity of CRL for the 9-HNA (53) over the other monomers, and the origin of this observation will be discussed in Chapter 4.

2.5.2: Polymer assembly of 8-HOA (52), 9-HNA (53), 10-HDA (42) and 11-HUA (43) by gel permeation chromatography.

Table 2.2 below shows the build up of molecular weight (M_W) through time for the four monomers, as measured by GPC. This relationship can be viewed in an alternative manner, by plotting the increase of molecular weight (M_W) with time, as shown in Figure 2.9.

Time /h	8-HOA	9-HNA	10-HDA	11-HUA
1	2903	1963	994	997
1	3254	1977	1129	1050
3	1403	6958	956	1095
3	1846	8585	1263	1323
5	1912	7423	1454	1089
5	2327	10105	1716	1235

Table 2.2. *Weight average molecular weight (M_W) with time.*

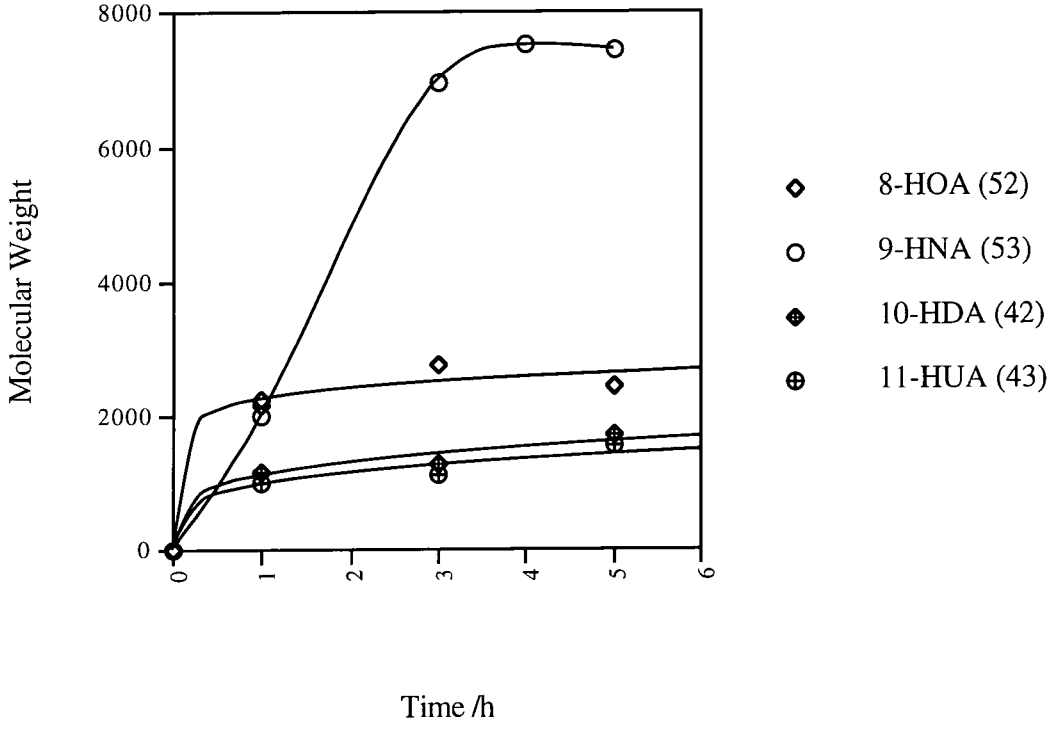


Figure 2.9. *The build up of molecular weight (M_W) through time for all four systems.*

Over a much longer time period (up to 14 days) the molecular weights continue to increase. However, this is due to the increase in very high molecular mass material (M_W of 35 000), and not to an increase in the length of every polymer chain. Indeed, the number average molecular weight remains almost constant at between 1000 and 2000 throughout the 14 days of the experiment. Figure 2.10 shows the molecular weight versus time relationship for 11-HUA (43) over 400 hours, for both the number (M_n) and weight (M_W) average molecular weights. Figure 2.11 is a GPC trace of 11-HUA (43) after 334 hours, showing that there is a large amount of high mass material, which increases the M_W value up to 7000, but significantly there is a larger proportion of the material at a low mass, which maintains the M_n value under 2000. Clearly a significant oligomer population persists, a characteristic of the reactions due to the molecular sieves.

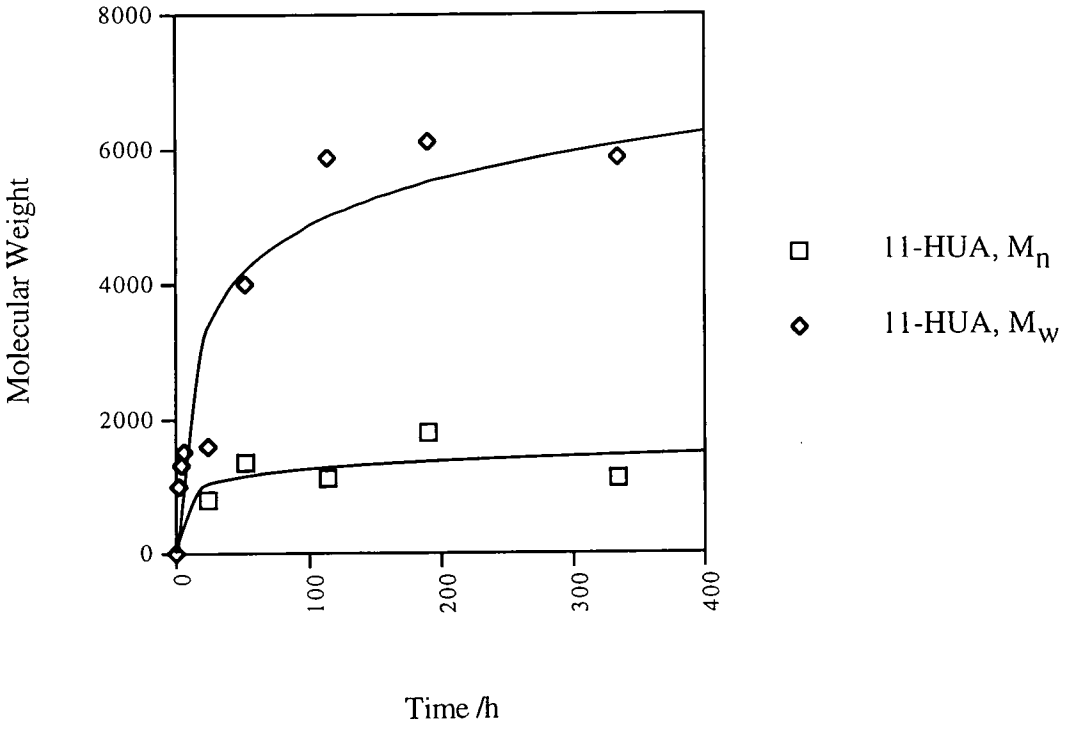


Figure 2.10. *Molecular weight (M_n and M_W) values for 11-HUA (43) over 400 hours.*

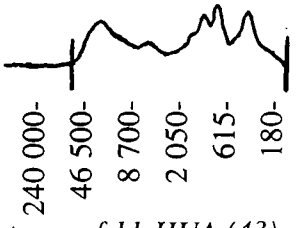


Figure 2.11. *GPC trace of 11-HUA (43) after 334 hours.*

2.6.1: Precipitation of the polyesters onto the molecular sieves.

An investigation was set up to explore deposition of the polymer onto the sieves. Polymerisation was initiated but stopped after 30 mins, and the reaction suspension and the molecular sieves were washed separately. After each work-up ^{1}H NMR was used to determine the monomer:oligomer ratio. In the event there was a ratio in the supernatant washing of 2:1, monomer to oligomer. When the molecular sieves washings were analysed by ^{1}H NMR there was a ratio of monomer to oligomer of nearly 3:1, showing a small but significant increase in deposition of the monomer onto the sieves, over the oligomer, in the early stages of the reaction (see Figure 2.12).

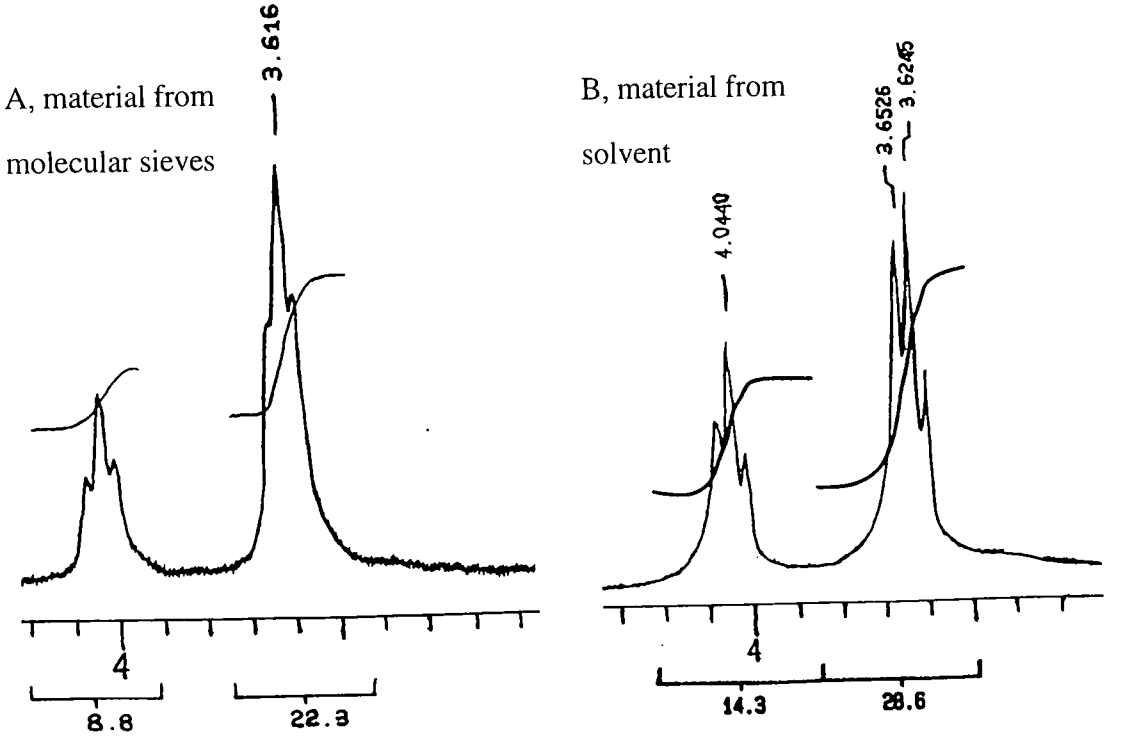


Figure 2.12. *^{1}H NMR spectra of partitioned material showing the ratio of monomer:oligomer in the solvent and on the molecular sieves.*

A second experiment, using 8-HOA (52), but without molecular sieves, demonstrated that there was a rapid consumption of monomer (5.5 hours), and build up of the oligomer in the early stages of the reaction (see Figure 2.13, the GPC trace of an experiment run without the use of molecular sieves). Thus from these preliminary

investigations it would appear that the molecular sieves play two opposing roles. They both assist oligomer assembly by removal of water during ester formation in the early stages of the polymerisation process, but suppress polymerisation by the adsorption of monomer onto the sieves. However removal of the molecular sieves from the system does not appear to suppress the initial rate of oligomer assembly.

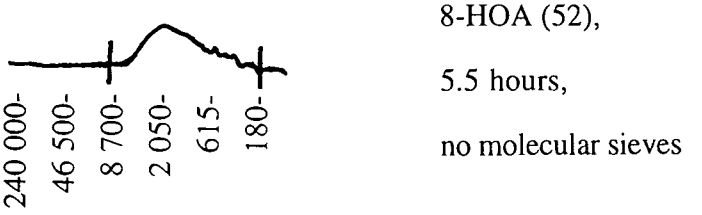


Figure 2.13. *GPC trace of CRL catalysed 8-HOA (52) polymerisation, after 5.5 hours, without molecular sieves.*

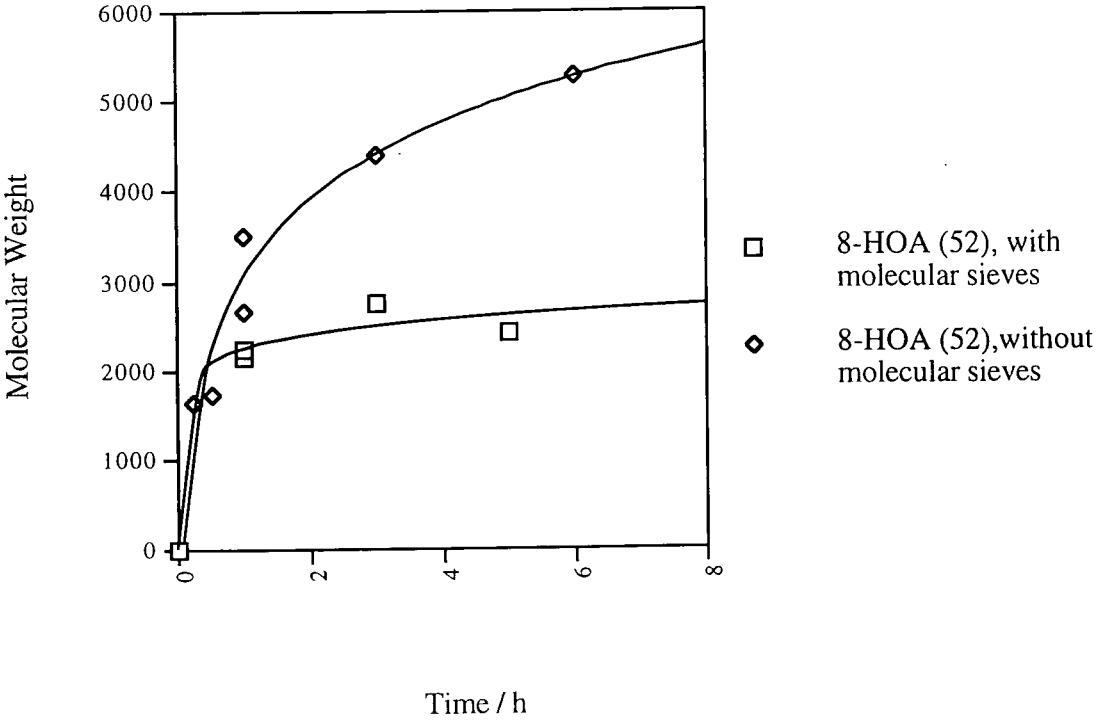


Figure 2.14. *Molecular weight (M_W) comparison of 8-HOA (52) polymer assembly with and without molecular sieves (in hexane at 55 °C).*

Figure 2.14 shows a molecular weight (M_W) comparison of the rates of polymer assembly for the two systems (with and without the molecular sieves), and that the increase in molecular weight of the polymer is more rapid in the initial phase when the sieves are not employed. The difference between the two systems for 8-HOA (52) was negligible over the first hour, but beyond two hours it became substantial. The marked contrast in the molecular weights over six hours prompted further investigations into the lipase catalysed polymerisations without molecular sieves. 10-HDA (42) and 11-HUA (43) were used for the study, and Figure 2.15 represents a plot of the results. Again a remarkable difference in the rates of assembly over eight hours is apparent with and without molecular sieves, and thus this would appear to be a general phenomenon.

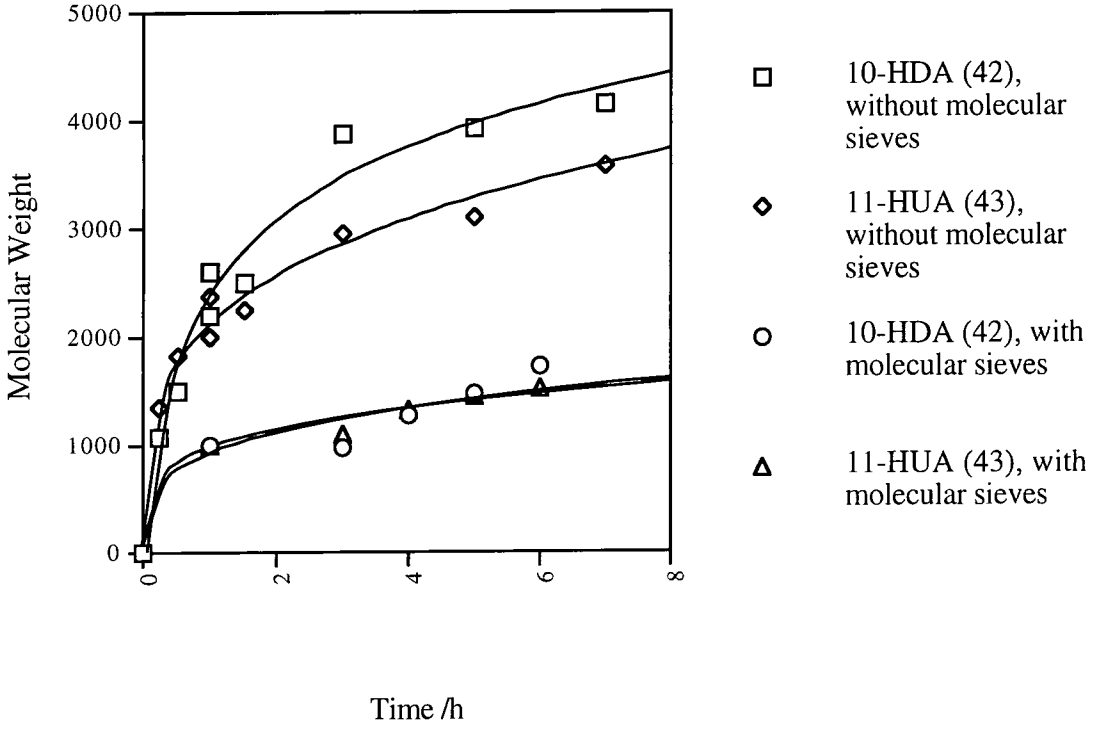


Figure 2.15. *Assembly of polymers by GPC analysis for 10-HDA (42) and 11-HUA (43), with and without molecular sieves.*

Further evaluation of the system with molecular sieves showed that there was great variation in the molecular weights and polydispersities achieved from one

experiment to the next using a given monomer. Figure 2.16 shows three GPC traces for 9-HNA (53) when molecular sieves were employed, each analysed three hours after polymerisation was initiated (marked A); and three GPC traces of 8-HOA, each from material isolated one hour after the polymerisation was initiated (marked B). These highlight the inconsistencies between different experiments when the sieves are employed, and the improved reproducibility when the sieves are not used.

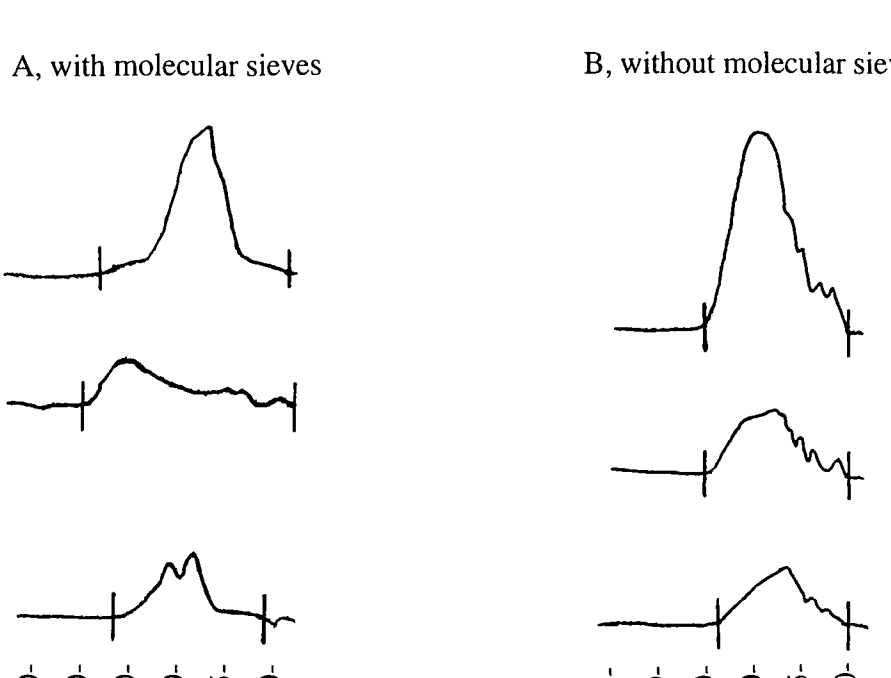


Figure 2.16. *Three GPC traces of 9-HNA (53) after three hours (marked A) and three GPC traces of 8-HOA (52) after one hour (marked B). This highlights the variability between experiments when molecular sieves are employed, which was not so apparent when the sieves were removed from the system.*

The high mass polymer, as well as the monomer, was also insoluble in hexane at 55 °C (see section 2.6.2), and thus another possibilty for the discrepancy between the different runs was that the high mass polymer deposits onto either the enzyme or the molecular sieves or both. An investigation was undertaken to determine this. Standard

reaction conditions were used (see section 2.2 and 6.2.1), but instead of analysing the material as a whole at the end of the experiment, the composition of the reaction was divided into three parts: the solvent; the enzyme in a small amount of solvent; and the molecular sieves. These three components were analysed separately by GPC, and the resultant data is shown in Figure 2.17.

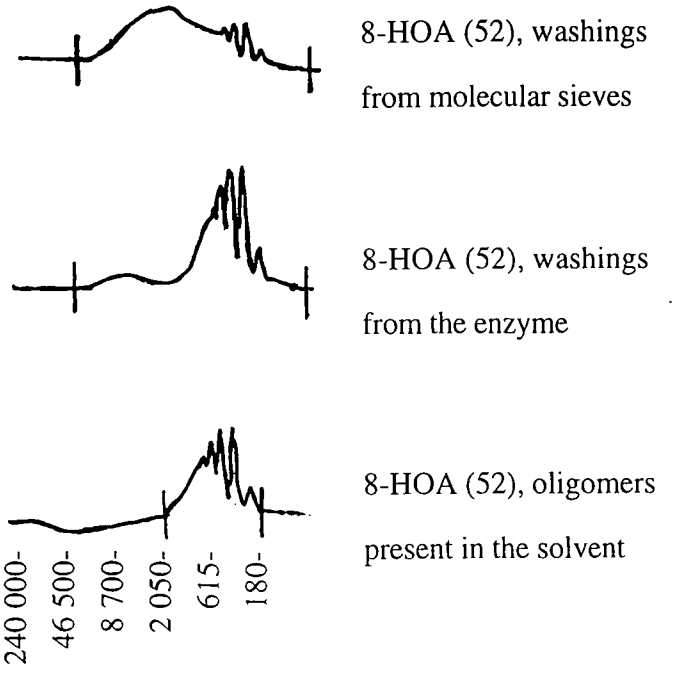


Figure 2.17. *GPC traces of the constituents of a polymerisation reaction after 50 hours.*

The results of this experiment clearly show that the polymer was largely insoluble in hexane, and that only the low mass oligomers remained in the solvent. Some of the polymer was deposited onto the enzyme, but the larger part of the higher mass polyester deposited onto the molecular sieves. Thus the molecular sieves offer a surface for polymer deposition, particularly as hexane is a poor solvent for the polyester. This material does not polymerise further, and thus the rate of polymerisation slows down. The deposition of both monomer and polymer onto the sieves would rationalise the variation between different runs of the same monomer. Furthermore, the adsorbed water within the molecular sieves may act to hydrolyse the polyesters depositing upon them, causing further variation in polydispersity (particularly with increased time) between

different experiments.

The results in this section demonstrate unequivocally that the molecular sieves play more than one role in the polymerisation process. They assist in removal of the water in the system, which helps the condensation reaction to progress. However, this advantage is outweighed by their ability to act as a surface onto which the polymer chains can deposit. Therefore, for future experiments it was decided to remove the molecular sieves from the the system, and to study the polymerisation reaction without a specific agent to adsorb the water (see Chapter 3).

2.6.2: Reprecipitation of monomeric and polymeric material.

An experiment was carried out on the monomeric material, using 11-HUA (43), to determine the solubility of the monomeric material in hexane at the reaction temperature. 11-HUA (43) (250 mg) was placed in hexane (30 ml) on a shaker at 200 rpm, at 55 °C for one hour. After hot filtration (55 °C) the recovered material was analysed by ^{1}H NMR and shown to be the monomer. The yield of the recovered monomer was low (60 mg, 24 %). Thus, with only this small quantity of monomer soluble in hexane, the monomer as well as the polymer, is mainly insoluble in the reaction medium.

The polydispersity of a CRL catalysed polymerisation of 8-HOA (52) over six hours varies from 1.6-1.8. However, if the reaction is allowed to proceed over the course of several days, the water generated in the condensation reaction begins to hydrolyse the polymer chains, generating low mass oligomers in addition to the high mass material (see sections 2.5.2 and 3.2.1). This results in a large polydispersity often up to 6.0 over several days. Therefore it proved desirable to assess if the polydispersity could be controlled at work up by selective dissolution in a solvent. Figure 2.18 illustrates a GPC trace of 10-HDA (42) after 96 hours. When the entire material was analysed the polydispersity was large (PD = 4.06). However, when only the high mass material in this trace was considered the polydispersity was 1.23. This high mass material could be separated by removing the low mass oligomers from the reaction

mixture. In a warm solution (55 °C) of hexane the higher mass material remained insoluble, and was obtained after filtration.

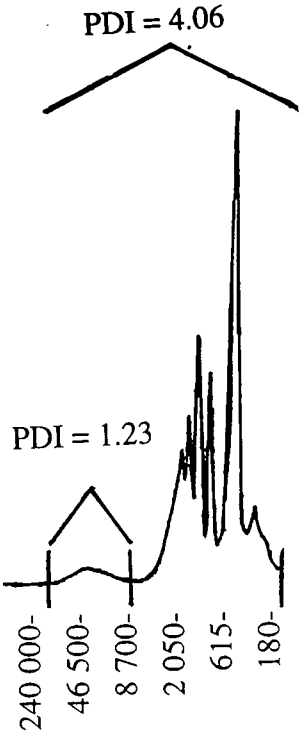


Figure 2.18. *GPC trace of 10-HDA (42) after 96 hours. Analysis of the whole material gives a high polydispersity, whereas the high mass polymer has a polydispersity of 1.23.*

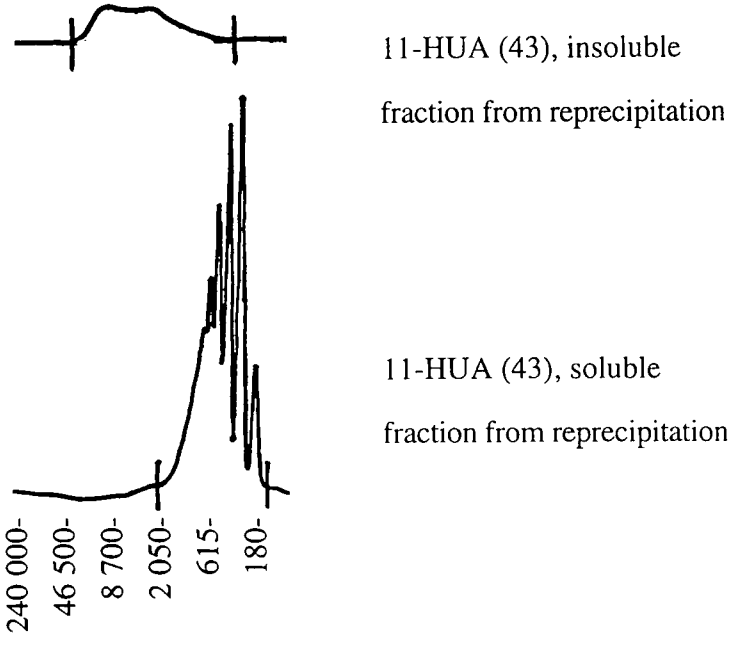


Figure 2.19. *GPC traces for the hexane soluble and insoluble polyester fractions after a reprecipitation experiment.*

Material from an 11-HUA (43) polymerisation reaction after five days (PD = 6.01), containing oligomers and polymers, was placed in hexane (20 ml) and shaken at 200 rpm at 55 °C for one hour and the suspension was filtered while warm. Both the filtrate and the residue were subjected to analysis by GPC, and the resultant GPC traces are shown in Figure 2.19, with the data displayed in Table 2.3. The polydispersity of the initial material was dramatically lowered after reprecipitation. In GPC trace A, which represents the solvent soluble fraction, oligomer is present with molecular weight (M_W) of 1597. GPC Trace B, shows that the polymer which is insoluble in hexane, with a molecular weight (M_W) of 16 309, supporting the results of the previous experiments.

	Mn	Mw	Polydispersity
Initial material	1001	6015	6.01
filtrate	1281	1597	1.25
residue	10089	16309	1.62

Table 2.3. *Mass and polydispersities of initial 11-HUA (43) polymer, filtrate and residue.*

2.7: Discussion.

2.7.1: Influence of the solvent.

Solvent	Dielectric Constant	Dipole Moment μ/D
Hexane	1.89	0.00
Cyclohexane	2.02	0.00
Carbon Tetrachloride	2.24	0.00
Toluene	2.38	0.38
Tetrahydrofuran	7.60	1.75

Table 2.4. *Dielectric constants and the dipole moments of the solvents investigated for the polymerisation reactions.*

Table 2.4 shows the dipole moments and the dielectric constants of the solvents investigated. The dielectric constant is a measure of the ability of a non-conducting molecule to shield charge. The dipole moment is a measure of how polarised a molecule is when it is not in an electric field. Hexane and cyclohexane proved the best solvents to generate polyester with the highest molecular weights. These are the least polar of the solvents studied and this observation is consistent with previous studies. For example, higher molecular weights of polyesters were found in the less polar solvents by Gutman[60], whose results were summarised earlier in Table 1.1.

Three solvents (CCl_4, THF and toluene) had similarly rapid initial rates over the first hour, before the reaction tailed off with no significant further increase in molecular weight over the time period studied. One hypothesis for this abrupt termination of the reaction is that these solvents strip water from the enzyme's surface as the reaction progresses, and deactivate it. This has been suggested for more polar solvents, such as acetonitrile[31].

2.7.2: Reactions without molecular sieves.

When the polymerisation reactions were performed without molecular sieves the

initial rates did not slow down as anticipated. The molecular weights attained were higher and thus there was little competitive hydrolysis of the polyester chains from released water. Clearly the amount of water released during this initial phase is low. This water could perhaps assist lubrication of the lipase and supplement the hydration layer surrounding the enzyme, thus increasing the catalytic prowess of the lipase.

Support for this hypothesis comes from a recent report in which a catalytic amount of methanol added to a rigoursly dried enzyme initiates a transesterification reaction[75]. Moore employed anhydrous subtilisin in dry hexane to transesterify propanol with acetylphenylalanine ethyl ester (APEE). After 50 minutes 5 μl of methanol was added to the reaction mixture. Immediately, rapid formation of the methyl and propyl esters commenced. Through other qualifying experiments it was determined that the methanol was activating the enzyme *via* a solvation process[75].

The results in this literature study indicate that the release of water in the polyesterification process could indeed activate the enzyme. Thus there is a precedent for the enzymatic reactions performed without the molecular sieves being more rapid relative to the reactions performed with molecular sieves.

2.7.3: Assembly of the polymer.

After an initial rapid phase (approximately six hours) of polymer assembly, the rate of assembly slows down (see figure 2.9). Correlation of the data from GPC with that from ^{1}H NMR, indicates that this initial rapid phase stops at the point when all of the monomer is consumed. The slower phase involves the linking of oligomer chains together. In a recent paper Russell *et al.*[71] lend support to this analysis stating that the enzyme is designed for the hydrolysis of smaller molecules, and thus when the smaller molecules are no longer present the efficiency of catalysis decreases. Russell concluded that a polymer mixture will be biased towards the low molecular weight materials, with a relatively low concentration of the high molecular weight component. This implies that the resultant polyester will have a relatively large polydispersity. However, in the reprecipitation experiments (see section 2.6.2), when the high molecular weight material

was separated from the oligomers and monomer, the higher mass polymer had a polydispersity of approximately 1.2. A statistical mass distribution, found in the classical step growth process of transesterification to polyesters, predicts a polydispersity of 2.0[76]. The developing polymer chain is insoluble in hexane, and the resultant polymer will not diffuse, remaining in close proximity to the enzyme, or even enveloping it. As each new monomer or oligomer unit is attached to the polymer the chain moves further round the enzyme, to a point at which no more polymerisation occurs, clearly due to the insolubility of the polymer. This would result in high mass polymer with a particular length of chain, and the observed narrow polydispersity.

With the reaction employing an enzyme:monomer ratio of 1:100, it was anticipated that with a smaller amount of enzyme present, and therefore fewer catalytic sites, there would be fewer polyester chains present, but that the chains would be of higher molecular weight, with a tighter polydispersity. In the event this was not apparent, and although there were high mass chains formed, the polydispersity of the material remained high (see section 2.3.2).

2.7.4: Specificity of the enzyme.

Analysis of the relative rates of polymer assembly by both ^{1}H NMR and GPC showed that 9-HNA (53) was the best substrate for the enzymatic polymerisation, both in terms of the rate of monomer consumption and molecular weight build up. 8-HOA (52) was the second best substrate in both categories, followed by 10-HDA (42), with 11-HUA (43) having the slowest relative rate of all four of the monomers studied. The length specificity of the enzyme will be discussed in greater detail in Chapter 4.

2.8: Conclusions.

From the studies performed in this chapter optimal reaction conditions for the enzymatic polyesterifications have been established. The optimal conditions for the ω-hydroxy acid polymerisations were in hexane, at 55 °C, without molecular sieves. CRL

appears to have a length specificity requirement for polymerisation of the ω-hydroxy acids. 9-HNA (53) was the most efficient monomer in the polymerisation reaction, followed by 8-HOA (52), 10-HDA (42) and 11-HUA (43). Long term polymerisation affords high mass polymeric material as well as oligomers, resulting in a material of large polydispersity. Reprecipitation of the polymeric material afforded the high mass fraction, with a molecular weight of Mw = 16 000, and a polydispersity of 1.6. The enzyme was shown to be active in catalytic quantities (in a mass ratio of 1:100, enzyme to substrate). The crude enzyme contains only about 1 % active protein,with the bulk of the material being precipitated salts.

CHAPTER THREE

PROGRESS OF ENZYMATIC POLYMERISATIONS

3.0: PROGRESS OF ENZYMATIC POLYMERISATIONS.

3.1: Aims and objectives.

In Chapter 2 the optimal conditions for CRL catalysed polymerisations of ω-hydroxy acids were established, and it was determined that the more rapid initial assembly of the polymer was afforded when molecular sieves were not employed. It was also observed that CRL had a length specificity, and that 9-HNA (53) was the favoured monomer. This was proceeded by 8-HOA (52), 10-HDA (42) and 11-HUA (43) respectively. Therefore the aims outlined in this chapter are to explore:

1) Comparison of six ω-hydroxy acids as substrates for polymerisation when molecular sieves are not employed.
2) Polymerisation of functionalised monomers.
3) Investigation into the sequence of assembly of the polyester.
4) Comparison of the enzymatic with a chemically ($Ti(OBu)_4$) catalysed process.

3.2.1: Progress of enzymatic reactions, without molecular sieves.

Reactions without molecular sieves were studied to assess the progress of polymerisation for various ω-hydroxy acids, and to establish the relative rates of monomer consumption, the rate of high mass polyester assembly, and the highest masses which could be achieved. Initially six ω-hydroxy acid monomers were investigated in the enzyme catalysed polymerisation, with chain lengths varying from 8 to 16 carbons.

Figure 3.1 shows the consumption of the monomers, determined by ^1H NMR. There is rapid initial consumption of all the monomers in the first hour of the reactions. As the reaction progresses to 100 % conversion it appears that 8-HOA (52) and 9-HNA (53) are the most rapidly consumed, with the longer chain systems condensing more slowly.

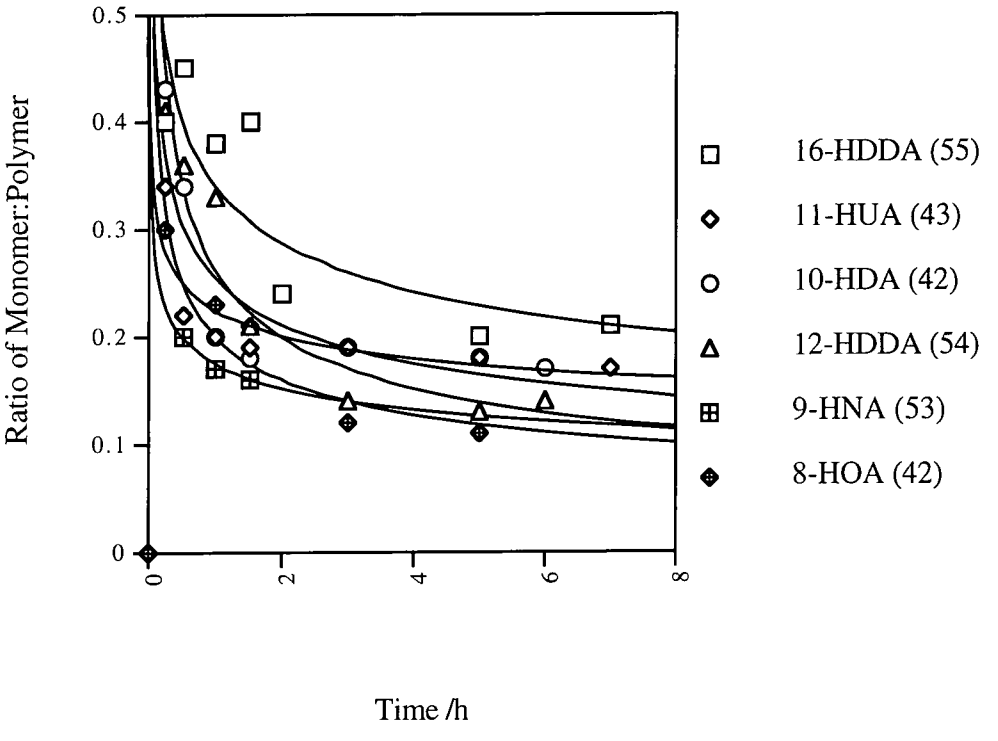


Figure 3.1. *Consumption of ω-hydroxy acid monomers with time by ^1H NMR analysis.*

Figure 3.2 shows the relative rates of polymer assembly, as determined by GPC (M_W), versus time. All six systems show a rapid initial assembly of oligomers, achieving molecular weights of about 1000 in approximately 30 mins. As the reaction proceeds into the slower phase of assembly, when the monomeric material is largely consumed, the molecular weights increase, and higher masses are obtained most rapidly in the cases of 8-HOA (52) and 9-HNA (53).

The start of the slower phase of assembly can be seen in both of the graphs, as the point at which the curves begin to level. By both NMR and GPC the initial phase of monomer consumption has finished after two hours, and the initial rate slows dramatically into the oligomer condensation phase.

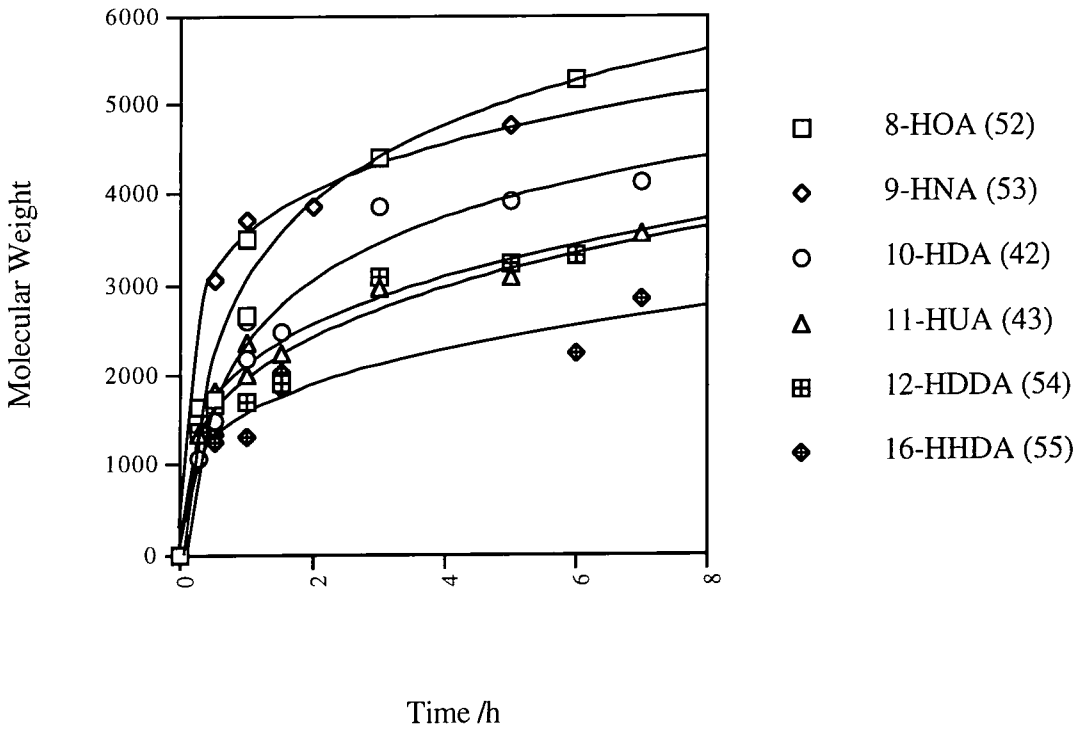


Figure 3.2. *The progress of polymerisation as measured by GPC (M_W) with time.*

After approximately 30 hours the water, released by the polycondensation process, became significant, and affected the equilibrium of the reaction. The enzyme began to hydrolyse the chains in a competitive process. The low molecular weight material became apparent in the GPC profiles of these reactions, and over time these began to dominate the GPC profiles (see Figure 3.3, GPC traces for 8-HOA (52) after 16, 24, 30 and 144 hours). The result of this was that after 144 hours the molecular weight (Mw) range came down to below 2000, which is lower than the mass observed after three hours, despite some very high mass material being formed (upto 35 000).

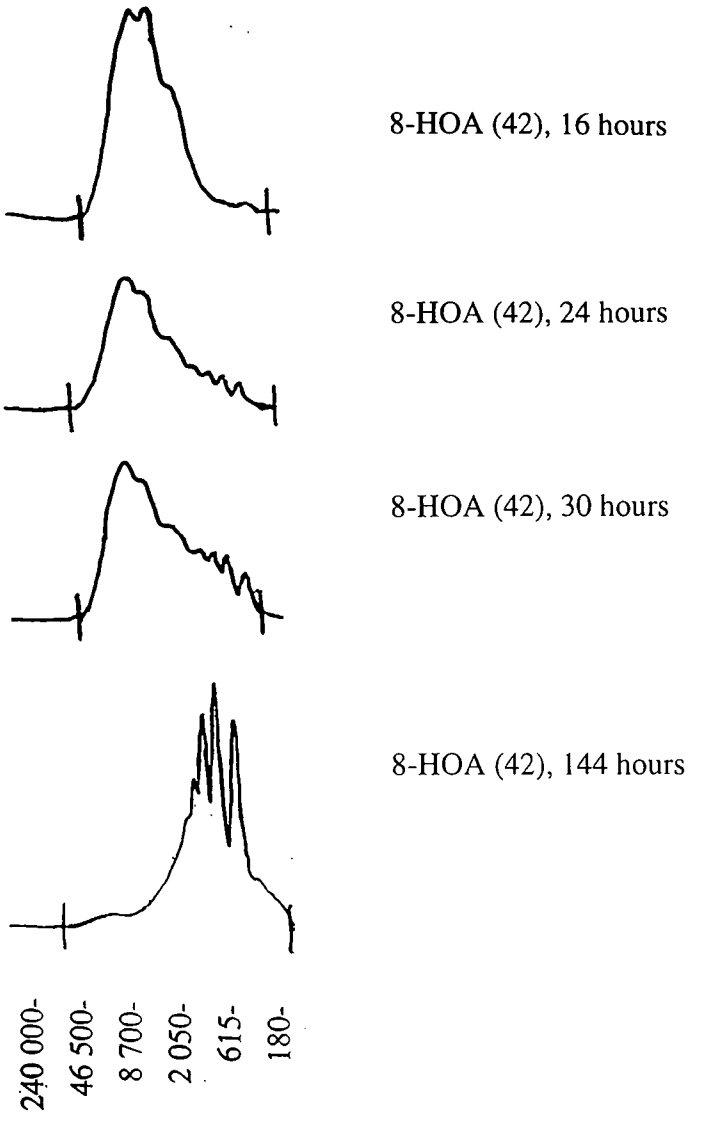


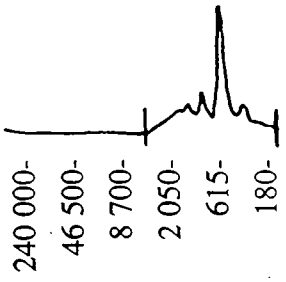
Figure 3.3. *GPC traces for the polymerisation of 8-HOA (52) in the middle and latter stages of the enzymatic reaction.*

Figure 3.3 shows very dramatically that the initially formed polymer is subsequently hydrolysed over time to an equilibrium mixture of low molecular weight oligomers. This hydrolytic reaction has been previously observed within this group when molecular sieves were employed in the polymerisation reaction[62]. 11-HUA (43) was polymerised over 196 hours, and although high mass material dominated the GPC profile in that case, low mass material was also present in the GPC traces. The dominance of the high mass material with molecular sieves is consistent with the molecular sieves retaining the water generated during the condensation reaction.

Without molecular sieves this water is free for hydrolysis, with the resultant effect that the hydrolytic reaction is much more rapid as seen in Figure 3.3.

3.2.2: Investigations into the diolide (56) formed from 16-hydroxyhexadecanoic acid (16-HHDA) (55).

The 16-HHDA (55) had perhaps the most unique polymerisation profile of all the monomers studied. Although high molecular weight material was observed over longer periods of time (several days), there was a dominant peak in the GPC trace (Figure 3.4) with an estimated mass of 520. It was previously shown in the group that 16-HHDA (55) formed the diolide (56)[77], and that this was the major product of the reaction with molecular sieves. The structure of the diolide was solved previously by X-ray analysis (see Figure 3.5).



16-HHDA (55), showing dilactone , after 5 hours

Figure 3.4. *The dimeric lactone (56), formed when 16-HHDA (55) is esterified[77].*

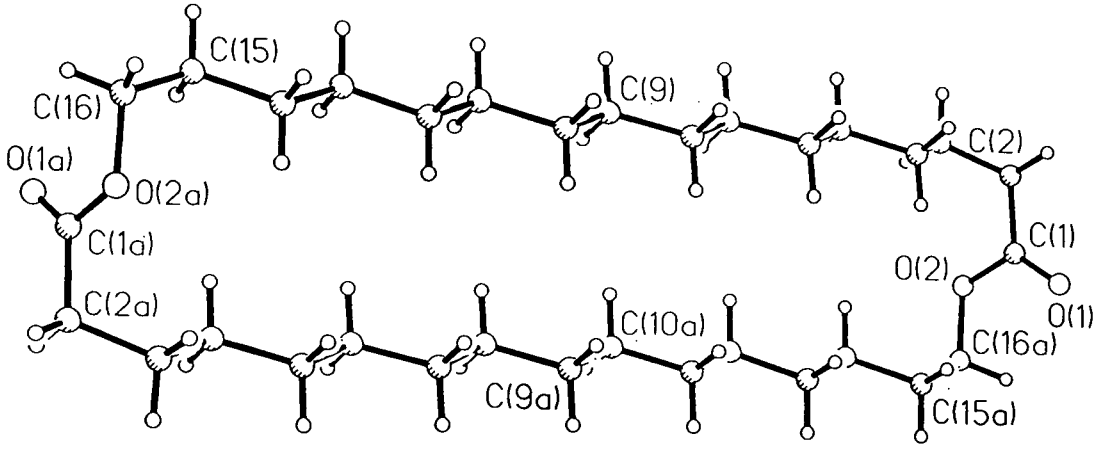


Figure 3.5. *Diolide (56) formed during 16-HDDA (55) polymerisation[77].*

Such diolides may be formed in the other ω-hydroxy acid systems, but they do not dominate the GPC profiles. The dimeric ring is perhaps more stable in this system, or perhaps there is a difference in mechanism which favours diolide over polymer assembly for such longer chain monomers.

3.2.3: Discussion of the assembly of the polymer.

The initial assembly of the polymers is most rapid for 8-HOA (52) and 9-HNA (53), and decreases with increase in chain length for 10-HDA (42), 11-HUA (43), 12-HDDA (54) and 16-HHDA (55) respectively. This may underly a preferred specificity of CRL for shorter chain lengths and this will be discussed in more detail in Chapter 4.

The consumption of the monomeric species was rapid in all cases, but particularly dramatic for 8-HOA (52) and 9-HNA (53) where oligomers of approximately 3000 were assembled within one hour and molecular weight of approximately 5000 within six hours. These rates are the fastest reported to date, and contrast most significantly with the lactone polymerisation of Kobayashi[54] (see section 3.4.1).

In the middle and late stages of the reaction (24 hours and 144 hours respectively) there is a different profile for the polymerisation. The system studied in most detail was 8-HOA (52). After 24 hours the water released during the condensation reaction begins to hydrolyse the polymers, presumably catalysed by the lipase. After 6 days the competitive hydrolysis reaction dominates to give an equilibrium mixture of oligomers, such that the M_W values for the materials are lower than that achieved after the first few hours. GPC indicates, however, that some high mass material remained[62].

3.3.1: An investigation into the sequence of polymer assembly.

A series of experiments was designed to determine the sequence of assembly of the polymer. Firstly the activity of the enzyme was tested, to determine whether the

enzyme remained active after 12 hours, or whether its activity decreased with time. Secondly, it was anticipated that the use of deuterium labelled monomers, added to the reaction vessel during an experiment, would shed some light on the sequence of assembly.

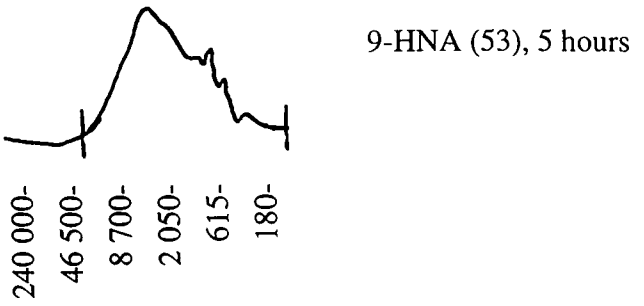
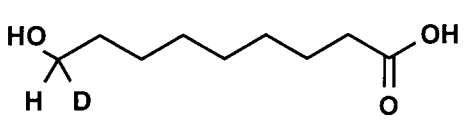


Figure 3.6. *The build up of oligomers after five hours for 9-HNA (53).*

9-HNA (53) was added to a suspension of the lipase in hexane, and oligomers were generated within five hours (see figure 3.6). After 12 hours additional monomer was added. However, using GPC it was impossible to determine the relative rate of consumption of the additional monomer, since a bimodal profile was not obvious. Thus a different approach was taken using deuterium isotope substitution and analysis by both ^{1}H and ^{2}H NMR.

The experiment was repeated with 9-HNA (53) as the monomer, and after 12 hours [9-$^{2}H_{1}$]-9-hydroxynonanoic acid (57) was added. The loss of the deuterated monomer was monitored by ^{2}H NMR. The rate of loss at this stage was similar to the initial rate of oligomer assembly 12 hours earlier. Figure 3.7 shows the rate of consumption of 9-HNA (53) as judged by ^{1}H NMR. After 12 hours the deuterium labelled monomer was pulsed into the reaction, and the consumption of this monomer was monitored by ^{2}H NMR. The graph shows clearly that the activity of the enzyme does not decrease with time, and that the monomer which is added to the reaction is consumed as rapidly as the monomer which was initially polymerised.

The use of ^{2}H NMR in this study was particularly useful for monitoring the loss of monomer, and determining the point at which all the monomer was consumed.



[9-$^{2}H_{1}$]-9-hydroxynonanoic acid

(57)

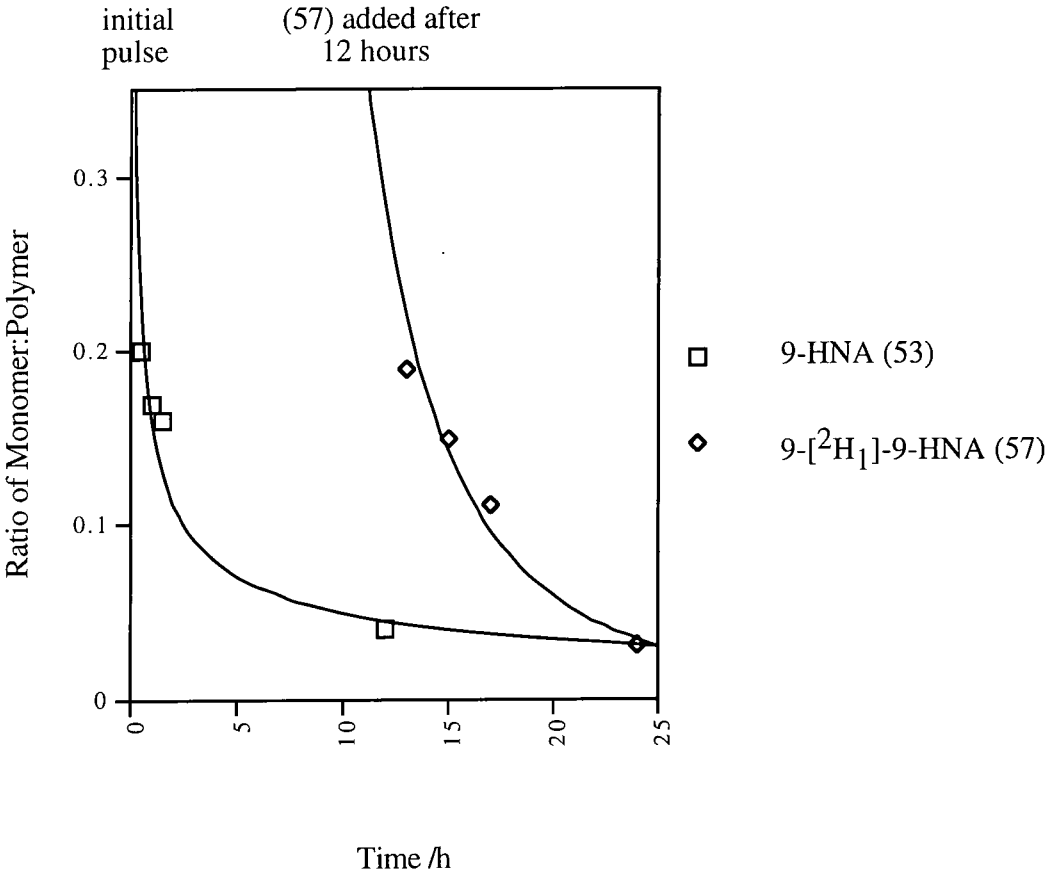
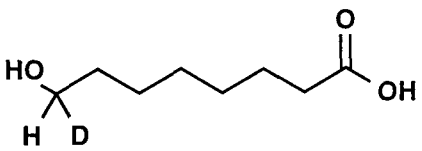


Figure 3.7. *Rate of consumption of 9-HNA (53), initially and after 12 hours when 9-[$^{2}H_{1}$]-9 HNA (57) was pulsed into the reaction.*

A separate experiment was performed using [8-$^{2}H_{1}$]-8-hydroxyoctanoic acid (58). However, the order of addition of labelled and unlabelled monomer was reversed. After 12 hours the oligomers derived from [8-$^{2}H_{1}$]-8-HOA (58) were assessed by ^{1}H and ^{2}H NMR. Unlabelled 8-HOA (52) was then added to the reaction vessel and the reaction continued for a further 12 hours. There was a peak at 4.0 ppm in the ^{2}H NMR spectrum, indicating the formation of the ester. A small peak also persisted at 3.6 ppm

throughout the experiment (see Figure 3.8). This peak could either be from the terminal end groups of oligomer or from residual monomer which has not esterified. However, this does impact on the mechanistic implications of polymer assembly, and will be discussed in more detail in section 3.3.2.



[8-$^{2}H_{1}$]-8-hydroxyoctanoic acid

(58)

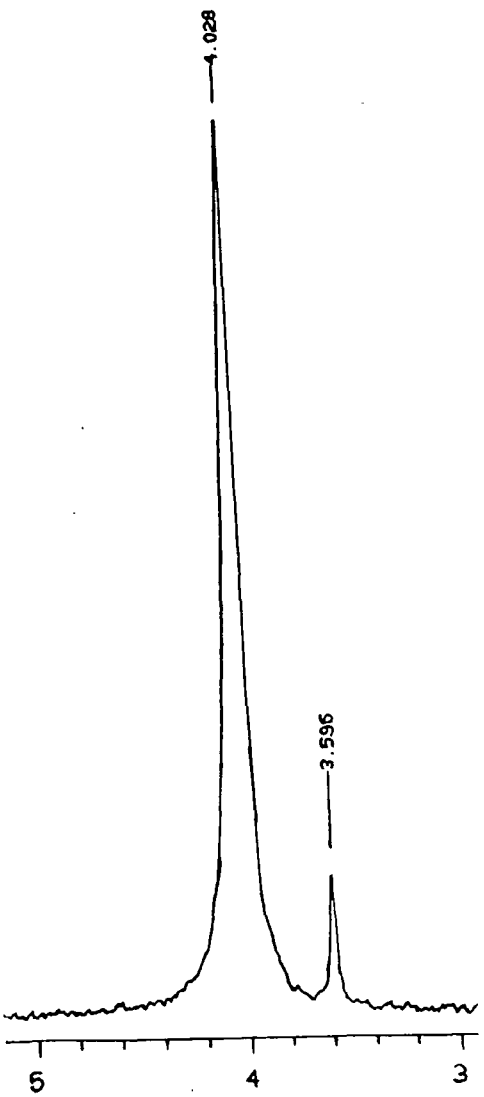


Figure 3.8. *^{2}H NMR spectrum, of 8-HOA (52) five hours after the unlabelled monomer was added to the deuterated polymer.*

3.3.2: Discussion of the sequence of polymer assembly.

The rate profiles shown in Figures 3.1 and 3.2 show that as the polymerisation proceeds the reaction slows down. The experiments in the previous section indicate that this was not due to loss of enzyme activity. Figure 3.7 illustrates that the enzyme retained its activity, over the time course of the reaction. There are several possible mechanisms for the assembly of the polymer. The enzyme could esterify monomer onto monomer, and to this dimer add more monomer, building up the chain one unit at a time in a stepwise manner. Alternatively the enzyme could add monomer onto monomer to generate a dimer, which could subsequently be condensed with another dimer to give a tetramer, and so on in a chain growth mechanism.

When the deuterated [8-$^{2}H_{1}$]-8-HOA (58) was studied a labelled oligomeric mixture was generated. Unlabelled material was then added after 12 hours. Figure 3.9 shows schematically three possible sequences (a), (b) and (c) of assembly of the polymer, starting with (a) the addition of the lipase to the deuterated monomer, (b) the assembly of the deuterated polymer, and (c) the randomisation of labelled and unlabelled monomer in the polymer.

The addition of the unlabelled monomer to the deuterated oligomer could have resulted in the terminal hydroxyl groups of the deuterated oligomers being capped, and thus a loss of the peak at 3.6 ppm in the ^{2}H NMR spectrum. However, the peak at 3.6 ppm in the ^{2}H NMR spectrum remained in the resultant material. This tends to indicate that the enzyme esterified the unlabelled monomers (as indicated by the disappearance of the peak at 3.6 ppm in the ^{1}H NMR), and was either not capping the existing oligomers (*i.e.* the new material was forming oligomers separate from the deuterated oligomers), or the enzyme was cutting up the chains as it added the non-deuterated material to the existing oligomers, and thus fresh deuterated end groups were constantly being exposed as the deuterated esters were being hydrolysed. Thus the experiment rules out scenario (a) but does not distinguish between (b) and (c).

Of (b) and (c) the more probable mechanism is (b). The relative rate of consumption of the monomer over this time period shown in Figure 3.7 indicates that the lipase is esterifying the monomer. Therefore the unlabelled monomers are

polymerised until the material has been consumed, at which point the lipase condenses the labelled and unlabelled oligomers.

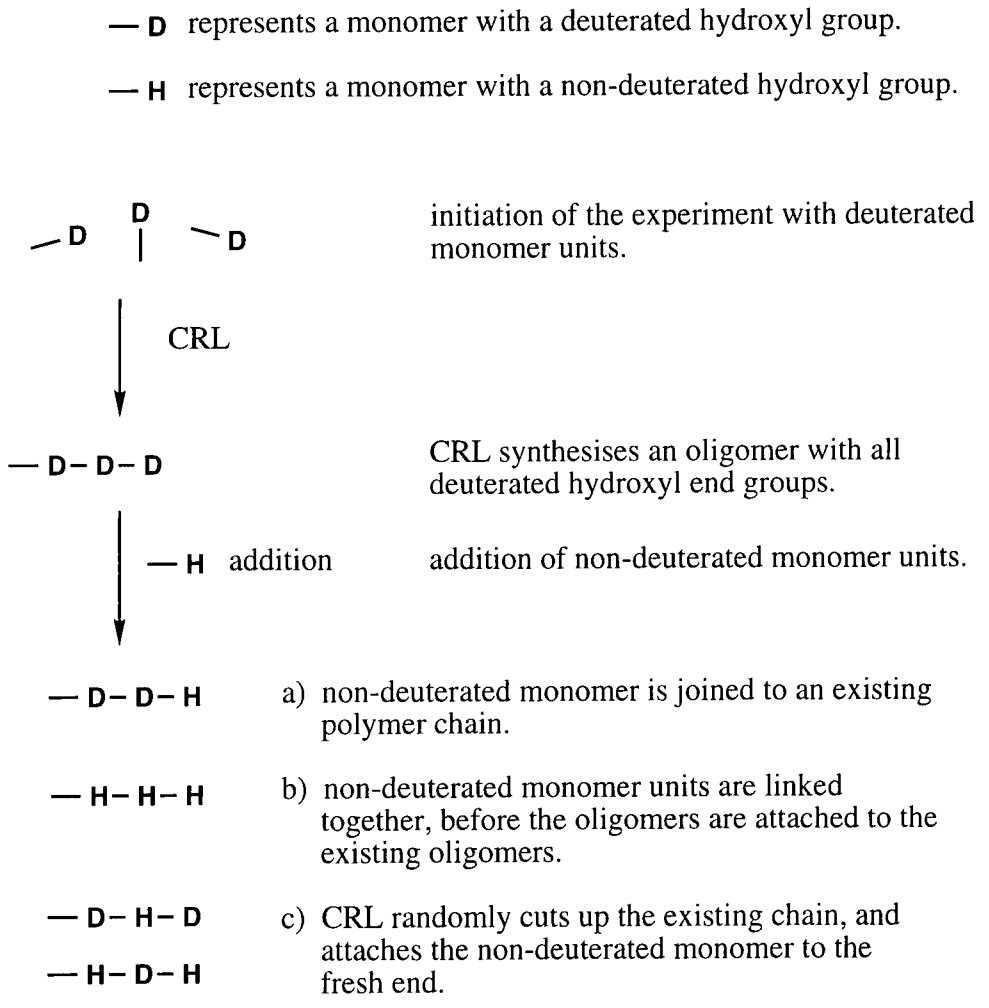


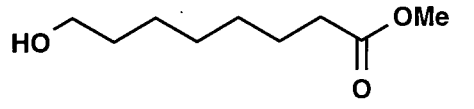
Figure 3.9. *Three possible sequences of polymer assembly.*

Mechanism (c) involving the lipase hydrolysing the deuterated polymer can be discounted in this scenario. The hydrolysis of the polymer chains is not observed until after several days of a reaction, whereas the time scale for this experiment was 24 hours. Thus randomisation of the chain has not yet occurred.

In the light of this experimental evidence it is probable that the lipase assembles the polymer by adding monomer to monomer, until all of the monomer is consumed. The oligomeric species are then condensed to form the higher mass polymers.

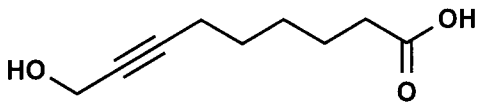
3.4.1: Polymerisations using other monomers.

An investigation was undertaken into the structural variation within the monomers which could be accomodated by CRL for polymerisation. Several derivatised ω-hydroxy acids or analogues were used as candidate monomers for polymerisation reactions, to compare with the resultant masses and rates of polymerisation of the ω-hydroxy acid reactions. The monomers studied were methyl 8-hydroxyoctanoate (59), 9-hydroxynon-7-ynoic acid (60), 10-hydroxyundecanoic acid (61) and methyl 11-aminoundecanoate (62).



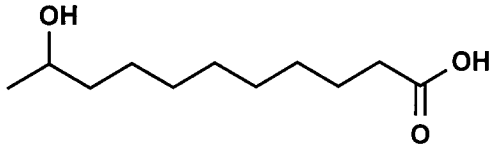
methyl 8-hydroxyoctanoate

(59)



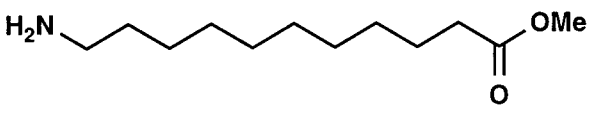
9-hydroxynon-7-ynoic acid

(60)



10-hydroxyundecanoic acid

(61)



methyl 11-aminoundecanoate

(62)

Monomer (59) was chosen to draw a comparison between an ω-hydroxy acid and an ω-hydroxy ester. The acetylenic hydroxy acid (60) was employed to determine if the lipase was able to accept a molecule with a linear segment. A considerable length of time was spent on the synthesis of (60), and this is discussed in section 5.3.6. Monomer (61) was employed as a substrate to allow a comparison in the rates of a primary and a secondary hydroxy acid polymerisation reaction. The amino ester (62) was chosen since the commercially available amino acid existed as a zwitterion, and as such is completely insoluble in hexane.

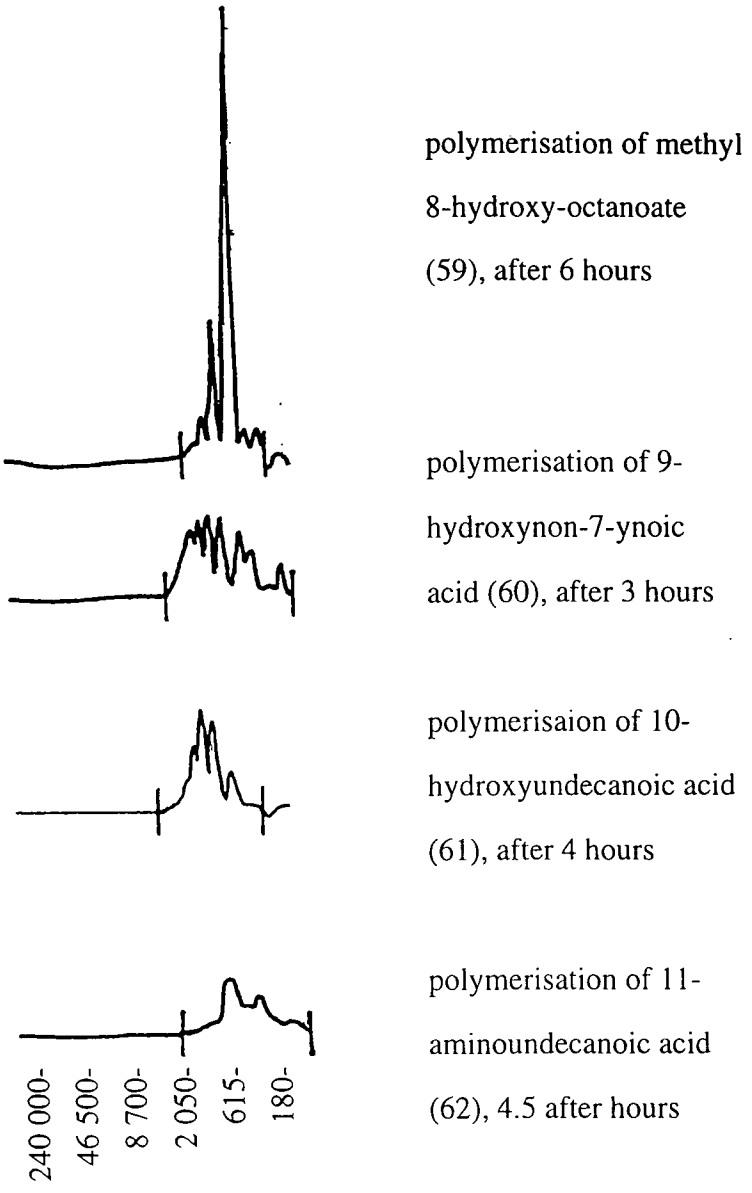
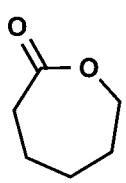


Figure 3.10. *GPC traces for the three ω-hydroxy acids (59), (60) and (61), and the ω-amino acid (62).*

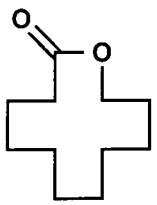
In the event all of these monomers were poor substrates and only oligomers were generated after 3-6 hours (see Figure 3.10). Thus CRL appears to be specific to aliphatic ω-hydroxy acid substrate. The oligomerisation with 10-hydroxyundecanoic acid (61), a chiral molecule, is studied in greater detail in Chapter 4, in an investigation of the stereospecificity of the lipase.

3.5.1: Comparison of ω-hydoxy acid with undecanolide polymerisation.

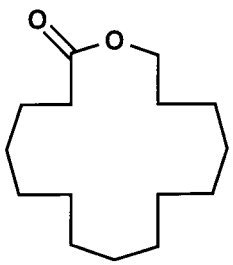
Kobayashi[52,53,54,55] has recently reported on the enzyme-catalysed ring-opening polymerisation of various lactones. Originally that work investigated smaller lactones, such as ε-caprolactone (18), using a lipase from *Pseudomonas fluorescens*. These reactions were conducted at 60 °C and achieved a polyester with molecular weight (M_n) of approximately 7000. This study of ring opening polymerisation was extended more recently[54] to incorporate larger macrolides. Accordingly 11-undecanolide (46) and 15-pentadecanolide (48) were studied as substrates, and the progress of the reactions were compared to the smaller lactones. Kobayashi has shown that the larger macrolides undergo enzyme catalysed polymerisation much faster than ε-caprolactone (relative rates of enzymatic polymerisation were not specified in this report)[54]. For example, neat undecanolide (46) at 75 °C with CRL generated a material of molecular weight (M_n) 21 900 after five days. The polymerisation of pentadecanolactone (48) generated only modest molecular weights (M_n = 6400)[54].



ε-caprolactone
(18)



undecanolide
(46)



pentadecalactone
(48)

A direct comparison of the relative rates of an ω-hydroxyacid with a lactone appeared appropriate. Therefore a lipase catalysed polymerisation of undecanolide (46) was studied in hexane using CRL, under the reaction conditions used for the polymerisation of ω-hydroxy acids (see section 6.2.1). Polymerisation of (46) occurred very slowly as judged by ^1H NMR, and far slower than the lipase catalysed ω-hydroxyacid polymerisations of our system, as can be seen from Figure 3.11.

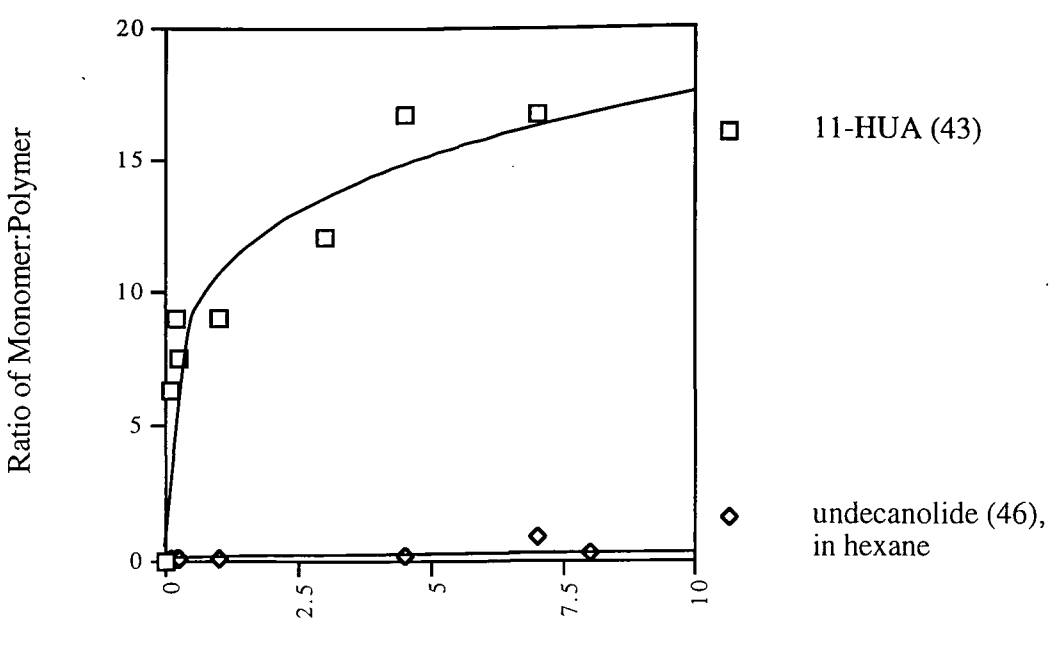


Figure 3.11. *The consumption of 11-HUA (43) and undecanolide (46) in hexane.*

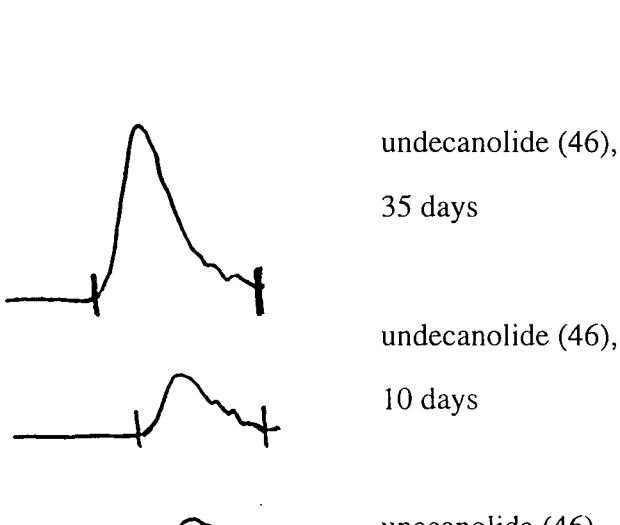


Figure 3.12. *GPC traces of neat undecanolide (46) polymerisation through time.*

Figure 3.11 shows the consumption of undecanolide (46) as a solution in hexane compared to the consumption of 11-HUA (43). For the undecanolide (46) reaction the consumption of the lactone is considerably slower than 11-HUA (43).

A neat polymerisation of undecanolide (46) (110 mg) was attempted, with enzyme (30mg) in a sealed tube. The GPC traces showed material of low overall molecular weight. Therefore, in comparison with the open chain ω-hydroxy acids, high molecular weights were achieved with undecanolide (46) only when the time scale for the polymerisation was considerably longer than for the ω-hydroxy acids. Figure 3.12 shows three traces of the neat undecanolide (46) polymerisation reaction.

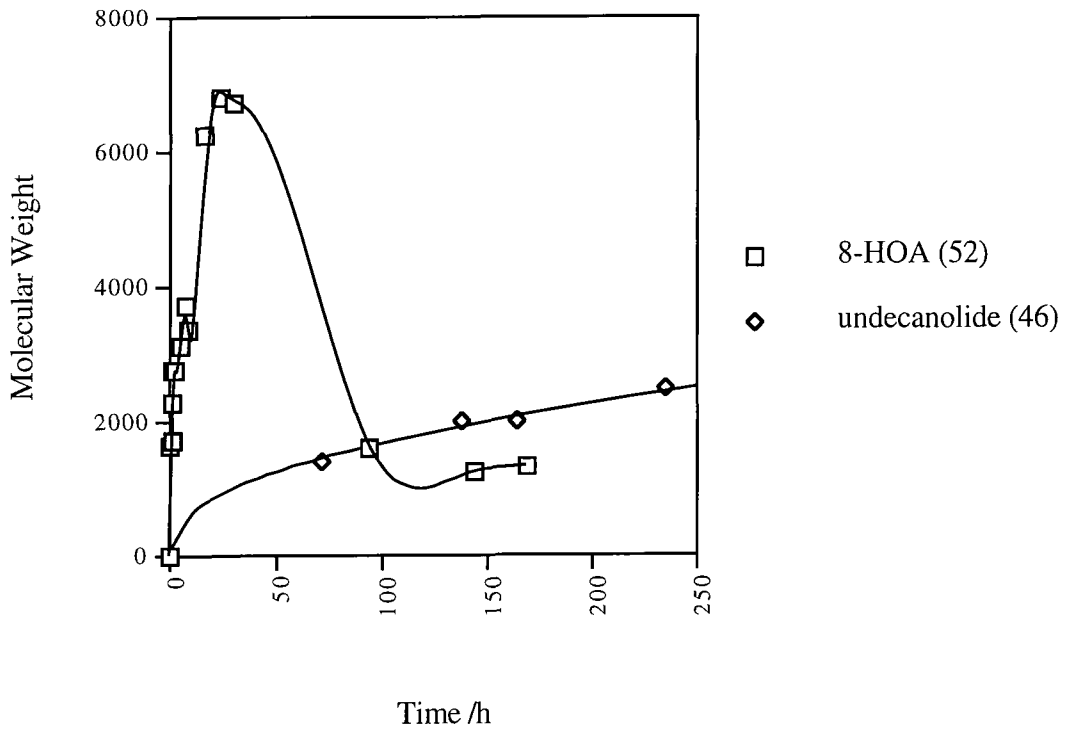


Figure 3.13. *The change in molecular weight (M_W) with time for 11-HUA (43) and neat undecanolide (46).*

Figure 3.13 shows the change in molecular weight (M_W) with time for 11-HUA (43) and neat undecanolide (46). The rate of assembly of the polymer from the lactone

is considerably slower than that from the hydroxy acid. However, undecanolide polymerisation is gradual, building up to high molecular weight over time, whereas the molecular weight of 11-HUA (43) increases over 24 hours, before the polymeric chains are then hydrolysed in the competitive reaction. Of course, no water is released in the undecanolide polymerisation.

3.5.2: Discussion of lactone polymerisation.

Two minimal mechanisms for the lipase catalysed esterification of undecanolide (46) which emerge as the most likely of a number of candidates, are shown in Figures 3.14 and 3.15.

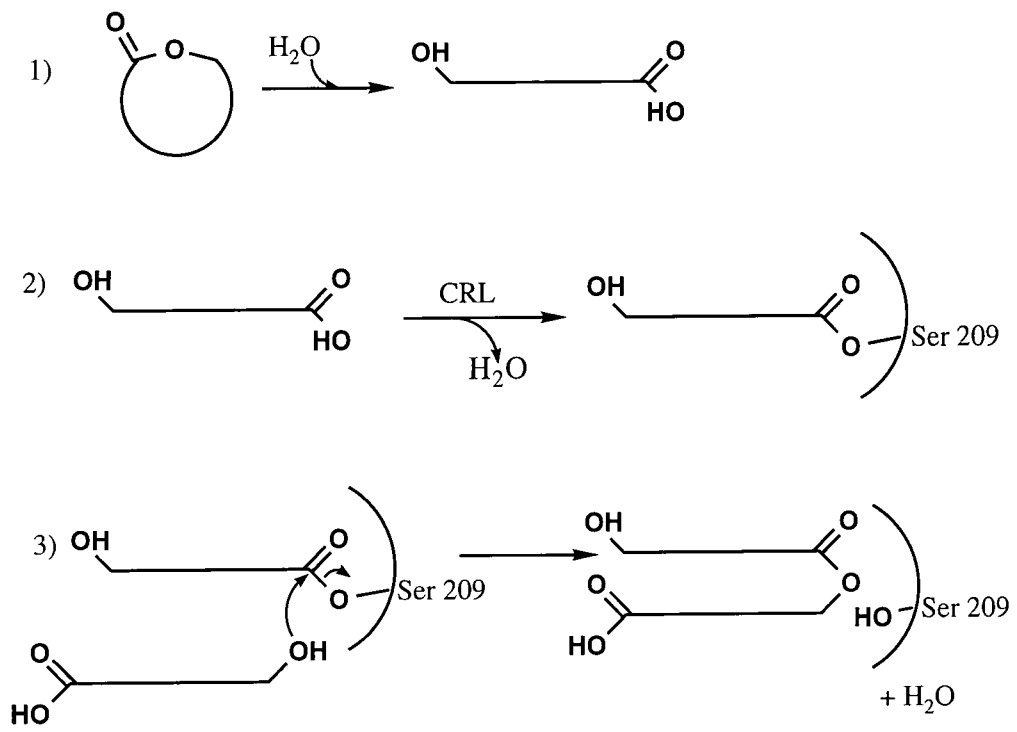


Figure 3.14. *Kobayashi's mechanism for the polyesterification of undecanolide*[54].

The mechanism proposed by Kobayashi (also shown in Chapter 1) requires a molecule of water to initiate lactone hydrolysis. Kobayashi also states that the slow

step of this reaction is the formation of the acyl-enzyme intermediate[54].

An alternative mechanism can also be envisaged, in which CRL is responsible for the ring opening of the lactone, to directly generate the acyl enzyme intermediate, now the slow step of the process. This activated species is then hydrolysed by a water molecule associated with the enzyme to release an ω-hydroxy acid. This species then attacks a second acyl enzyme intermediate to form a dimer, and return CRL to its original state.

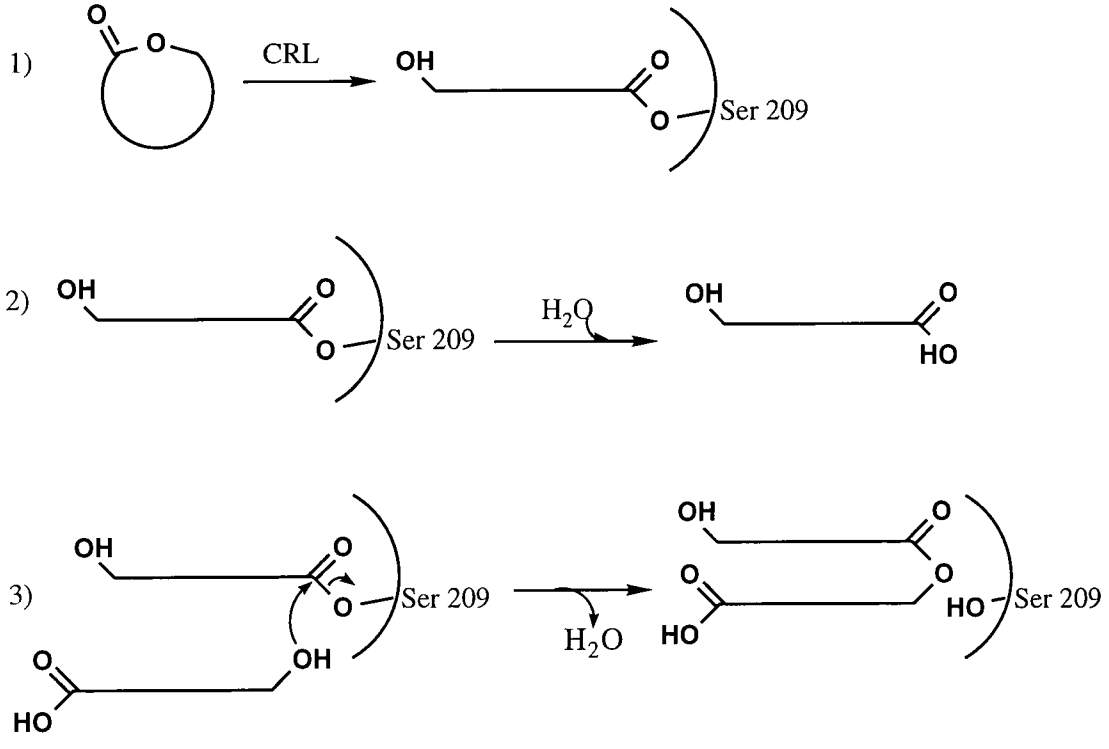


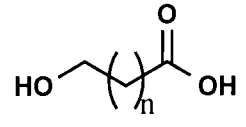
Figure 3.15. *A second mechanism for the lipase catalysed polymerisation of undecanolide.*

The ring strain of the larger lactones is considerably less than that for ε-caprolactone (18). For example, dipole moments for ε-caprolactone (18), undecanolide (46) and pentadecanolide (48) are 4.45, 1.86 and 1.86 respectively[54]. The rate of NaOH catalysed hydrolysis of the larger macrolides is 300 times slower than that of the seven membered ring, caprolactone (18)[54]. This is a different situation to the enzyme catalysed ring-opening polymerisation where the larger lactones react faster, suggesting

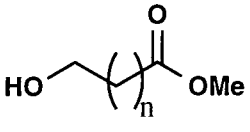
a very different mechanism from non-enzymatic hydrolysis. Kobayashi proposes that the rate determining step of the reaction is the formation of the acyl-enzyme intermediate, and that the larger lactones mimic the conformation of the lipases' natural substrates, triacylglycerides[54]. Therefore the formation of this intermediate is preferred for the larger lactones, and thus the enzyme-catalysed polymerisation of these lactones is faster for the macrolides.

The lipase has a role in the polymerisation of both smaller and larger lactones, although the conformation adopted by the larger lactones is favoured by the enzyme, and thus they are hydrolysed more rapidly. Nonetheless, these rates are relatively very slow compared to the ω-hydroxy acids which are polymerised much more rapidly than the larger lactones. This could be due to the more rapid formation of the acyl-enzyme intermediate, which is necessarily slower for undecanolide (46) than for the ω-hydroxy acids, due to esters being less reactive than acids.

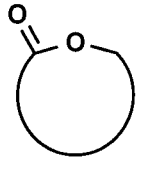
An interesting point to emerge from these studies as regards the specificity of CRL is the relative rate of enzyme catalysed polymerisation of the ω-hydroxy acid, the ω-hydroxy ester and the lactone. The free acid polymerises considerably more rapidly than either of the two other substrate shown in Figure 3.16. The formation of the ester slows the polymerisation process down, with the straight chain ester and the cyclic ester forming polymers only comparatively slowly. Thus CRL has been shown to much prefer the free ω-hydroxyacid over either of the esters.



ω-hydroxy acids



ω-hydroxy esters

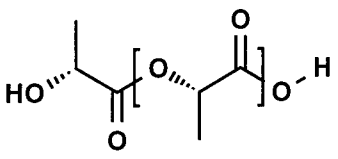


lactones

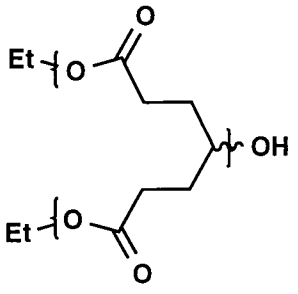
Figure 3.16. *Structures of ω-hydroxy acids, ω-hydroxy esters and lactones.*

3.6.1: Polymerisation using Ti(OBu)4.

Polyhydroxy acids, such as poly (*L*)-lactide (see Figure 3.17), have been widely investigated in recent years for their use as biodegradable materials[69]. This synthetic polymer has been explored for use in such varied fields as drug delivery systems[78], as a temporary scaffold for tissue restructuring[79] and to improve the physical properties of materials employed in bone replacements[80].



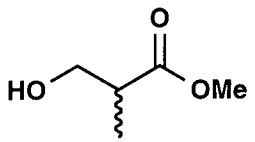
poly (*L*-lactide)



poly (diethyl 3-hydroxyglutarate)

Figure 3.17. *Poly (L-lactide) and the hyperbranched poly (diethyl 3-hydroxy glutarate).*

Ti(OBu)4 has been used in polymerisations reactions to synthesise hyperbranched polymers such as poly (diethyl 3-hydroxyglutarate) (see Figure 3.16). The reaction vessel was heated in an oil bath up to 125 °C, and the reaction allowed to proceed for up to six hours. A vacuum was applied to the reaction vessel for the last 30 minutes to aid the removal of the ethanol[81]. This catalyst has also been employed to polymerise the enantiomers of methyl β-hydroxyisobutyrate (51)[69]. The optimal temperature for this reaction was 150 °C, with a significant drop in mass if the reaction was not performed at the optimal temperature. These transesterification reactions used 0.05 molar equivalents of the titanium catalyst[69].



(*R*) and (*S*) methyl β hydroxybutyrate
(51)

To compare the molecular weights and polydispersities of the enzyme catalysed polymerisations with a synthetic polymerisation, various transesterification reactions were performed. The synthetic method used neat methyl and ethyl esters of hydroxy acids, with the catalyst, Ti(OBu)4. The reactions were performed under a constant flow of nitrogen, and at the end of some of the reactions the nitrogen flow was shut off and a vacuum applied to drive the reaction to higher molecular weight by removing the alcohol generated. The reactions were performed at high temperatures, being initiated at 100 °C, with the temperature steadily increased upto 150 °C at a rate of 10 °C per minute. Figure 3.18 shows an illustration of the equipment used for these reactions.

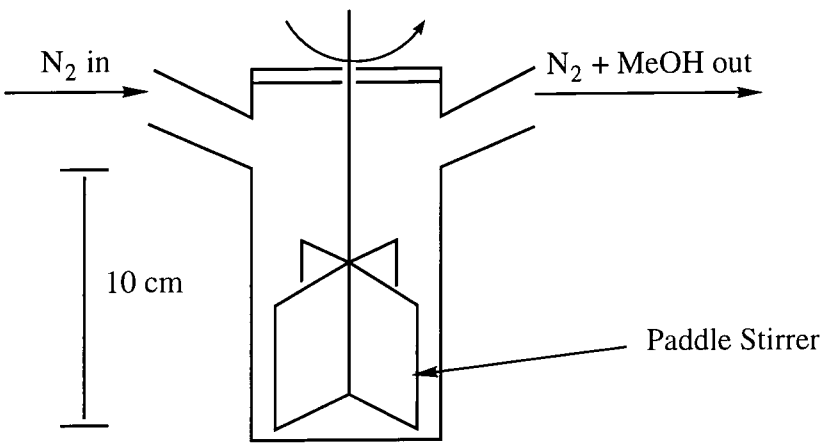
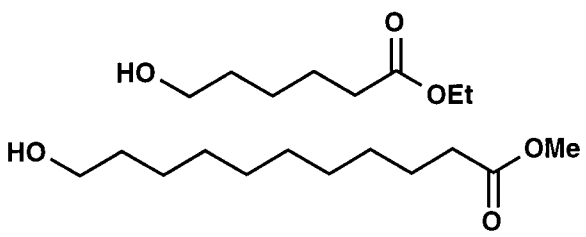


Figure 3.18. *The equipment used in the transesterification reactions using Ti(OBu)4 as the catalyst*[69].



ethyl 6-hydroxyhexanoate
(63)

methyl 11-hydroxyundecanoate
(64)

The GPC traces of the transesterification materials show very uniform profiles (see Figure 3.19). The molecular weights (M_W) vary between 2700 and 7100, and the polydispersity ranges from 1.7 to 2.6. Several of the GPC traces show a "stepping" pattern on the lower mass edge of the peak. This pattern can be attributed to individual oligomeric units, ie, dimer, trimer, *etc*, until the peaks can no longer be resolved. The stepping pattern is less pronounced when the vacuum has been applied to the system, and indeed the lower mass units are not as prevalent.

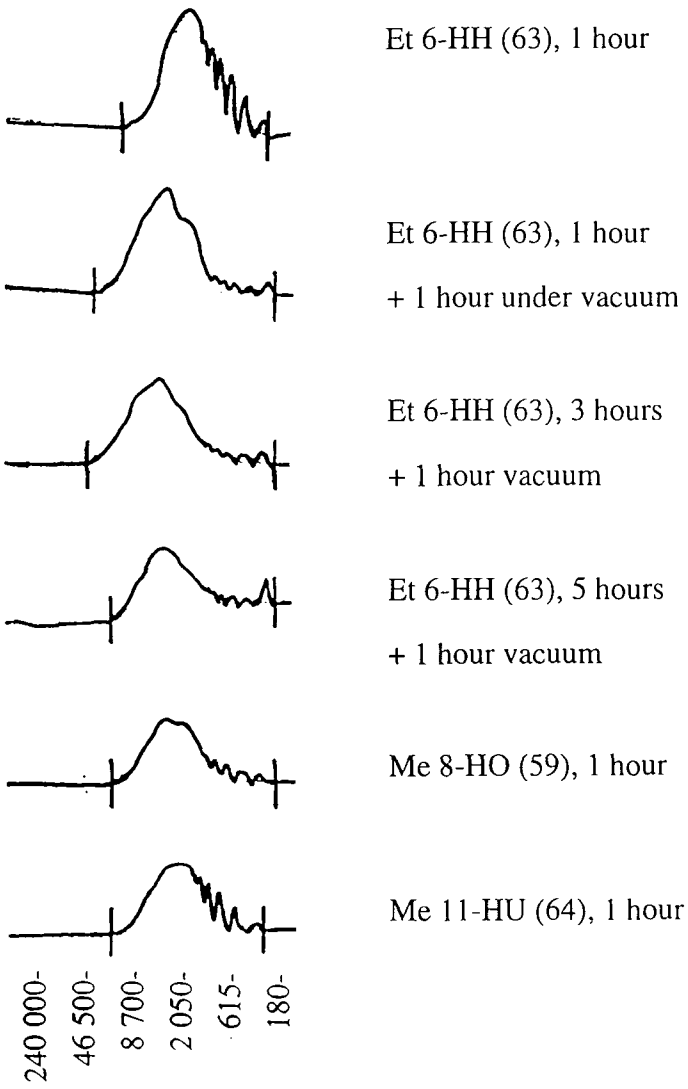


Figure 3.19. *GPC traces from the Ti(OBu)4 mediated polymerisations.*

Table 3.1 gives the data for the progress of the Ti(OBu)4 mediated polymerisations for the three substrates, ethyl 6-hydroxyhexanoate (Et 6-HH) (63), methyl 8-hydroxyoctanoate (Me 8-HO) (59), and methyl 11-hydoxyundecanoate (Me 11-HU) (64), employed in the polymerisations.

Material	Time /h	Vacuum /h	Mw	PDI
Et 6-HH	1	-	2760	1.71
Et 6-HH	1	1	5833	2.14
Et 6-HH	3	1	7008	2.57
Et 6-HH	5	1	5496	2.60
Me 8-HO	1	-	4693	2.01
Me 11-HU	1	-	4695	1.86
Me 12-HS	1	-	3959	1.71
Me 12-HS	6	-	5070	1.65
Me 12-HS	4	4	4125	1.68

Table 3.1. *Mass (M_W) against time in the Ti(OBu)4 transesterification experiments.*

Time /h is the time each experiment was placed under nitrogen; Vacuum /h is the time each experiment was placed under high vacuum.

Figure 3.20 is a graphical representation showing a comparison of two enzymatic polymerisations, performed without molecular sieves, with a Ti(OBu)4 mediated polymerisation of Et 6-HH (63). The Ti(OBu)4 polymerisation proceeds at a rate similar to the enzymatic polymerisation over the early part of the experiment, with similar molecular weights (M_W) at comparable stages of the reactions. This again illustrates how efficient the lipase is as a polymerisation catalyst, in that it competes favourably with the rate of the chemical process.

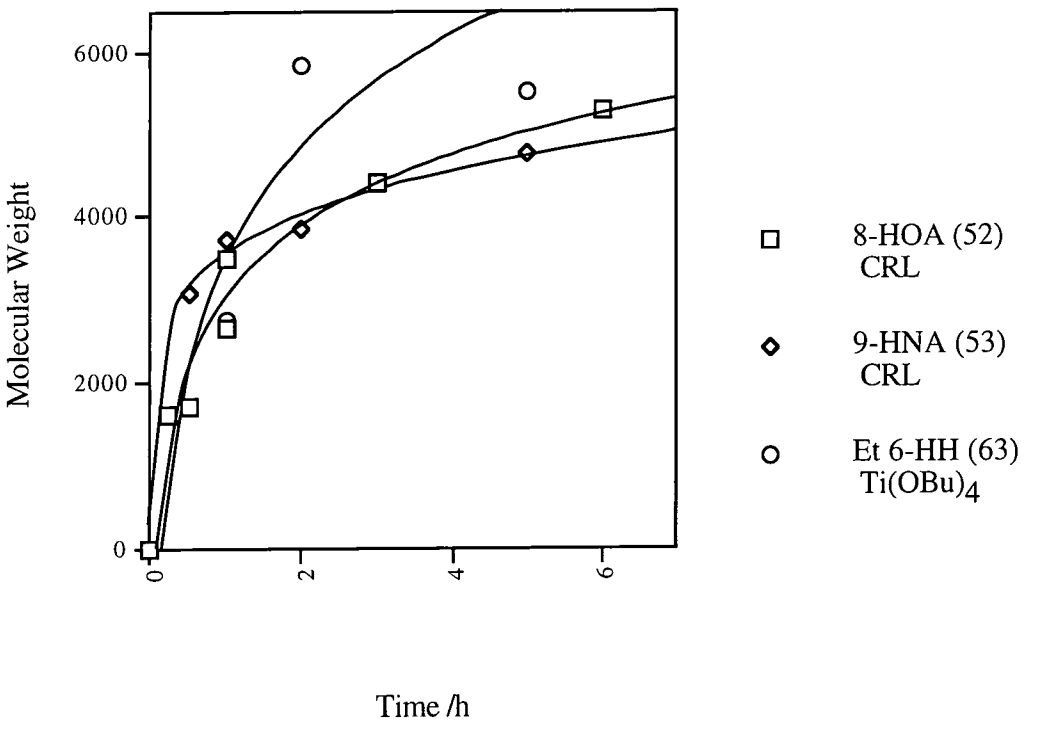


Figure 3.20. *Comparison of the molecular weight (M_W) build up for the polymers from an enzymatic reaction and a transesterification reaction.*

From Figure 3.19 it can be seen that the GPC profiles for all of the reactions catalysed by Ti(OBu)4 show a bell shaped curve, with a stepping pattern on the low molecular weight side of the curve, which has been attributed to the individual oligomer peaks . This profile is very similar to the four GPC traces of the polymer from the enzymatic reaction, which are shown in Figure 3.21, with polydispersities varying from 1.8 to 4.2. These four traces do not, however, show the stepping pattern observed in the Ti(OBu)4 catalysed reaction of Figure 3.19. This is attributed to the enzymatic preference for the esterification of the monomeric over oligomeric material, thus all monomer and low oligomer is consumed. On the other hand in the Ti(OBu)4 polymerisation, a much more classical stepgrowth assembly operates, with a statistical distribution of species including low molecular weight oligomer.

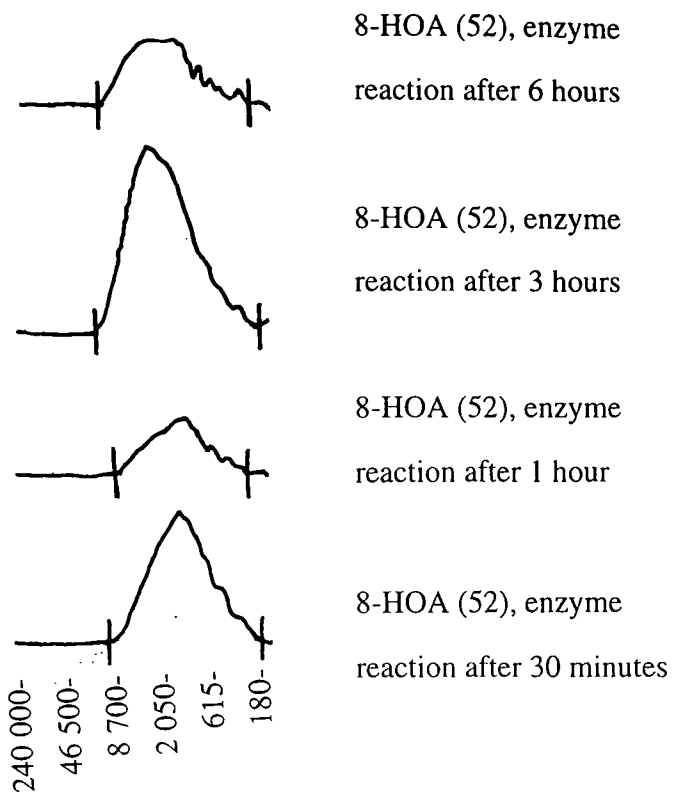


Figure 3.21. *Four GPC traces for the enzymatic polymerisation of 8-HOA (52), from which the data in Figure 3.20 is derived.*

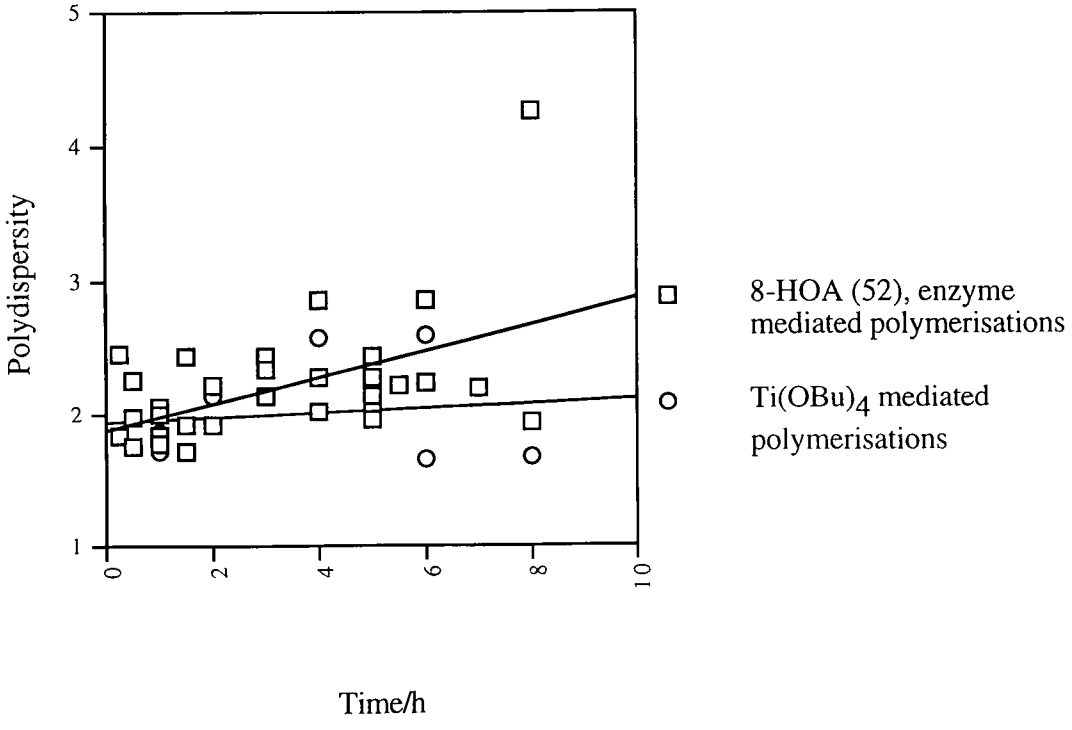


Figure 3.22. *Comparison of the polydispersities for the enzymatic and Ti(OBu)4 mediated polymerisations.*

Figure 3.22 shows a graphical representation of the polydispersities of the enzymatic and Ti(OBu)4 mediated polymerisations over eight hours. Figure 3.22 shows that the range of polydispersities is larger for the enzymatic reactions, and that the average polydispersity increases with time, whereas that of the Ti(OBu)4 reactions is maintained with an average value close to two.

	Enzymatic reactions	Ti(OBu)4 reactions
Polydispersity	1.8 - 4.2	1.6 - 2.6
Molecular weights (M_W)	similar	
Relative rates	similar	

Table 3.2. *A comparison of the enzymatic and Ti(OBu)4 mediated polymerisation reactions.*

The enzymatic reaction has a slightly larger polydispersity range than the Ti(OBu)4 mediated polymerisations, but does compare very well overall with the inorganic catalyst in terms of the relative rates of the reactions and the molecular weights (M_W) achieved. This can be seen in Table 3.2.

3.6.2: Polymer assembly followed by MALDITOF analysis.

A sample of the Ti(OBu)4 mediated polymerisation from Et 6-HH (63) after one hour, was subjected for analysis by MALDITOF MS (see Figure 3.24). The spectrum has been taken above a mass cut-off of 600, to exclude peaks from the dihydroxybenzoic acid matrix, in which the sample was dissolved before analysis. The repeat unit for this polymer has a mass of 114, and this is readily identifiable from the peak masses. An interesting phenomenon obvious in this spectrum is the presence of two peaks for each monomer step. This arises as each oligomer has an end group, which is either the ethyl group, from the starting material, or a butyl group, which has

transesterified from the catalyst (see Figure 3.23). Each oligomer has a counter-ion, usually sodium or potassium, which is derived from the matrix. For example, in Figure 3.24, the peak at 890.3 is the heptamer, with the butyl end group and a potassium counter-ion, whereas the peak at 861.4 is the heptamer with the ethyl end group, and the potassium ion.

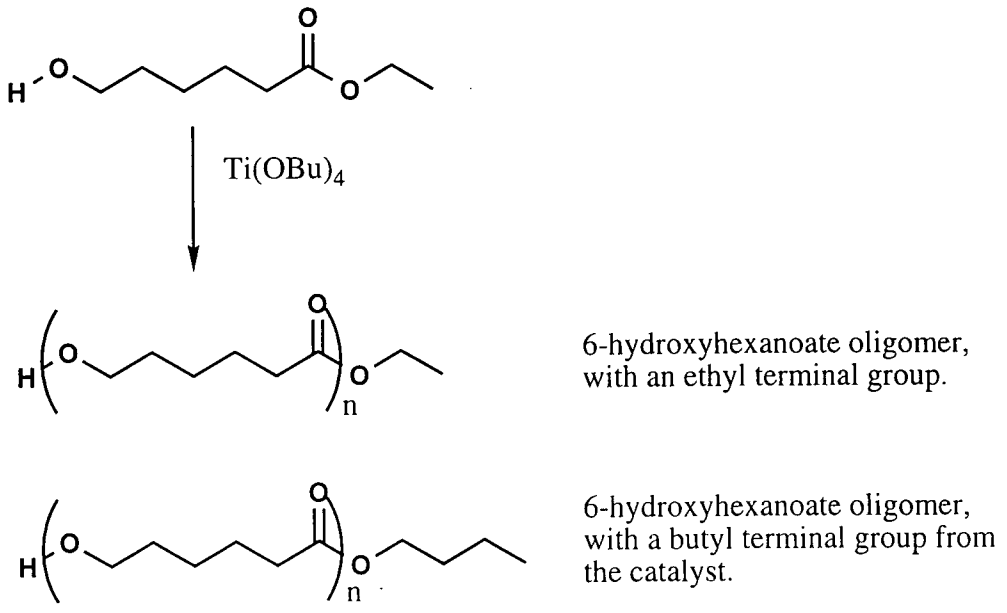


Figure 3.23. *Oligomer formation using Ti(OBu)4 as the catalyst, with ethyl or butyl terminal groups.*

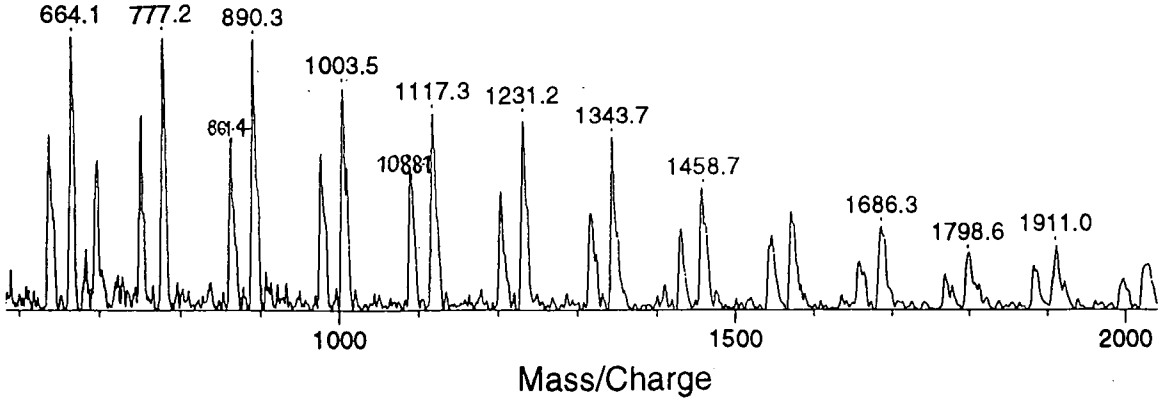


Figure 3.24. *MALDITOF MS spectrum of Ti(OBu)4 catalysed polymerisation product of Et 6-HH (63) after one hour.*

3.6.3: Polyesterification reactions using secondary alcohols.

All of the monomers used in the Ti(OBu)4 catalysed polymerisations were achiral primary alcohols. A chiral secondary alcohol presents the possibility of differences ocurring in the properties between the racmic and the enantiomerically pure materials. For example, it is known that polymers synthesised from racemates usually have a lower level of crystallinity relative to polymers synthesised from enantiomerically pure monomers[70]. This is shown in Figure 3.25 for methacrylate derivatives.

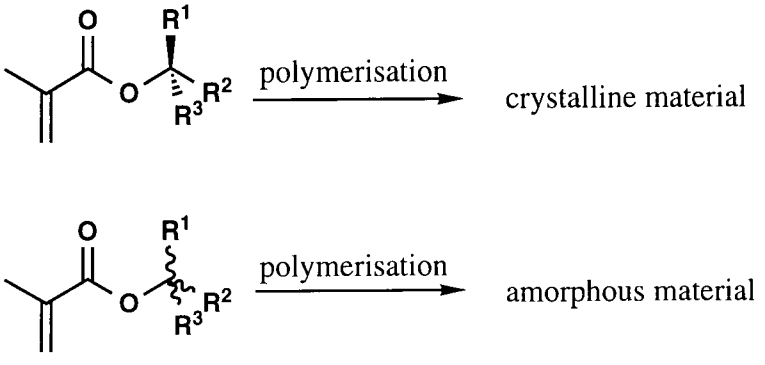


Figure 3.25. *Polymers synthesised from enantiomerically pure and racemic materials can have different physical properties.*

Thus an investigation into the physical properties of polymers synthesised from chiral secondary alcohols was undertaken. (*R*)-Ricinoleic acid, which is derived from castor oil, is used commercially in the synthesis of (*R*)-12-hydroxystearic acid (commercially available). Esterification of this acid generated enantiomerically pure methyl (*R*)-12-hydroxystearate [Me (*R*)-12-HS] (65) (see section 5.7.1). The racemate of this material was also synthesised by oxidation of the enantiomerically pure material to the ketone and reduction to the alcohol (see Figure 3.26). Thus the physical properties of the polymers derived from the racemate and the enantiomerically pure material could be compared. Interestingly the secondary hydroxyl group did not appear to suppress polymerisation under Ti(OBu)4 catalysis, and similar masses were achieved for this monomer in comparison to the polymers derived from the primary hydroxy

esters (see Figure 3.27).

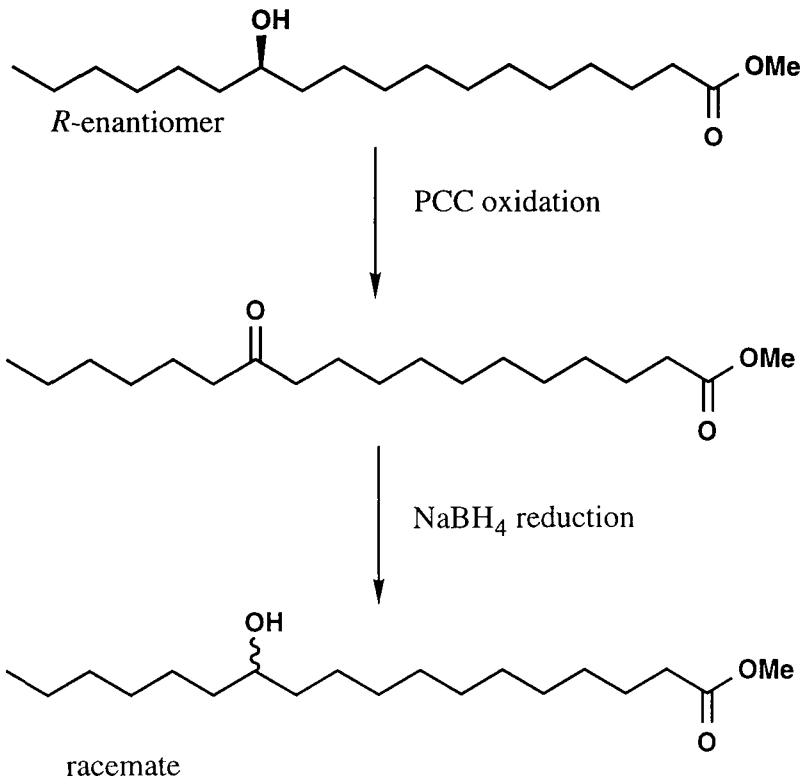


Figure 3.26. *Racemisation of Me (R)-12-HS (65) by oxidation and reduction.*

Both the enantiomerically pure and racemic polymers which were synthesised in the reactions were oils, and differed from the products of the shorter chain primary alcohol esters, which were all solids at room temperature. Polymer samples of racemic Me 12-HS (65) and (*R*)-(65) were analysed by differential scanning calorimetry (DSC), to probe the phase behaviour of the polymer in the liquid-crystalline state. However, no clear phase transitions were observed in each case.

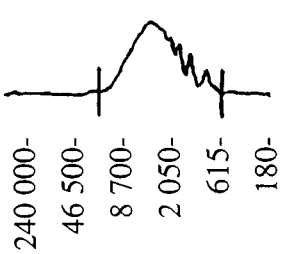
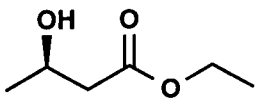


Figure 3.26. *GPC trace of a polymer from Me* (R)*-12-HS,* (R)*-(65) with a secondary alcohol after three hours.*



(*R*)-ethyl
3-hydroxy butyrate
(66)

The transesterification reaction was then repeated with ethyl 3-hydroxybutyrate (Et 3-HB) (66), with both the *(R)*-enantiomer, and the racemate. Polymerisation of these materials differed leading to a low melting solid for the (*R*)-enantiomer, but an oil for the racemate.

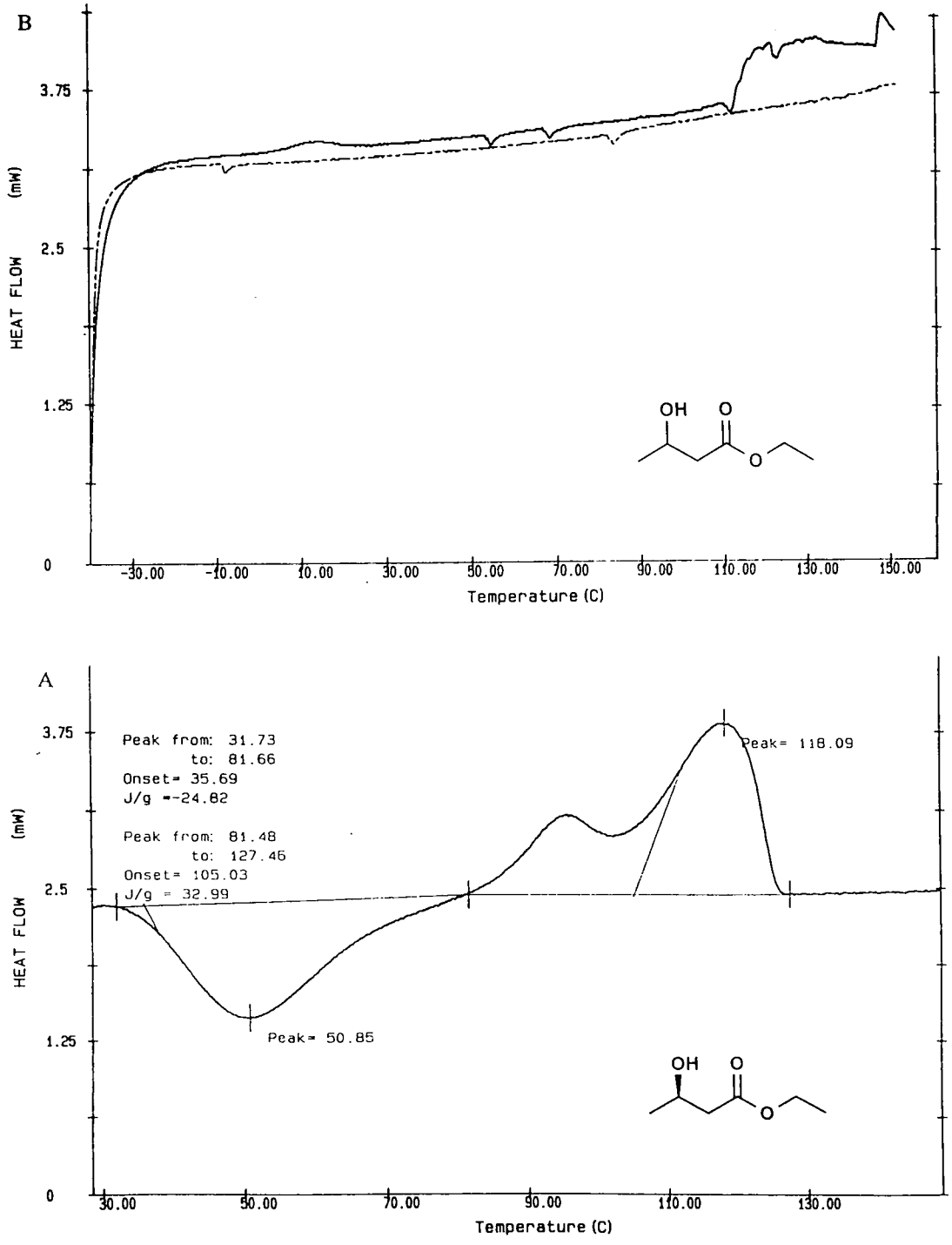


Figure 3.28. *DSC traces of the polymers synthesised from racemic Et 3-HB (66) trace A, and from Et* (R)*-3-HB* (R)*-(66) trace B.*

The racemic mixture showed no phase transitions between -30 °C and 150 °C, and was thus essentially an amorphous material (melting point -22.35 °C). The polymer made from the optically pure material did show a phase transition, with an endothermic reorganisation followed by a melt at 114.70 °C. There was an exothermic crystallisation, shown by the negative peak at 36.75 °C (see Figure 3.28).

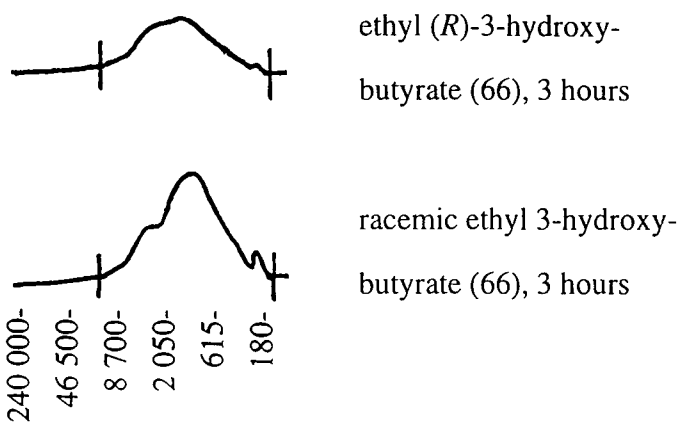


Figure 3.29. *GPC traces of Et 3-HB (66) after the transesterfication reactions.*

Figure 3.29 shows the GPC traces for the polymers from the enantiomerically pure and racemic starting materials. Both traces show similar profiles to the other polymeric materials synthesised using the Ti(OBu)4 catalyst, and analysed by GPC. Thus neither the short chain length nor the secondary alcohol had a significant effect on the polymerisation process.

3.6.4: Discussion of the polymerisations performed using Ti(OBu)4.

The highest masses generated by the Ti(OBu)4 mediated transesterification were approximately 20 000. This can be compared to 35 000-40 000 for the enzyme-catalysed reaction over the same time period. These values did not change even after high vacuum was applied to the system. The distinct stepping pattern which was observed on the lower molecular weight edge of the GPC traces and attributed to

individual oligomers was not apparent at all in the enzymatic polymerisations of ω-hydroxy acids (see section 3.6.2). The polymerisation with $Ti(OBu)_4$ follows a step growth assembly. The enzymatic polymerisation tends towards a step growth assembly with time, but in the earlier stages this is a clear tendancy towards monomer consumption, with similar relative rates and molecular weights to the $Ti(OBu)_4$ polymerisation.

The polymer of racemic ethyl 3-hydroxybutyrate (66) was judged to be amorphous. However, when the enantiomerically pure material was polymerised the material showed clear phase transitions after DSC analysis, suggesting a much more crystalline material.

3.7: Summary.

This chapter has desribed the progress of the enzyme catalysed polymerisation reaction with ω-hydroxy acids, without the use of molecular sieves. 8-HOA (52) and 9-HNA (53) achieved high molecular weights most rapidly. The rate profile for these reactions showed two phases of assembly, an initial phase in which all of the monomer was consumed, and a slower phase in which the oligomers were condensed to form polymers. These were followed after several days by a competitve process which hydrolysed the polymeric chains.

The predominant product of the esterification of 16-HHDA (55) is known to be a dimeric lactone[97]. This molecule was observed in the GPC trace of the polymer formed from this monomer. The lipase catalysed polymerisation of analogues of ω-hydroxy acids and various derivatives was also investigated, and the results of these experiments were compared to the results of the ω-hydroxy acids polymerisations. However, generally these analogues were poor substrates for polymerisation.

An experiment was performed which was designed to determine the sequence of polymer assembly by incorporating deuterium labelled monomers into the polymerisation reaction. This showed that the new monomeric material was polymerised onto other fresh monomers, rather than the existing oligomeric chains.

The progress of the ω-hydroxy acid polymerisations were compared to the ring opening polymerisation of undecanolide (46). The polymerisation of the lactone achieved high mass, but was not nearly as rapid as the ω-hydroxyacid polymerisations.

The enzyme catalysed polymerisation was then compared to the polymerisations of ω-hydroxyesters using the non-enzymatic catalyst, $Ti(OBu)_4$. These polymerisations had similar initial rates to the enzyme catalysed reactions of ω-hydroxyacids, with the GPC traces all showing similar profiles. An investigation into the polymerisation of substrates with secondary alcohols was also undertaken using the $Ti(OBu)_4$. Ethyl 3-hydroxybutyrate (66) was polymerised as both the racemate and in enantiomerically pure (*R*) form, which gave an oil and a solid respectively on polymerisation. Thses polymers showed different characteristics when analysed by DSC.

CHAPTER FOUR

ACTIVE SITE STUDIES

4.0: ACTIVE SITE STUDIES.

4.1: Introduction and aims

The structure of the *Candida rugosa* lipase has been determined at 2.06 Å resolution by Cygler in 1993[11]. This study revealed that the active lipase exists in an open conformation, in which the active site is accessible to the solvent. The flap, which covers the active site in the closed conformation, opens almost perpendicular to the protein surface, exposing the binding site. The key catalytic residue, serine 209, is situated at the bottom of the exposed region, which consists almost entirely of hydrophobic residues (both aliphatic and aromatic). There are only two hydrophilic residues in the vicinity of the active site, glutamic acid 208 and serine 450, and these play a strategic role in hydrogen bonding to the carbonyl groups of the triglyceride substrate.

Histidine 449 and glutamic acid 341 are adjacent to serine 209, and together these three residues comprise the catalytic triad. Several residues in the vicinity of the active site triad help to stabilise the acyl-enzyme intermediate which is formed during the hydrolytic process. The NH atoms of glycine 123, glycine 124 and alanine 210 all form hydrogen bonds with the acyl-enzyme intermediate, as shown in Figure 4.1.

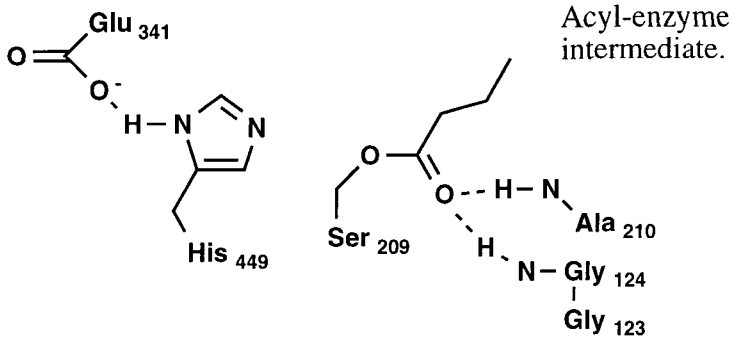
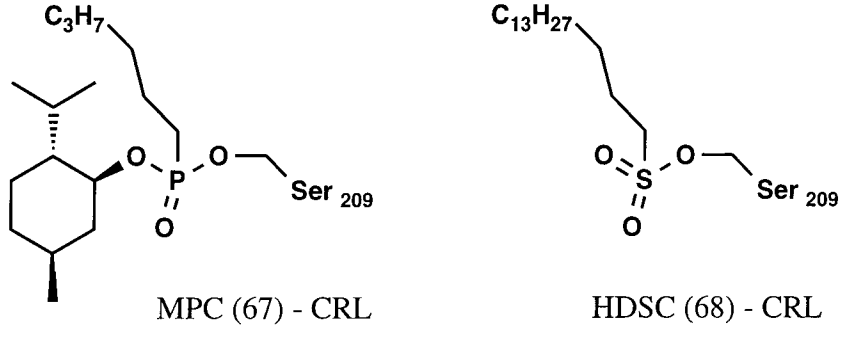


Figure 4.1. *The acyl-enzyme intermediate and the active site residues of CRL. The hydrogen bonding between the substrate and the residues Gly 123, Gly 124 and Ala 210 is also shown.*

The inhibitors *O*-menthyl hexylphosphonochloridate (MPC) (67) and hexadecanesulphonyl chloride (HDSC) (68) (shown in Figure 4.2 bound to the serine 209) were employed in the X-ray study of the enzyme[12,20]. The phosphorus or sulphur atoms became covalently bound to serine 209 through oxygen and the hydrophobic chain was located in a hydrophobic tunnel[12].



MPC (67) - CRL HDSC (68) - CRL

Figure 4.2. *MPC (67) and HDSC (68) bound to serine 209[12,20].*

These studies[20] demonstrated that there is a narrow, hydrophobic tunnel within the enzyme, into which the scissile fatty acyl chain can enter. The tunnel extends towards the centre of the lipase, then bends towards the protein surface, forming an "L" shape. There is minimal conformational change in the positions of the amino acid side chains lining the tunnel when the substrates enter, and the tunnel can accommodate fatty acids of up to 18 carbons in length.

It was observed for HDSC (68) that the hydrophobic chain adopted predominantly anti-zig-zag conformations, with three *gauche* conformations to allow the molecule to bend round leucine 302, and head along the tunnel toward the surface of the protein. In the case of MPC (67) the chain was too short to reach the bend in the tunnel. The menthyl moiety of MPC (67) is bound in a crevice above the catalytic site, which is created in the open structure of CRL by movement of the flap.

In Chapter 3 it was demonstrated that polymerisation activity varied with the length of the monomer. 8-HOA (52) and 9-HNA (53) showed the most rapid relative

rates of assembly. With these studies in the recent literature, and this observation made concerning CRL and the length specificity earlier in this thesis, the objective of this chapter was to investigate how the monomer may bind to the enzyme. Specifically, the following points were addressed:

1) Evaluate the conformation of 9-HNA within the hydrophobic cavity using molecular modelling.

2) Design and investigation of affinity labels to try to inhibit polymerisation.

Further investigations are also discussed in this chapter relating to the mechanistic aspects of substrate binding to the enzyme, and the enantioselectivity of CRL.

4.2.1: Models of the active site.

Norin *et al.*[82] used molecular modelling to investigate the substrate-binding regions of three lipases (including CRL). They concluded that there were two different components for binding, the first of which showed high affinity for aliphatic groups (the binding site for fatty acid chains) while the second site was a hydrophilic region. The authors claimed that this latter region should be important for the hydrolytic activity of the enzyme. Linko[46] used molecular dynamics simulations and electrostatic potential calculations to find an energy minimised lipase-substrate complex for the *Rhizomucor miehei* lipase with sebacic acid derivatives (AA-monomers) as the substrates. Hydrogen bonds were established within the complex, with the polarity of the active site amino acids assisting the orientation of the substrates, and the surface electrostatic potential of the complex helping the lipase to orientate itself for interfacial activation.

From the results outlined in Chapter 3 the enzyme catalysed polyesterification using CRL was most efficient in the early stages of the polymerisation, with 8-HOA (52) or 9-HNA (53) as the monomer. From Cygler's studies[8,11,12,20] it was anticipated that the monomer would be accommodated within the active site tunnel which accepts the hydrocarbon chain. Alternatively however, the ω-hydroxyl end

group may be refused entry into the hydrophobic tunnel, and be located only at the tunnel entrance. From our own studies we can conclude that a rapid assembly of oligomer occurs in the early stages of reaction until the monomer is consumed. The reaction then slows down, which suggests a specificity of binding of the monomer over oligomer. Thus it was an objective of this study to establish if the monomer could be located within the hydrophobic tunnel as shown schematically in Figure 4.3.

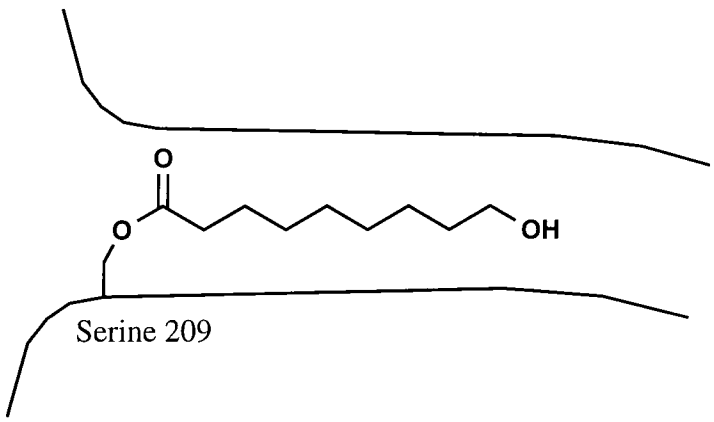


Figure 4.3. *9-HNA (53) in the hydrophobic cavity of CRL?*

4.2.2: Computational studies

The X-ray coordinates of the *Candida rugosa* lipase deposited in the Brookhaven Protein Database were were used to locate the hydrophobic cavity by establishing the solvent accessible surface of the enzyme. The coordinates which were used were taken from Cygler's study[20] of the open conformation of CRL, with the phosphate inhibitor MPC (67) covalently bound to serine 209.

These studies were performed computationally by creating a sphere with a diameter of 2.0 Å to represent a water molecule, and allowing this molecule to roll around the surface of the protein. The resultant profile, which is a measure of the ability of this small (solvent) molecule to penetrate into the protein, revealed the active site and the hydrophobic tunnel. A photograph of the solvent accessible surface of the protein is shown in Figure 4.4. In this photograph it can be seen that there are mainly hydrophobic residues in the tunnel beyond the active site residues. There are only two

hydrophilic residues in the region of the tunnel, the catalytic residue, serine 209 is highlighted in yellow, and serine 247 is highlighted in red. The cavity is composed entirely of hydrophobic residues, except for these two hydrophilic serines, which are 14.55 Å apart. It is intriguing that this distance of 14.55 Å is close to the length of the 9-HNA (53) monomer.

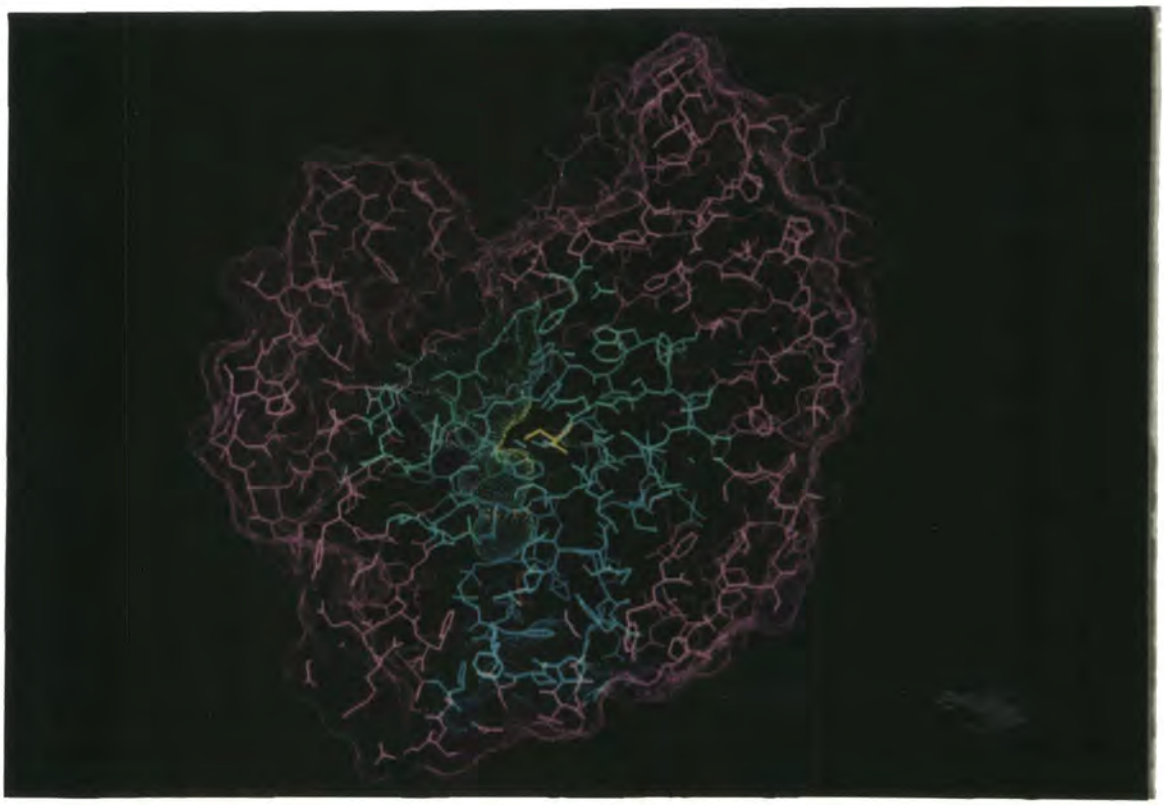


Figure 4.4. *Photographic representation of the solvent accessible surface in CRL.*

The hydrophobic tunnel is shown clearly in Figure 4.5. All of the green residues are within 6.0 Å of serine 209, which is coloured yellow. The angle represented in this Figure shows the entrance to the cavity and looks down the length of the cavity.

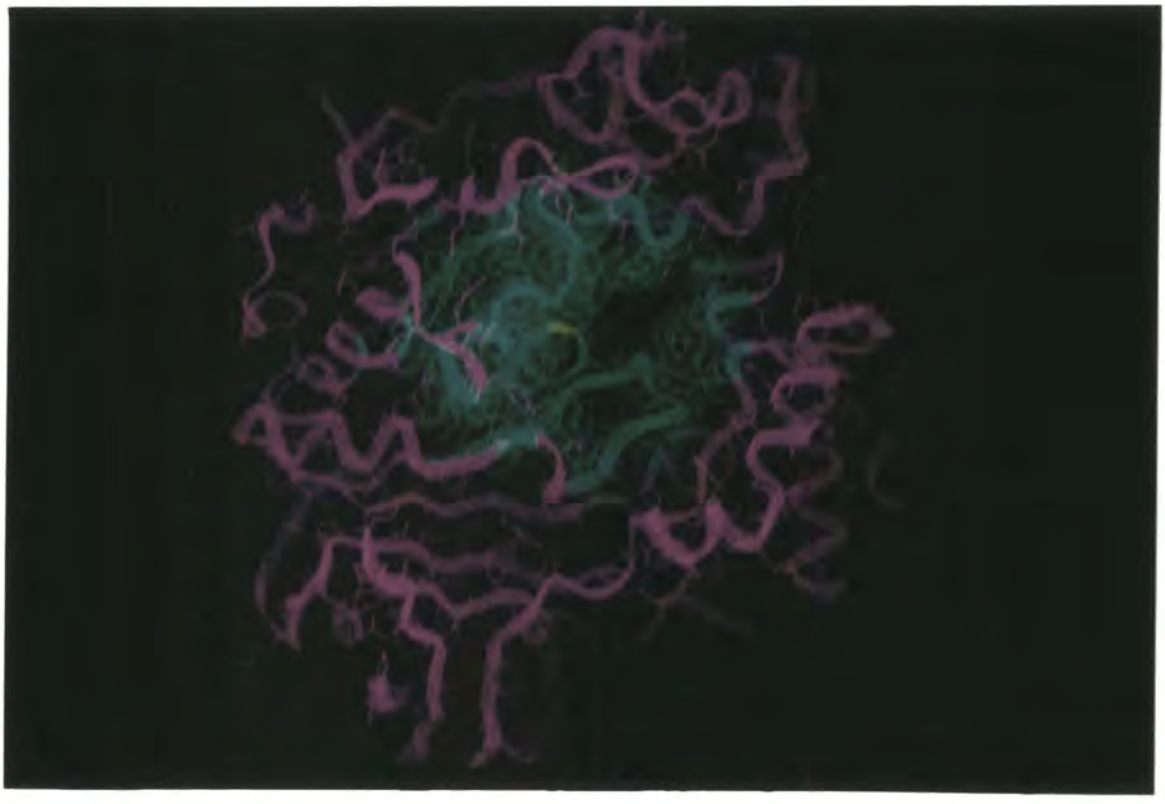


Figure 4.5. *Ribbon view of CRL, with the residues within 6.0 Å of serine 209 highlighted in green.*

Following this experiment a molecule of 9-HNA (53) was covalently attached to the serine 209 residue to simulate the acylated enzyme intermediate. The hydroxyl end group of the chain was then located within the cavity. A computational programme (using software programmes from Biosym/MSI of San Diego) was used to find a minimum energy conformation for the entire chain. This required that the conformational energy be minimised with respect to the atoms of the amino acid residues which formed the cavity, both for steric and electrostatic interactions, and with respect to the other carbon atoms in the chain, so that unfavourable conformations for the chain were disgarded.

The result of these studies was the generation of a conformation of 9-HNA (53) bound to the active site, with eight of the C-C bonds adopting a *trans* antiparallel

conformation, and C_8 adopting a *gauche* conformation to avoid coming into contact (within 2.0 Å) with the lining of the cavity.

Remarkably the hydroxyl group approached within hydrogen bonding distance of the hydroxyl group of serine 247. The distance measured of 2.66 Å is the O - O distance between the oxygens of serine 247 and the terminal hydroxyl group of the monomer. The H...O distance is therefore approximately 1.66 Å and is an acceptable distance for the formation of a hydrogen bond (O-H...O). The hydroxyl group of the substrate was able to approach even more closely to the serine hydroxyl, in certain conformations. However, these conformations were of high energy and were thus disregarded. Figure 4.6 shows 9-HNA (53) (in yellow) in the hydrophobic tunnel, bound to serine 209 (in pink) and coming within 2.66 Å of serine 247 (also in pink).

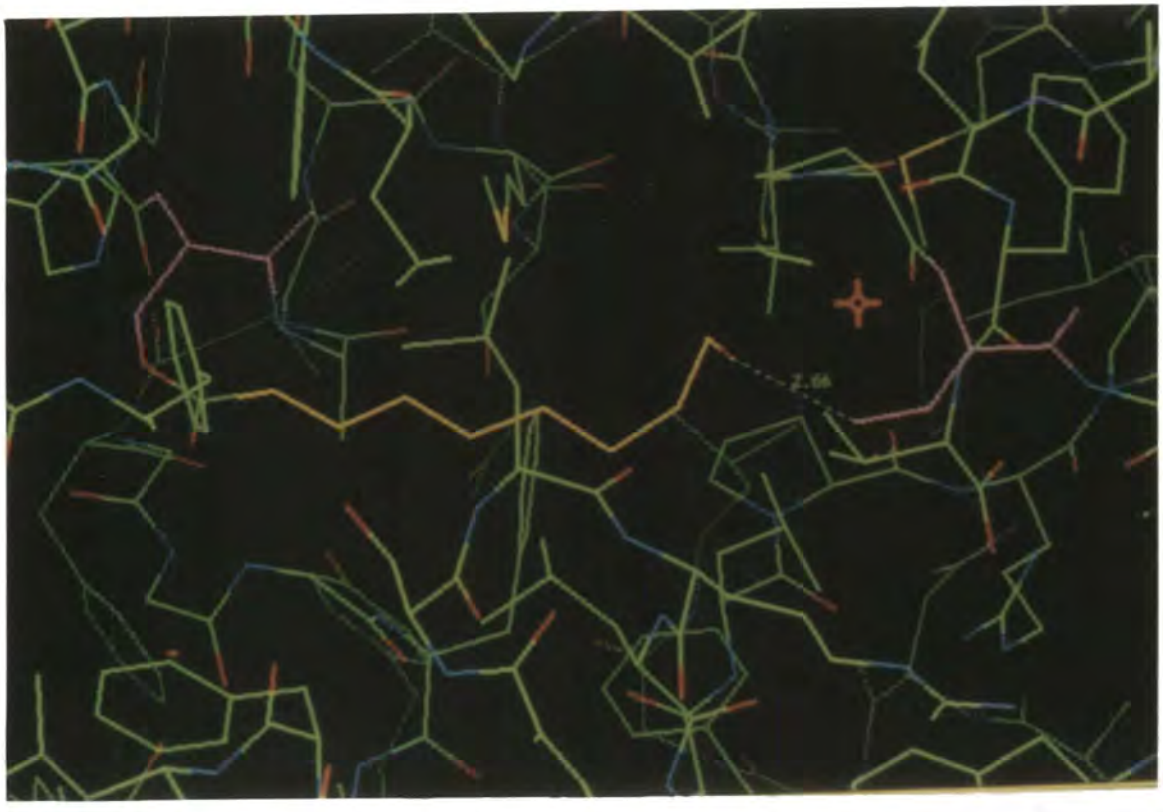


Figure 4.6. *The distance (2.66 Å) between the terminal hydroxyl group of 9-HNA (53) and the hydroxyl group of serine 247.*

The exact location of the serine 247 hydroxyl hydrogen atom is unknown. The hydrogen atoms are not resolved by X-ray analysis, and their locations are simulated. Therefore it is not clear where the hydroxyl hydrogen of serine 247 is located in this structure. Either the hydrogen of the serine residue is free to take part in hydrogen bonding to the substrate oxygen, or the substrate hydrogen forms a hydrogen bond to the oxygen of serine 247. During the molecular modelling simulations[46] hydrogen bonds within a system (enzyme) are not destroyed. Thus it appears reasonable that it is the hydrogen of the substrate which forms the hydrogen bond to the oxygen of the serine residue. Furthermore, this would allow the hydrogen of the serine hydroxyl group to bond quite reasonably to another hydrogen bond acceptor atom within the active site cavity. Figure 4.7 shows a schematic representation of 9-HNA (53) bound within the active site, and the hydrogen bond formed between serine 247 and the terminal hydroxyl group.

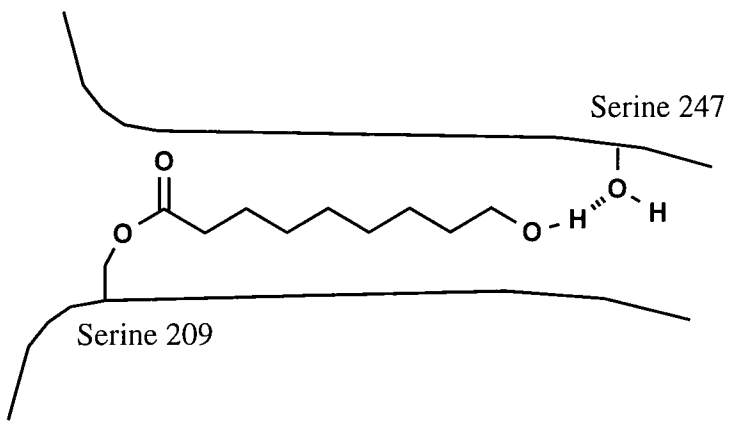


Figure 4.7. *The proposed role of serine 247 in hydrogen bonding to the hydroxyl group of 9-HNA (53).*

For these calculations the atoms of the enzyme remain as a fixed set of points derived from the X-ray coordinates. However, the enzyme does have significant conformational flexibility, and these dynamics may realise transient conformations which allow the substrate to approach more closely to the serine hydroxyl group. Thus during binding the serine residue could perhaps form a stronger hydrogen bond to this

hydroxyl group.

The observation that there was a serine residue at such a location in the hydrophobic tunnel, coupled with the study locating 9-HNA (53) in the cavity, suggested that the hydroxyl group of this serine could hydrogen bond with the hydroxyl group of 9-HNA (53). This clearly could stabilise 9-HNA (53) and accommodate its binding during the esterification process, leading to the rapid initial polymerisations which have been observed experimentally.

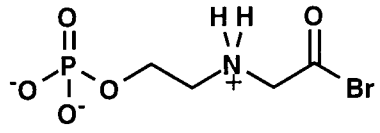
4.2.3: Discussion on length specificity aspects of polymerisation.

9-HNA (53) and 8-HOA (52) undergo enzyme catalysed polymerisation at similarly rapid rates (see section 3.2.1). The assembly of the polymers from these monomers is more rapid relative to the longer chain lengths which were studied. One explanation for this observation is that the terminal hydroxyl group of these chains forms a hydrogen bond to the hydroxyl group of serine 247 on binding, and that this stabilisation increased the initial rate of polymerisation.

However, if 9-HNA (53) and 8-HOA (52) come within hydrogen bonding distance of the hydroxyl group of serine 247, which would account for the parity in the progress of their esterification reactions, then the 10-, 11- and 12-ω-hydroxy acids should react at a similar but slow rate, since no such specific bonding can be expected in these cases. 10-HDA (42) reacts faster than both of the longer chains (which polymerise at essentially the same rate), and the assembly of the 16-HHDA (55) derived polymer has the slowest relative rate of all six systems. Since this is the case we must also consider the possibility of a surface interaction which governs the esterification reactions rather than a process governed by the substrate entering into the active site cavity as described above.

4.3.1: Affinity labelling.

There are many reports[83] in the literature describing the use of affinity labels to deactivate an enzyme by covalent modification of a particular amino acid residue. This can happen in two ways. Either modification of a residue within the active site (competitive inhibition), or alternatively modification of a residue remote from the active site, but one whereby the modification prevents the enzyme from performing its catalytic function (non-competitive inhibition). For example, N-bromoacetylethanol-amine phosphate (BAEP) (69) has been used to competitively inhibit fructose kinase enzymes. The inhibition was shown to be irreversible, and dependant upon the concentration of the inhibitor.



(69)

On the basis of the binding hypothesis which emerged after the computational fitting of 9-HNA (53) into CRL, a range of affinity labels were designed as candidates to inhibit the polymerisation of 9-HNA (53). The use of various brominated substrates was anticipated to covalently modify the hydroxyl group of serine 247. An ω-bromo acid incorporates a good leaving group onto the chain at a position optimal for nucleophilic attack by the hydroxyl oxygen of serine 247. If on binding these functional groups become close then they may react together. Thus such ω–bromo acids may covalently modify the protein and suppress catalytic activity (see Figure 4.8).

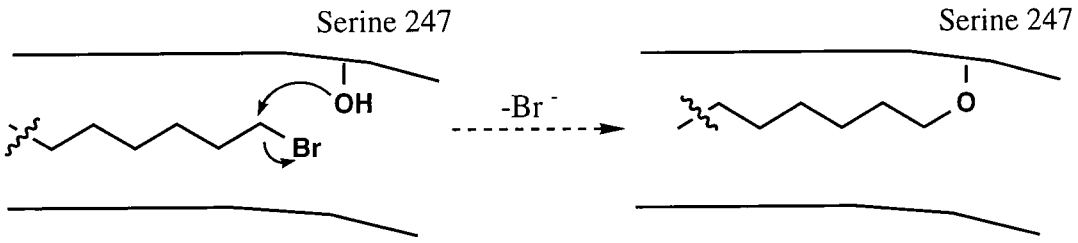
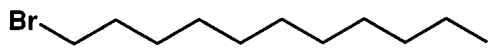
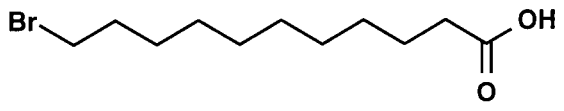


Figure 4.8. *Anticipated modification of serine 247 using an ω-bromo acid.*

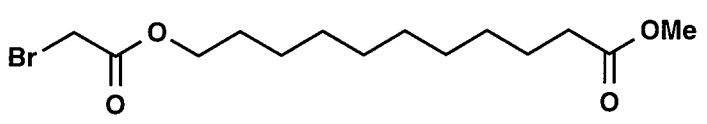
The target molecules for the affinity labelling experiments all incorporated a primary alkyl bromide. Initially 1-bromoundecane (1-BrU) (70) and 11-bromoundecanoic acid (11-BrUA) (71) were used to test the inhibition hypothesis. Subsequent experiments employed bromoacetoxy derivatives of ω-hydroxy methyl esters such as (72) and (73) as it was anticipated that the more electrophilic bromoacetoxy group would increase the efficiency of nucleophilic attack from serine 247 onto a more electropositive carbon. Figure 4.9 shows the candidate affinity labels which were used in this study. Molecules (69) and (70) were commercially available, however, compounds (71) and (72) had to be prepared, and their synthesis is discussed in section 5.10.1.



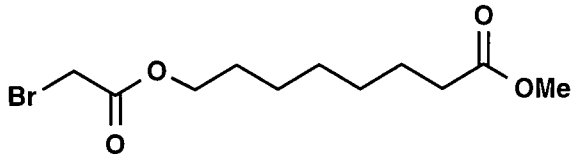
1-BrU (70)



11-BrUA (71)



Me 11-BrAcU (72)



Me 8-BrAcO (73)

Figure 4.9. *Candidate affinity labels for the inhibition reactions.*

An experiment was set up in which the enzyme was pre-incubated in hexane with 11-BrUA (71) (50 mg). After 24 hours 9-HNA (53) (200 mg) was added to the reaction. This was compared to a control reaction without 11-BrUA (71). Figure 4.10 shows the ratio of monomer to polymer through time (as judged by ^{1}H NMR) for these two reactions. From the graph it is clear that the monomer is consumed far quicker in the control experiment than in the reaction with 11-BrUA (71). Although this result

was encouraging it was inconclusive as there were several possible explanations for the differing rates of 9-HNA (53) consumption.

1) Irreversible inhibition was taking place in a specific manner.

2) Non-specific inhibition was taking place.

3) The inhibitor was involved in transesterification reactions with the monomer.

The third rationale considers the possibility that 11-BrUA (71) is a substrate forming an acyl enzyme intermediate. This species could then be attacked by an incoming 9-HNA (53) molecule, and form an ester. This would slow down the rate of polymerisation relative to the control, due to the subsequent absence of an attacking nucleophilic species.

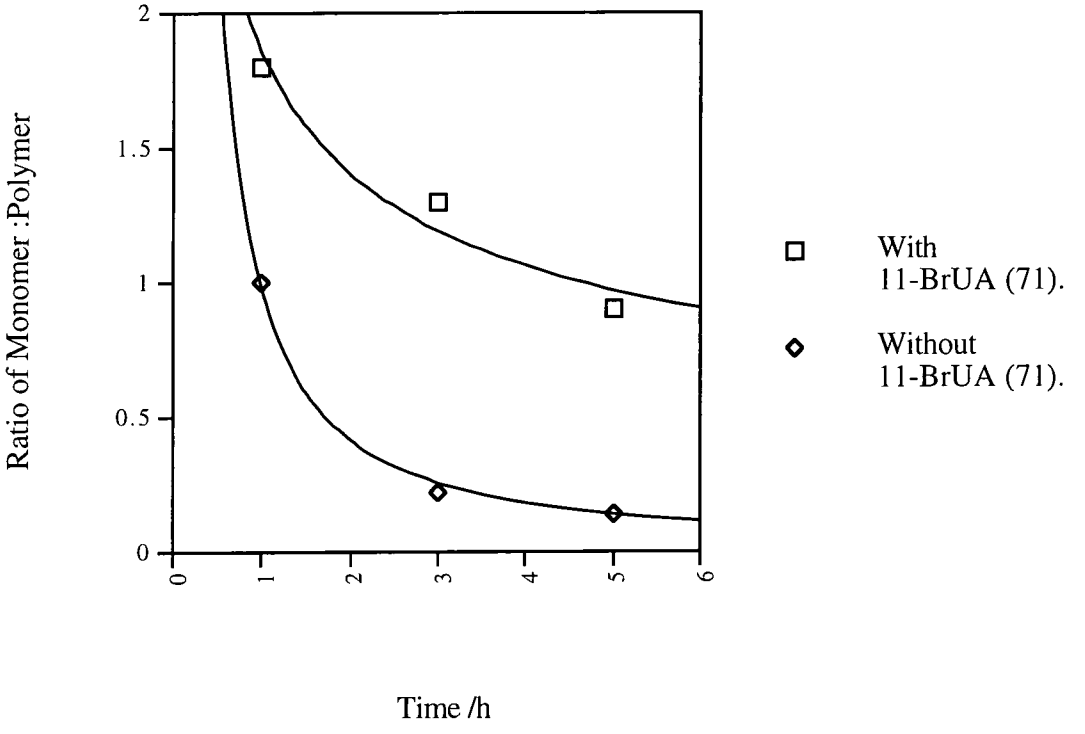


Figure 4.10. *The consumption of 9-HNA (53) as a ratio of monomer: polymer, with and without 11-BrUA (71).*

An experiment with 1-BrU (70) was judged appropriate to evaluate the system

further. Since there is no carboxylate group on (70) this molecule can not take part in esterification reactions, and should only act as an inhibitor if there is a covalent modification. Accordingly a reaction was conducted where the enzyme was incubated for 24 hours with 1-BrU (70) (50 mg) prior to adding 9-HNA (53) (200 mg). A control reaction was also conducted for direct comparison. Several experiments were performed with the alkyl bromide as the potential inhibitor. However, as judged by ^1H NMR the rate of monomer consumption varied, from comparable to the control, to slower than the control. Thus these results were not reproducible and this set of experiments was deemed inconclusive. In view of this the experiment was altered to study the effect of the inhibitor on the enzyme in a different, more amenable reaction, and this is described below in section 4.3.2.

4.3.2: Inhibition of transesterification reactions.

The candidate inhibitors were evaluated in transesterification reactions of 2,2,2-trichloroethyl butyrate (TCEB) (74) with 1-hexanol (75). Such transesterifications have been studied[28,84] previously in organic solvents, and the lipase is known to catalyse the formation of the hexyl butyrate (76) in a rapid reaction between TCEB (74) and 1-hexanol (75) (shown in Figure 4.11).

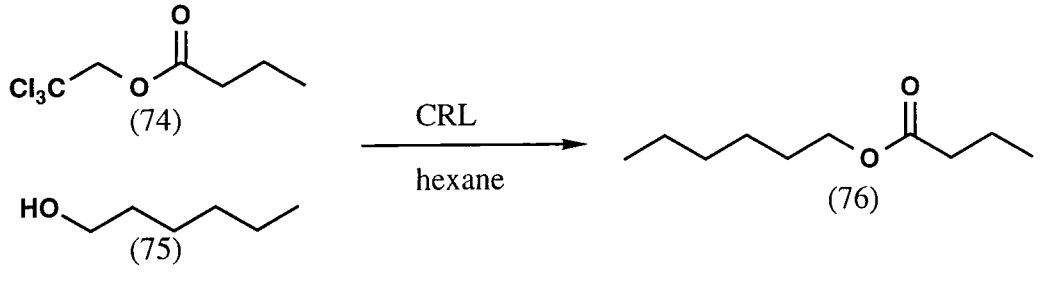


Figure 4.11. *Enzyme catalysed formation of hexyl butyrate (76) from TCEB (74) and 1-hexanol (75)[28,84].*

A control reaction was set up with CRL and equimolar quantites of TCEB (74) and 1-hexanol (75) in diethyl ether (5 ml). ^{t}-Butyl-benzene was added as an inert internal standard for quantitative analysis by GC. GC analysis was selected since it gave accurate ratios of the reagents and products, against the internal standard. Aliquots could be taken and stored (-78 °C) until they could be assayed by GC.

Once a reaction profile for the control was established, reactions were then carried out using the candidate inhibitors. Figure 4.12 and 4.13 show the relative rates of consumption of TCEB (74) and the build up of hexyl butyrate (76) respectively, as judged be GC analysis. The inhibitor (25 % by mass) was added to each reaction. The reaction with 1-BrU (70) showed no inhibition at all, with a reaction profile for the transesterification comparable to the control reaction. 11-BrUA (71) appeared to enhance the progress of the reaction relative to the control and reaction rates for Me 11-BrAcU (72) showed only a small deviation from the control, appearing to induce only a low level of inhibition of the enzyme catalysed reaction.

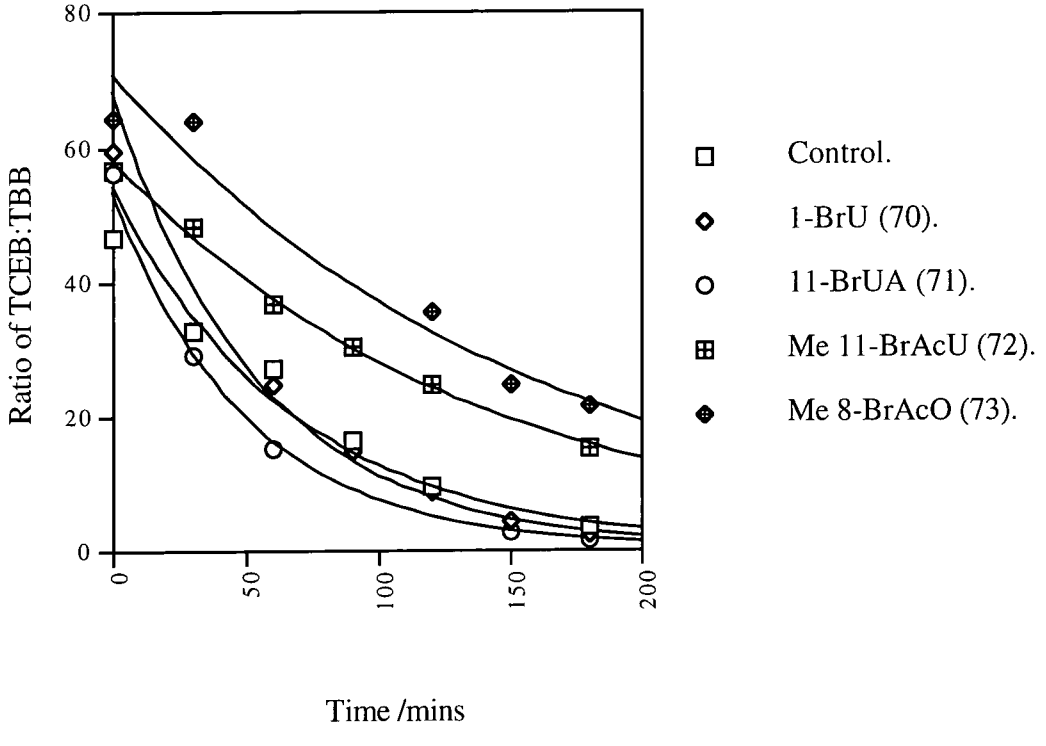


Figure 4.12. *The rate of consumption of TCEB (74) in all the systems used.*

When Me 8-BrAcO (73) was studied, the loss of TCEB (74) was slower than that of the control, and the Me 11-BrAcU (72) reaction. The build up of hexyl butyrate (76) (see Figure 4.13) showed a small deviation from the rate of build up in the control reaction. Thus it was judged that Me 8-BrAcO (75) slowed the transesterification reaction, but was not an inhibitor of the lipase. This system proved a more reliable assay method, but despite some variations in the reaction profiles, no dramatic effects were observed to suggest that specific inhibition was occurring.

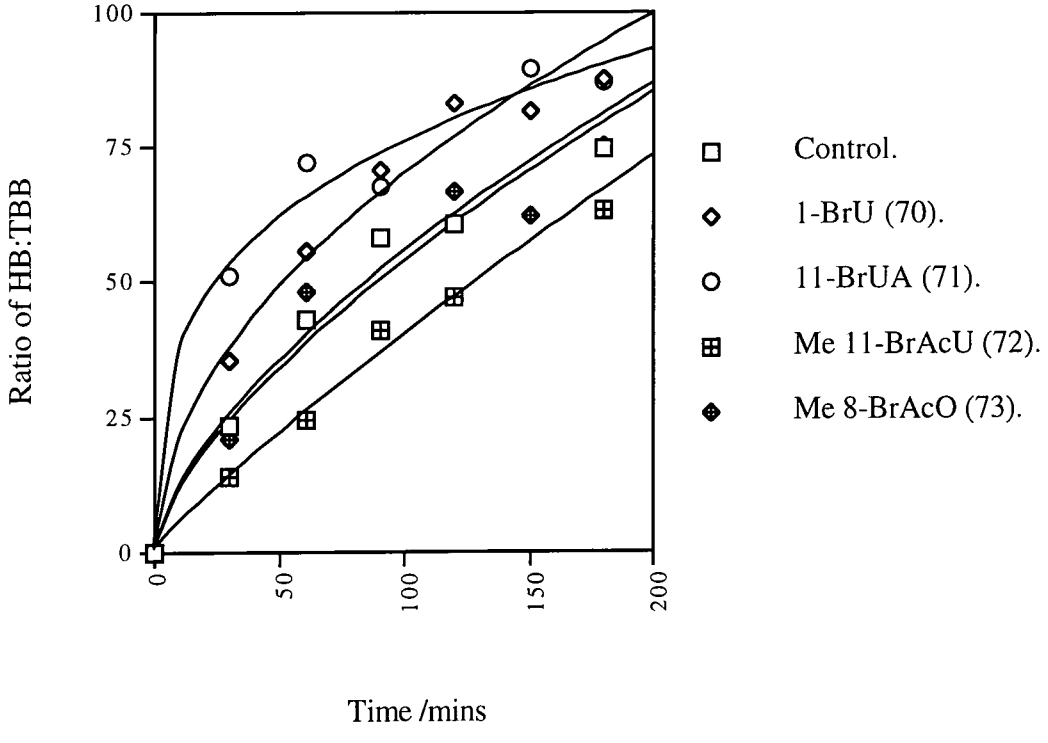


Figure 4.13. *The rate of build up of hexyl butyrate (76) in all the systems used.*

4.3.3: Discussion of the affinity labelling studies.

Several brominated molecules were incubated with CRL to investigate the possibility of the hydroxyl group of serine 247 attacking the alkyl halide, and causing covalent modification of the enzyme. An initial study showed that there was some inhibition of polymerisation using 11-BrUA (71). However, when this affinity label was studied as an inhibitor in the reaction between TCEB (74) and 1-hexanol (75) there

was no significant loss of activity of the enzyme. Thus it was concluded that 11-BrUA (71) was not an inhibitor.

Following this, several candidate compounds were synthesised which incorporated a primary alkyl bromide α to the carbonyl of an ester group. It was anticipated that this group would offer a better electrophile, and would undergo nucleophilic attack from the hydroxyl group of serine 247 more readily. The first molecule which was prepared, Me 11-BrAcU (72), showed only a small deviation in the reaction profile of TCEB (74) consumption and HB (76) build up from the control, and was thus not considered to be an inhibitor of the enzyme.

However, if the hypothesis suggesting a hydrogen bond between the hydroxyl group of serine 247 and 9-HNA (53) is correct, then Me 11-BrAcU (72) would extend further than 14.55 Å into the hydrophobic cavity, beyond the hydrophilic residue, and may not be correctly situated for nucleophilic attack by the serine residue. Thus the shorter affinity label methyl 8-bromoacetoxy octanoate (Me 8-BrAcO) (73), which appeared to offer a more appropriate chain length, was considered a better candidate and was synthesised. However, in the event this compound did not induce any significant deviation in the reaction profile of the transesterification reactions relative to the control system.

There are several possible explanations for the lack of inhibition occurring in these systems. The hydroxyl group of serine 247 may not be nucleophilic enough to attack the alkyl bromide, despite the presentation of the bromoacetoxy group. Secondly the bromoacetoxy group may be too large to enter into the cavity, and therefore no covalent modification would occur as predicted.

In summary, the results in this section were not consistent with covalent modification of the enzyme as none of the proposed affinity labels significantly inhibited both the polymerisation and the transesterification reactions studied.

4.4.1: Stereoselectivity of the Candida rugosa *lipase.*

Bhalerao *et al.*[85] drew some conclusions on the three dimensional shape of the binding site of the *Candida rugosa* lipase. In particular they investigated how far an acetoxy ester could be separated from the terminal ester while, during hydrolysis of an ester, the enzyme maintained enantiomer recognition. In a remarkable study they were able to show that the enantioselectivity increased to 99 % ee as the number of carbon atoms between the ester and the chiral centre was *increased* to eight. This selectivity was improved further when an n-butyl rather than a methyl ester was used as the substrate. This is the only example known where a resolution is achieved in a lipase hydrolysis and with functional groups so far apart. The substrate employed in the Bhalerao study was chosen for its potential role as an intermediate in the synthesis of natural products, and is shown in Figure 4.14.

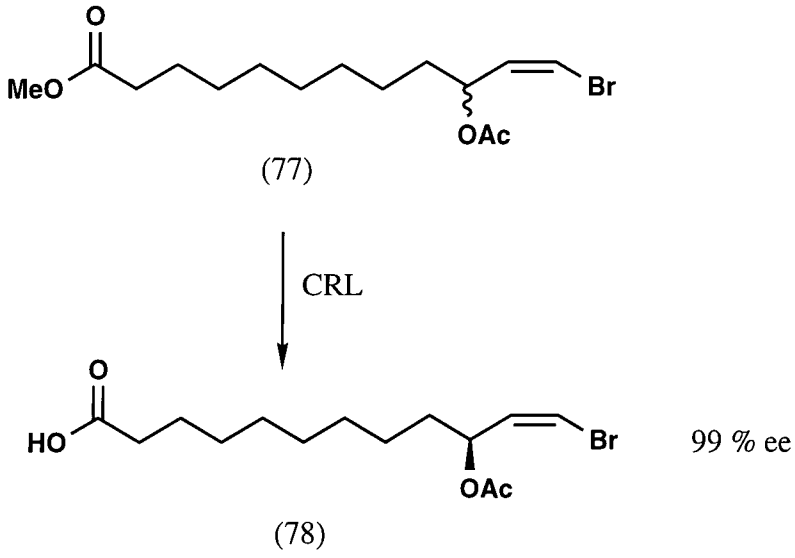


Figure 4.14. *Bhalerao's resolution of a molecule with eight carbons separating the two ester groups*[85].

From these observations an active site model was proposed for the *C. cylindracea* lipase incorporating two hydrophobic binding sites near the active site, and an ability of the enzyme to recognise a substrate with a chiral centre remote from the site of the reaction[85].

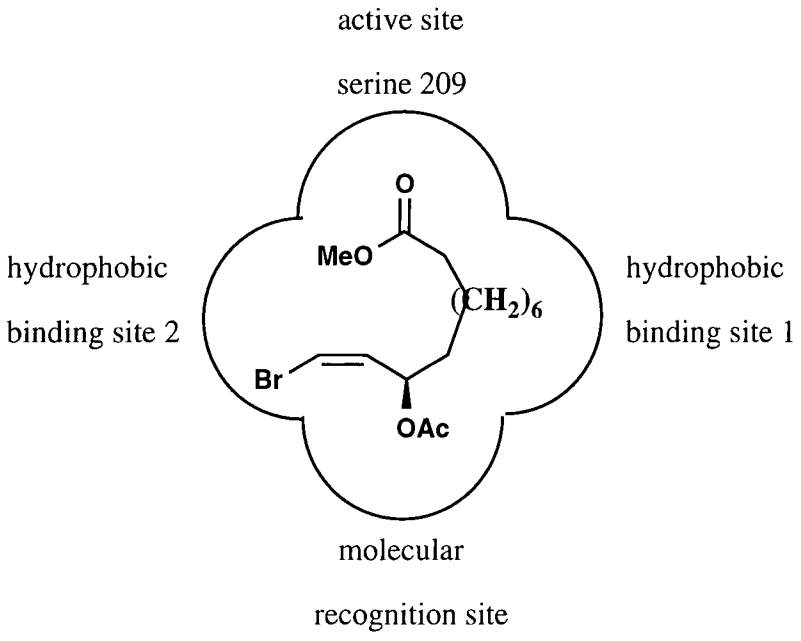


Figure 4.15. *Bhalerao's active site model and substrate*[85].

The model[80] (shown in Figure 4.15) proposed that both hydrophobic ends of the chain are in the hydrophobic binding site 2, and the carbon chain between the functional groups are in hydrophobic binding site 1. The methyl ester would become bound to serine 209 and the chiral centre would be placed in a hydrophobic recognition site. After hydrolysis of the methyl ester group, the configuration of the acetoxy ester adjacent to the bromovinyl group was evaluated, and it was found that the enzyme favoured the resultant hydroxy acid in which the stereogenic centre had the (*R*) absolute configuration[85]. This was in good agreement with other reports[27,86,87]. Bhalerao proposed that the (*R*) ester (*R*)-(78), which was hydrolysed preferentially over the (*S*) ester (*S*)-(78), acted as a competitive inhibitor to (*S*) ester hydrolysis. Thus the (*S*) enantiomer was not hydrolysed until the (*R*) enantiomer had been consumed.

Kazlauskas[88] and Cygler[12] predicted the enantioselectivity of lipase enzymes on the basis of a series of experiments, determining how the lipases reacted with various substrates. They found that the enantioselectivity increased for the substrates as the difference in the size of the groups at the chiral carbon was increased. Since the crystal structure of CRL is solved, and the models of the active site binding regions

proposed, many investigations have been undertaken to test these models in a predictive manner. The model which emerged was based on the size of the substituents at a chiral centre (which contains a secondary alcohol) in a cyclic system (containing either one, two or three rings). The model correctly predicted with greater than 93 % accuracy which enantiomer would react faster on this basis, for the lipase enzymes from *Candida rugosa* and *Pseudomonas cepacia*, and *Cholesterol esterase*[88]. Figure 4.16 shows the steric requirements for the enantiomer which will react faster according to the proposed model.

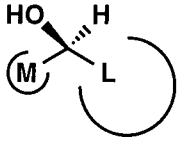


Figure 4.16. *The steric profile of the enantiomer of a secondary alcohol which will bind preferentially with CRL. M represents a medium and L represents a large substituent*[12, 88].

Wallace and Morrow[41] generated the first lipase catalysed optically active polymer. In their study they used porcine pancreatic lipase with 1,4-butanediol (22) and bis(2,2,2-trichloroethyl)(±)-3,4-epoxyadipate (23).

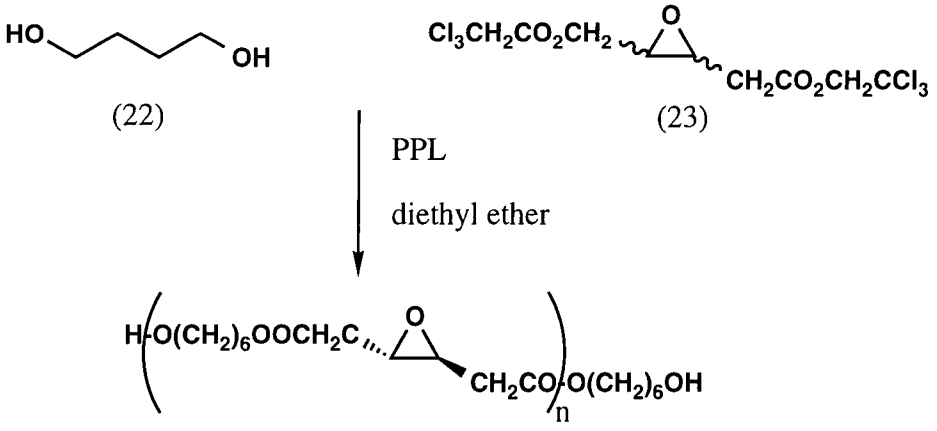


Figure 4.17. *Reaction scheme of Wallace and Morrow in their investigation into the synthesis of optically active polymers*[41].

They realised that an enzyme would preferentially select one enantiomer over another in a racemate[41]. Thus, in their AA - BB system if the racemate of epoxyadipate (23) is used one of the enantiomers should be incorporated preferentially into the polymer. This idea was realised with a 25:1 predominance of one enantiomer over the other in the polymer representing a 96 % ee. However. the study did not reveal the absolute stereochemistry of the preferred monomer. This remains the only reported study where a lipase catalysed polyesterification has generated an optically enriched material[41].

4.4.2: Synthesis of an optically enriched polyester.

An investigation was therefore undertaken to determine if the *Candida rugosa* lipase would assemble an enantiomerically enriched polymer, if presented with a racemic hydroxy ester. A secondary alcohol seemed the most straightforward way to introduce a chiral centre, and it was desirable to maintain a ten carbon relationship between the ester and the alcohol functionalities. Thus 10-hydroxyundecanoic acid (61) emerged as a candidate substrate, particularly as it could be synthesised readily . A synthesis was developed by oxymercuration of methyl undecylenate (79), and this is discussed fully in section 5.6.1. Furthermore, the polymerisation of this monomer had been studied in Chapter 3, where it was demonstrated that low molecular weight oligomers could be generated.

Accordingly another polymerisation study was carried out on 10-hydroxy-undecanoic acid (61), and stopped at approximately 50 % conversion, as judged by ^1H NMR. The GPC trace of the resultant polymer/oligomer from (61) after five hours is shown in Figure 4.18.

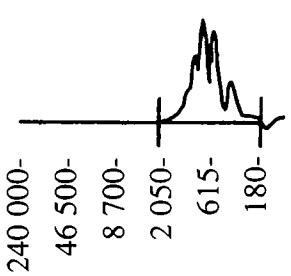


Figure 4.18: *GPC trace of a polymer of 10-hydroxyundecanoic acid (61).*

The oligomeric material was separated from the residual monomer acid by column chromatography, and the monomer acid fraction derivatised with (*R*) Mosher's acid (2-methoxy-2-trifluoromethyl-2-phenylacetic acid), forming a diastereomeric mixture. From the same sample work up the oligomers were hydrolysed with NaOH, and the products extracted into diethyl ether after acidification, before being derivatised with (*R*) Mosher's acid. The enantiomers of the secondary alcohol can not of course be distinguished by ^{1}H NMR, and thus derivatisation with (*R*) Mosher's acid became necessary, now enabling the differentiation of the diastereoisomers, by chemical shift non-equivalence in the ^{1}H NMR spectra, as shown in Figures 4.19 and 4.20.

The resultant ^{1}H NMR spectra shown in Figure 4.20 indicated a 3:1 ratio of the diastereoisomers in each case. This ratio of (*R*) to (*S*) is opposite in each case as expected. Thus the enzyme had polymerised one of the enantiomers preferentially, and did exhibit stereoselectivity towards the secondary alcohols.

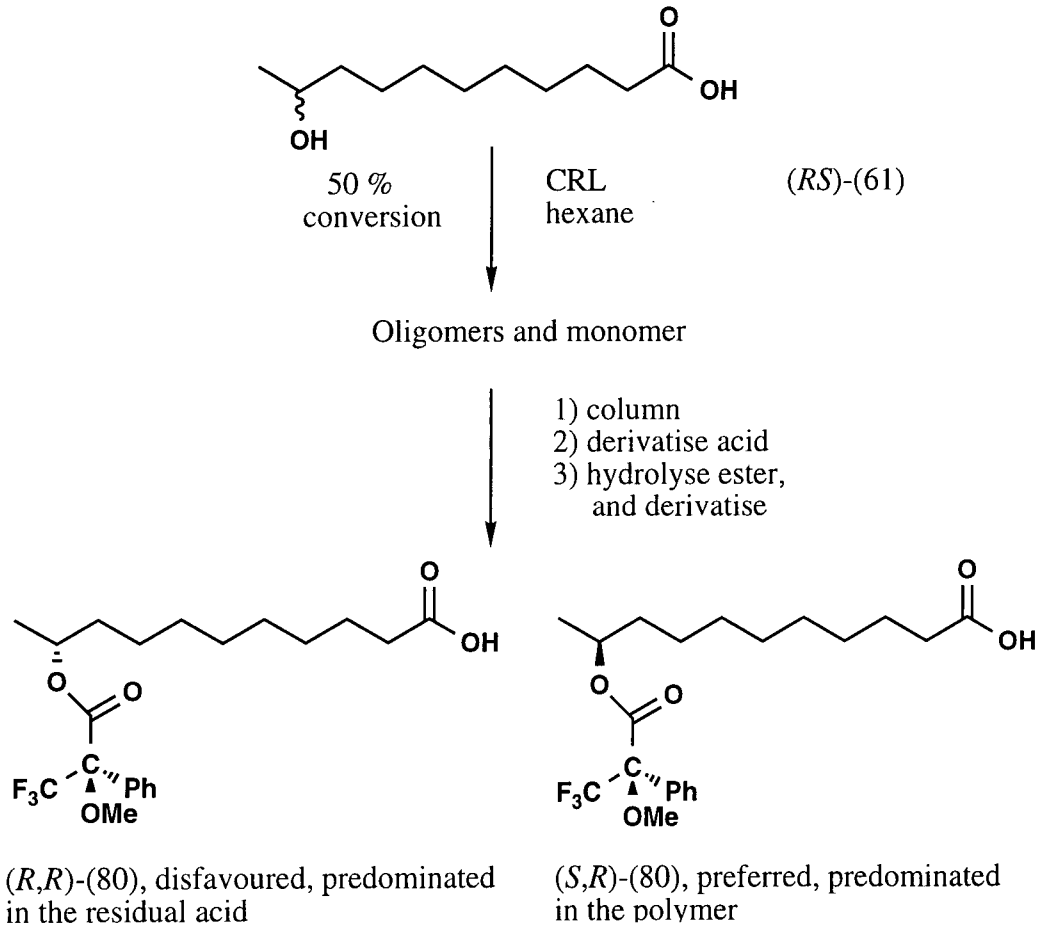


Figure 4.19. *Strategy for determining stereoselectivity of enzymatic preferential.*

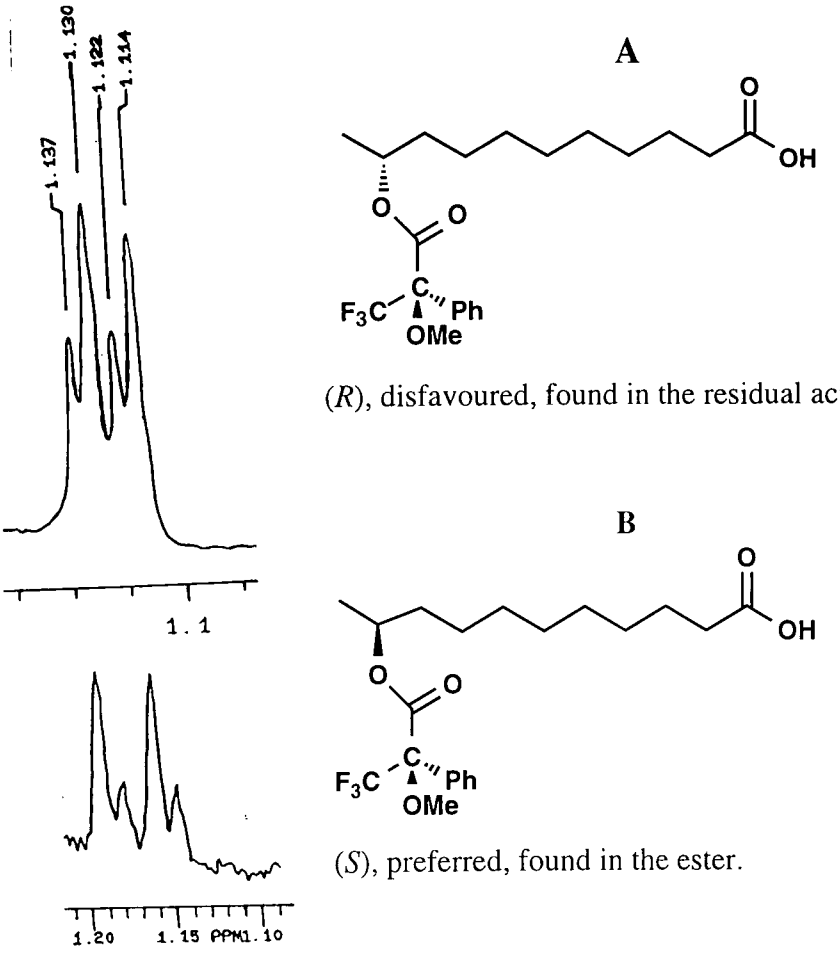
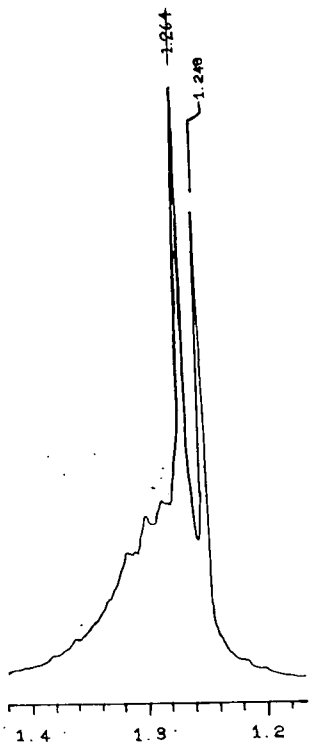
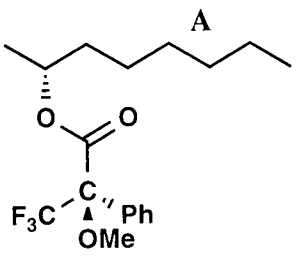


Figure 4.20. *Section of the ^1H NMR (250 MHz) spectra of Mosher's acid ester derivatives of 10-hydroxyundecanoic acid (61), from A) the residual monomer, and B) the hydrolysed oligomer.*

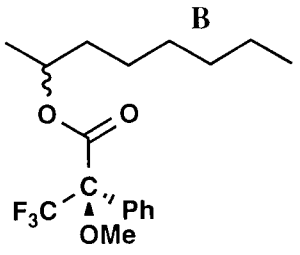
In order to assign the absolute stereochemistry to the preferred enantiomer in each of the fractions, an appropriate reference compound was required. This was achieved by preparing (*R*) Mosher's acid esters of (*R*)-(-)-2-octanol (*R*)-(82) and the racemic 2-octanol (*RS*)-(82). 2-Octanol (82), which is commercially available, was judged to provide a system sufficiently similar to the 10-hydroxyundecanoic acid (61) to provide a reliable model for our system, as the alkyl functionality of the monomer should not significantly perturb the chemical shift of the methyl group. Enantiomerically pure 2-octanol (*R*)-(82) generated only one diastereoisomer as expected after derivatisation, as shown in Figure 4.21 (A). Of course, the racemic mixture gave a 1:1 ratio of two diastereoisomers, also as expected, a situation which is

clearly observable at the methyl signals (1.1 ppm) in the resultant ^{1}H NMR spectrum, shown in Figure 4.21 (B). By comparison with the racemic 2-octanol (82) derived spectra, we can confidently assign the methyl doublets to each diastereoisomer. It follows that the (*S*) enantiomer of the hydroxy acid (*S*)-(61) was the preferred one utilised by the enzyme, while the (*R*) enantiomer (*R*)-(61) was disfavoured and present in excess in the residual acid.





2-(*R*)-octanol, derivatised with Mosher's acid



racemic 2-octanol, derivatised with Mosher's acid.

Figure 4.21. *Section of the ^{1}H NMR (400 MHz) spectra of Mosher's acid ester derivatives of A)* (R)*-2-octanol (83), and B)* (R,S)*-2-octanol* (R,S)*-(83).*

The spectra for the 2-octanol derivatives (83) in Figure 4.21 were recorded on a 400 MHz NMR instrument, whereas the spectra for the 10-hydroxyundecanoate derivatives (80) in Figure 4.20 were recorded on a 250 MHz NMR instrument, which accounts for the difference in signal overlap between Figures 4.20 and 4.21.

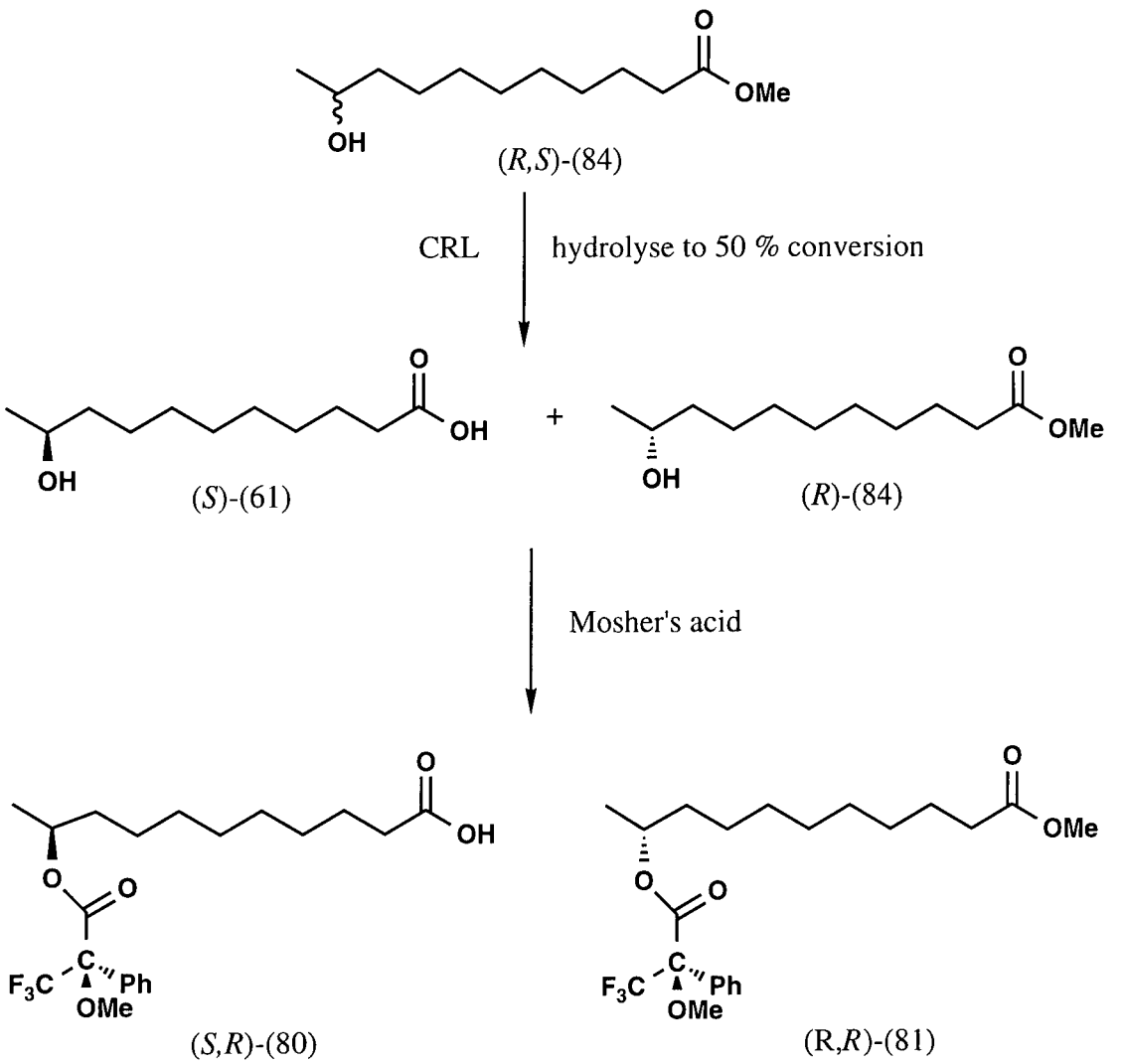


Figure 4.22. *The proposed enzymatic hydrolysis of a secondary alcohol.*

At this point an additional study was carried out, to investigate if there was any enantiomeric preference during the hydrolysis of the methyl ester of 10-hydroxyundecanoate (84). In an aqueous buffer it was anticipated that the enzyme would hydrolyse the ester, and that if the reaction was stopped at a halfway point, then

both the acid and the residual ester could be analysed to determine their respective enantiomeric excesses. This again could be accomplished by ^{1}H NMR analysis after preparation of Mosher's acid esters of the resultant materials as shown in Figure 4.22. This study appeared appropriate as Bhalerao had observed that the ester hydrolysis could result in resolution of a stereogenic centre up to eight carbons from the ester.

Accordingly CRL was incubated with (*R,S*)-(84) until approximately 50 % of the monomer had been consumed, as judged by ^{1}H NMR analysis. Figure 4.23 shows the methyl region of the resultant ^{1}H NMR spectrum of the Mosher's acid derivatised acid (80), after column chromatography. There is no significant excess of one diastereoisomer over the other in Figure 4.23, as the ratio of the equivalent sets of doublets is close to 1:1. The broad peak at 1.20 is from CH_2 groups in the alkyl chain. The residual ester (84) was also derivatised with Mosher's acid, and again there was no significant excess of one diastereoisomer over the other (spectrum not shown). Thus the enzyme did not display any stereoselectivity in this case.

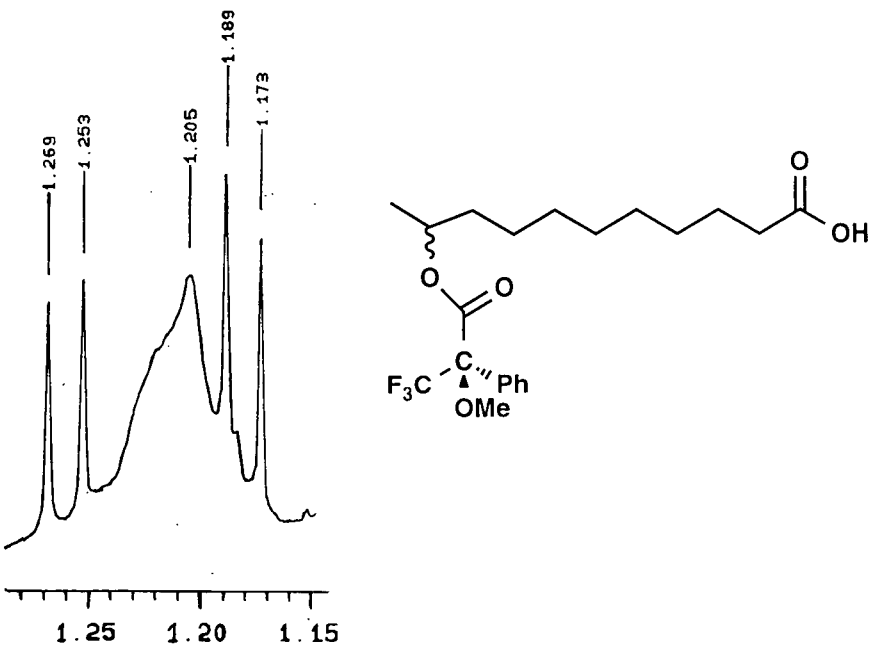


Figure 4.23. *Section of the ^{1}H NMR spectrum of the Mosher's acid derivatised acid (80).*

The results of this hydrolysis experiment tend to indicate that the stereoselectivity of CRL observed in the oligomerisation reaction is not due to binding of the molecule within the hydrophobic tunnel. This contrasts with Bhalerao's observation of resolution during a hydrolytic reaction, when the stereogenic centre was eight carbons distant from the centre of hydrolysis[85]. If there was stereoselective binding in this case, then this hydrolytic reaction would be expected to reveal a similar selectivity to that observed in the oligomerisation reaction. However, no enantiomeric excess was observed.

The stereoselectivity exhibited during the oligomerisation reaction can therefore be attributed to the esterification event. However, this does not exclude the possibility that the substrate is binding within the tunnel, but that this binding is not stereospecific. During the esterification reaction the acylated enzyme then has an enantiomeric preference for the (*S*) enantiomer of the incoming monomer. Thus the ester which was formed showed predominantly the (*S*) configuration. Figure 4.24 shows the three possible regions where stereoselectivity may be expressed when CRL binds to 10-hydroxyundecanoic acid (61).

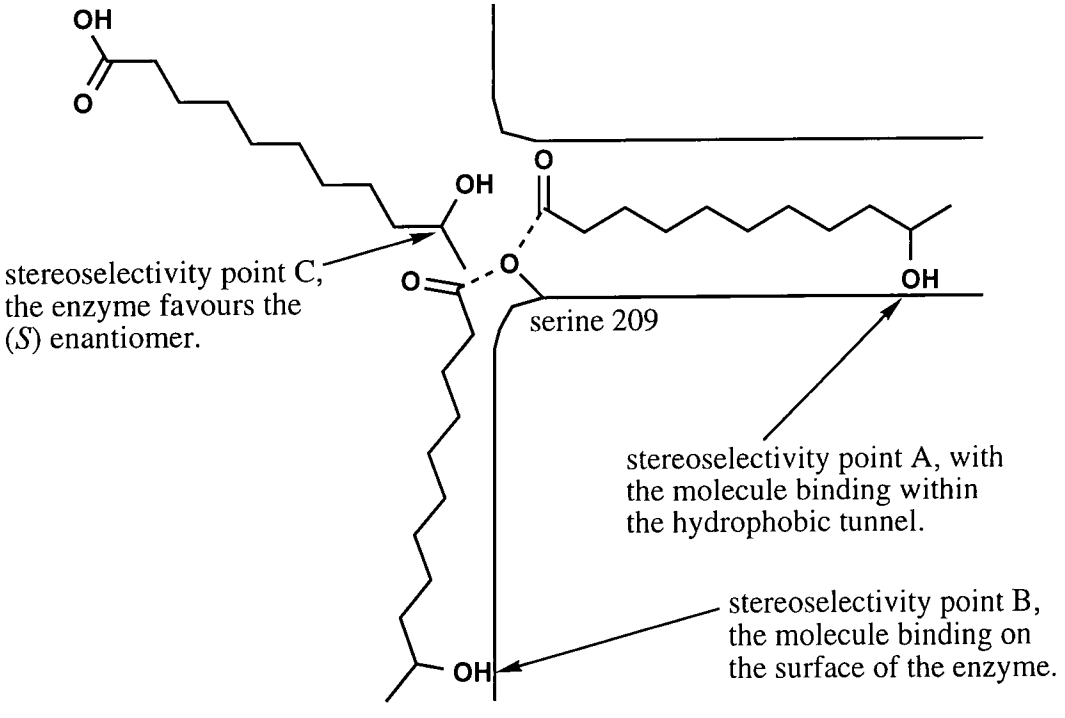


Figure 4.24. *Three possible sites where stereoselectivity could be induced during the oligomerisation of 10-hydroxyundecanoic acid (61).*

4.5.1: Discussion of binding in CRL.

Two general hypotheses were described at the beginning of this Chapter for the binding of the substrate to CRL. Either the substrate can enter the hydrophobic tunnel in a similar manner to the hydrocarbon chain of a triglyceride, or alternatively, the substrate can not enter the active site tunnel because of a lack of compatability between the terminal hydroxyl group and the hydrophobic tunnel. Therefore the substrate remains on the surface of the enzyme. A schematic representation of these two possibilities is shown in Figure 4.25.

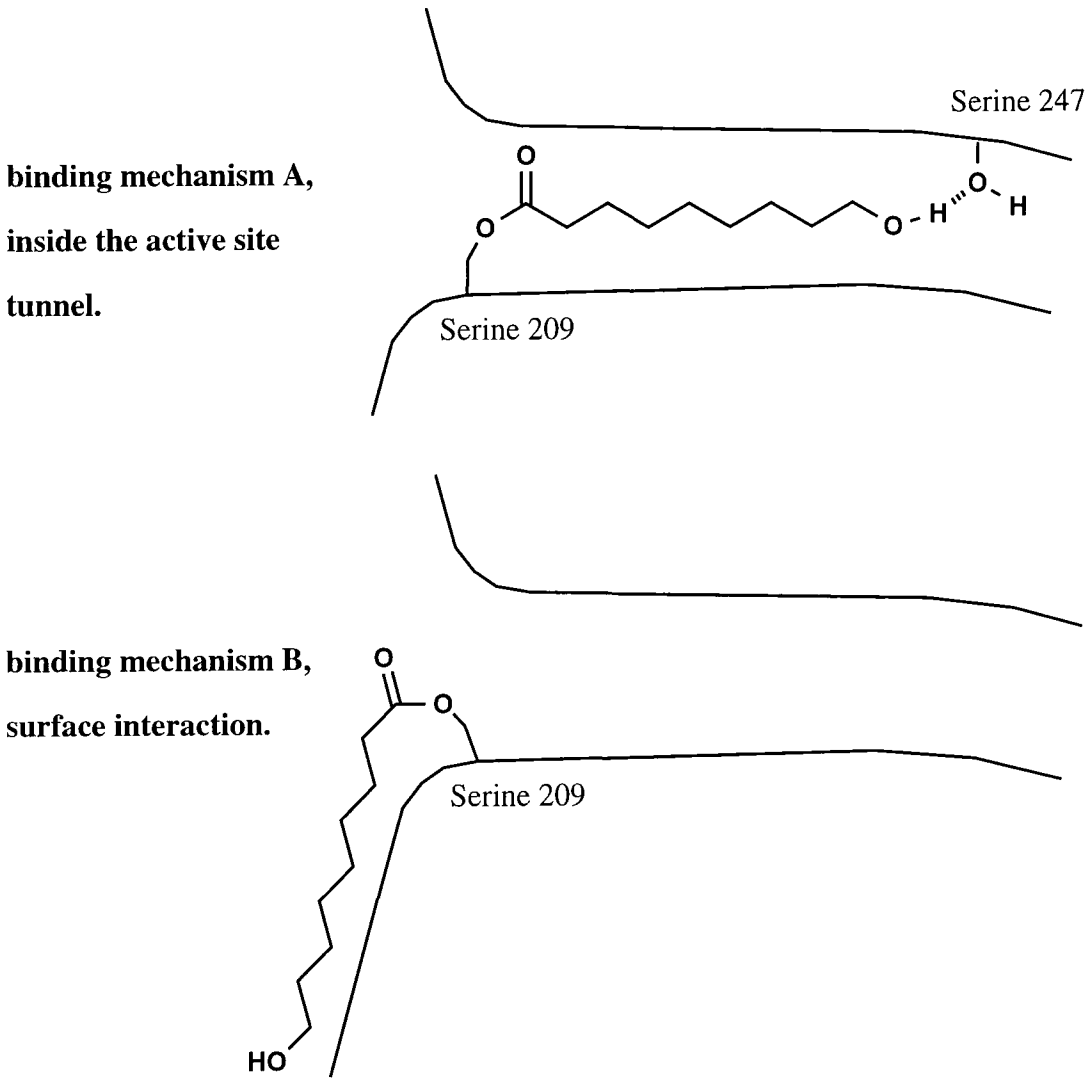


Figure 4.25. *Two binding hypotheses, A, the substrate is within the hydrophobic tunnel, and B, a surface interaction.*

The results discussed in this Chapter provide evidence both for and against these hypotheses.

Binding mechanisms.

Comparison of the initial rates of lipase mediated polymerisation of ω-hydroxy acids indicated that there was a preferential reaction with 8-HOA (52) and 9-HNA (53) over the longer chain monomers. This implied that there was a specific interaction which favoured polymerisation of these monomers. Analysis of the hydrophobic tunnel used in the binding of triglycerides revealed the hydroxyl group of a serine residue which presented the possibility of a hydrogen bonding interaction between the enzyme and these monomers, if they bind in this tunnel. This hypothesis is strengthened by the computer modelling studies, in which it was shown that the terminal hydroxyl oxygen of 9-HNA (53) could come within hydrogen bonding distance of serine 247 hydroxyl group, when the chain conformation was minimised. Thus the computer modelling studies were compatible with the substrate entering the hydrophobic tunnel and binding to the hydroxyl group of serine 247.

However, the tunnel binding hypothesis was not reinforced by the study with the alkyl bromides, which were anticipated to be inhibitors of the enzyme. No significant inhibition was observed in this study. There are a variety of explanations for the lack of inhibition, for example, the brominated substrates may have been too large to enter the hydrophobic tunnel; the substrates may not have located the electrophilic carbon close enough to the hydroxyl group of serine 247 for nucleophilic attack to occur; the serine hydroxyl group may not have been nucleophilic enough to covalently modify the enzyme as anticipated. Therefore, these experiments employing alkyl bromides remain somewhat inconclusive.

A protein is a three dimensional molecule, and as such the surface of the tunnel has a specific shape. Thus preferential binding of one enantiomer of 10-hydroxyundecanoic acid (61) over the other was anticipated. However, this was not observed in the hydrolysis of methyl 10-hydroxyundecanoate (84).

When the material comprising the polymer and the residual acid (61) was derivatised and analysed, the ^{1}H NMR spectrum of the polymer favoured one

diastereoisomer, while the ^{1}H NMR spectrum of the residual acid favoured the other diastereoisomer. This suggests that the resolution (3:1) occurs only when the secondary alcohol acts as a nucleophile for deacylation, not when it is bound to the protein, as shown in Figure 4.26.

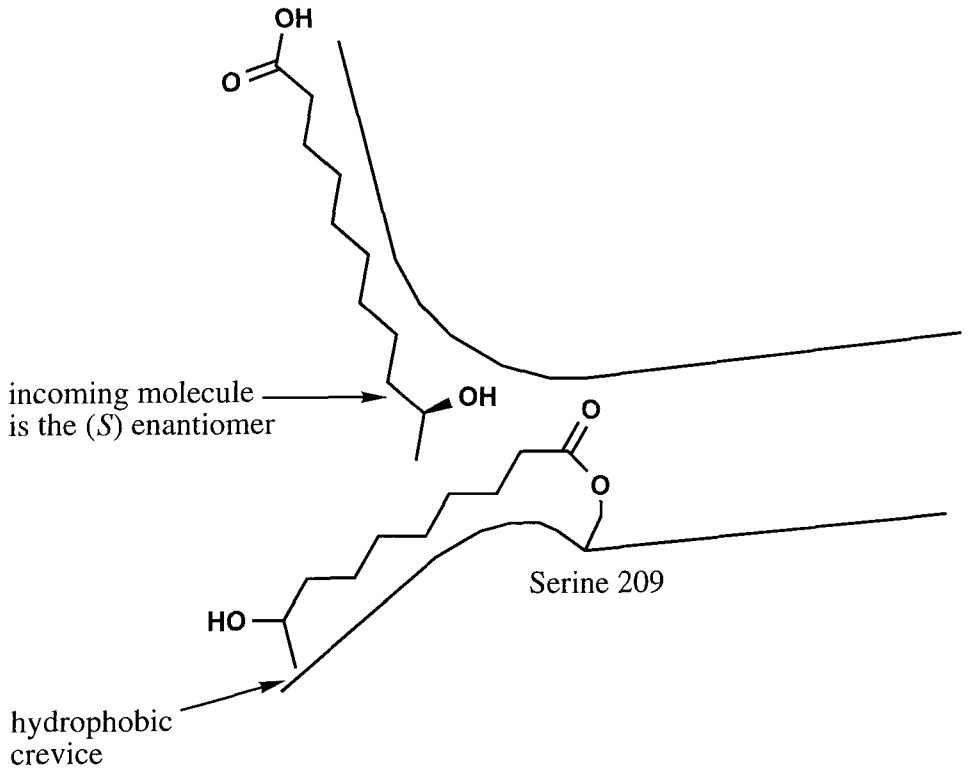


Figure 4.26. *A molecule of 10-hydroxyundecanoic acid (61) bound in the active site of CRL and in the hydrophobic crevice. The amino acids around the active site direct the incoming molecule, with the (S) enantiomer preferred.*

4.5.2: Summary of the binding hypotheses.

The orientation of the hydrocarbon chain in the enzyme-substrate complex during polymerisation is not fully understood. Two hypotheses have been discussed in this chapter, both presented with valid but inconclusive arguments for the particular mode of binding by the substrate to the enzyme. Thus at this point the exact binding mode of the monomer remains unknown, but the balance of the evidence favours surface binding rather than tunnel binding.

CHAPTER FIVE

SYNTHESIS

5.0: SYNTHESIS.

5.1: Synthesis of monomers for Chaper 2.

In Chapter 2 various monomers were required for the polymerisation reactions. 10-HDA (42) was commercially available. 8-HOA (52) and 11-HUA (43) were synthesised in a straight forward manner by hydrolysis of the respective ω-bromo-acids[89]. However, the synthesis of 9-HNA (53) was much more involved. There are various routes to this compound in the literature, for example, from the reduction of the monomethyl ester of azelaic acid (85)[90], or from ozonolysis of oleic acid (86)[91]. These syntheses were attempted, together with several other routes to 9-HNA (53). The last route discussed in this Chapter, involving the generation of a nine carbon species from propargylic alcohol (87) and diiodohexane (88), and the synthesis involving OsO_4 and NMO proved to be the best in terms of the quantity of material prepared, despite involving more synthetic steps than any of the other routes.

5.2.1: 8-HOA (52) and 11-HUA (43).

The synthesis of 8-HOA (52) (see section 2.5.1) started from 8-bromooctanoic acid (89) following the method of Ahmed *et al.*[89]. Accordingly a solution of the bromoacid (89) in aqueous potassium hydroxide was heated under reflux for 7 days. After acidification and work up this gave the desired 8-HOA (52) in 65 % yield.

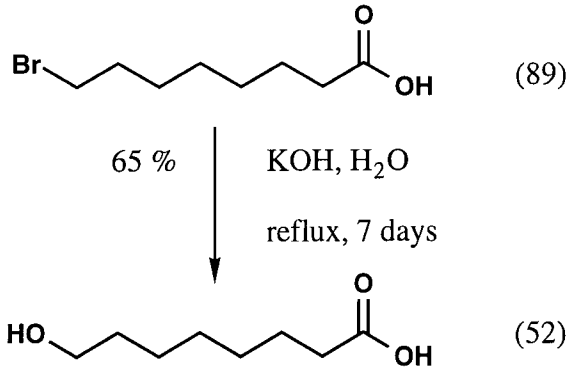


Figure 5.1. *Hydrolysis of 8-bromooctanoic acid (89)*[89].

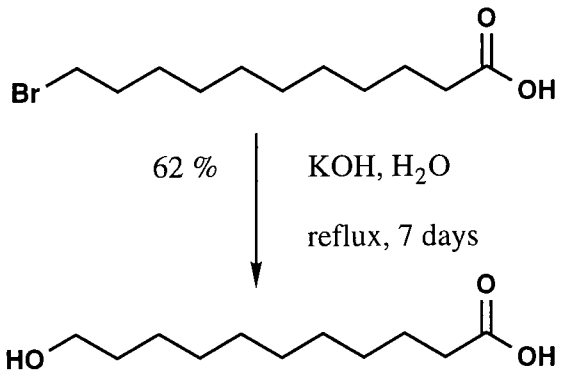


Figure 5.2. *Hydrolysis of 11-bromoundecanoic acid (71)*[89].

11-HUA (43) (see section 2.5.1) was also prepared by this method, starting from 11-bromoundecanoic acid (71), and gave the corresponding acid in 62 % yield.

5.3.1: Synthesis of 9-HNA (53).

This proved the most difficult of the ω-hydroxy acid monomers to prepare. Several routes towards 9-HNA (53) (see section 2.4.1) were considered. Methyl azelate (85), the monoester acid, was dissolved in monoglyme and was selectively reduced using sodium borohydride with lithium chloride as a catalyst[90]. The desired 9-HNA (53) was isolated, albeit in low yield (11 %). Unreacted starting material and diol were also recovered from this reaction.

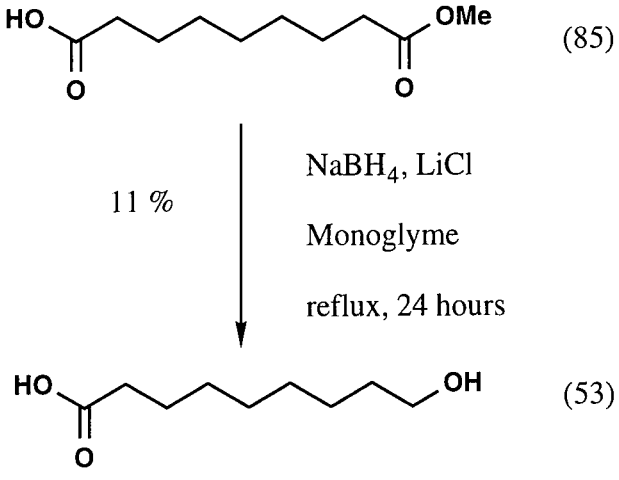


Figure 5.3. *Reduction of monomethyl azelate (85)*[90].

5.3.2: Two of the routes to 9-HNA (53) involved the oxidative cleavage of the olefinic bond of oleic acid (86)[91,92]. Oleic acid (86) was esterified in refluxing acidic methanol. Ozone was then bubbled through a solution of the resultant methyl ester (90) in DCM, until the solution turned blue[91]. Once the olefin had been consumed the reaction was quenched with dimethyl sulfide, and the resultant aldehyde (91) was then reduced with sodium borohydride in methanolic NaOH at 0 °C. However, this one pot reaction was inefficient, and gave methyl 9-hydroxynonanoate (92) in a low yield (5 %) after chromatography. The ester was then hydrolysed to 9-HNA (53) by refluxing in methanolic NaOH in moderate yield (51 %). However, the low yield of the penultimate step rendered this route impractical.

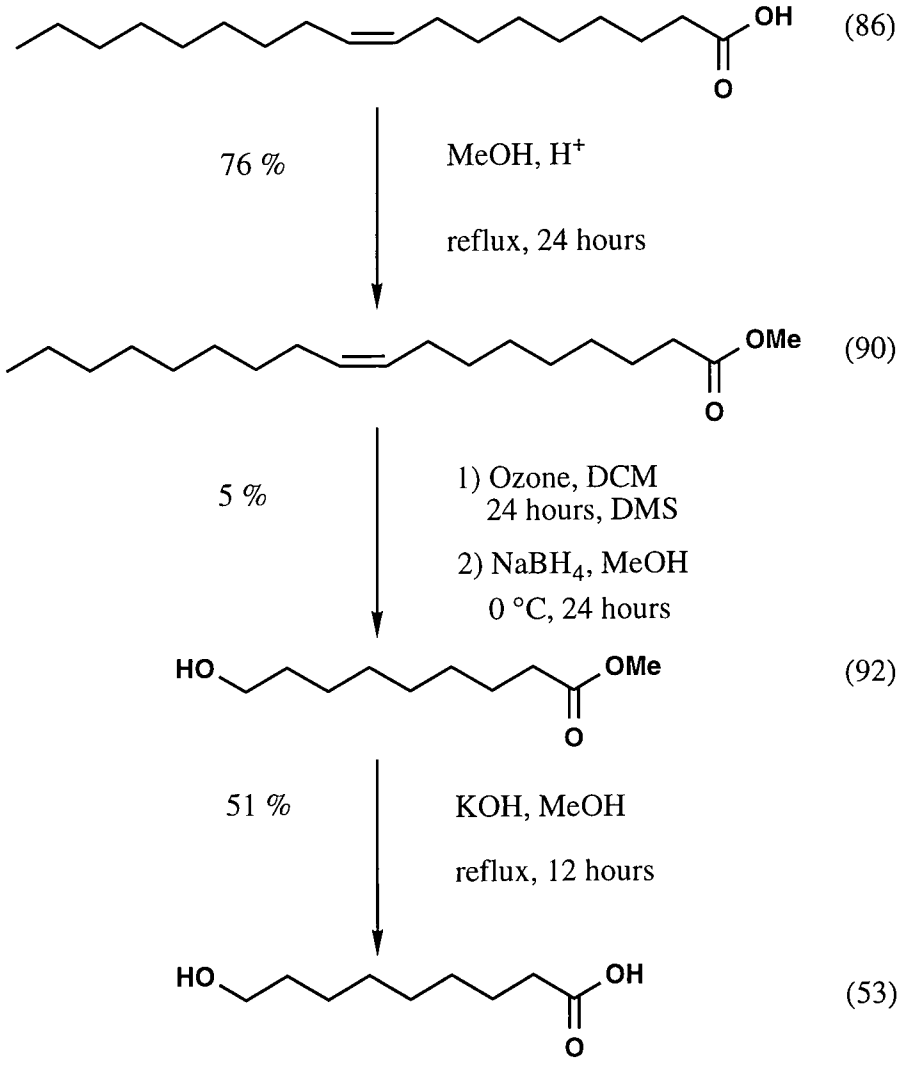


Figure 5.4. *9-HNA (53) synthesis from oleic acid (86) using ozone[91].*

5.3.3: The next route to 9-HNA (53) also used oleic acid (86) and involved treatment of methyl oleate (90) with a catalytic amount of osmium tetroxide and NMO (one equivalent)[92]. Due to the stereospecificity of this reaction the *cis*- double bond generates the *erythro*-diol (93) after osmylation, and this compound was recovered in good yield (88 %).

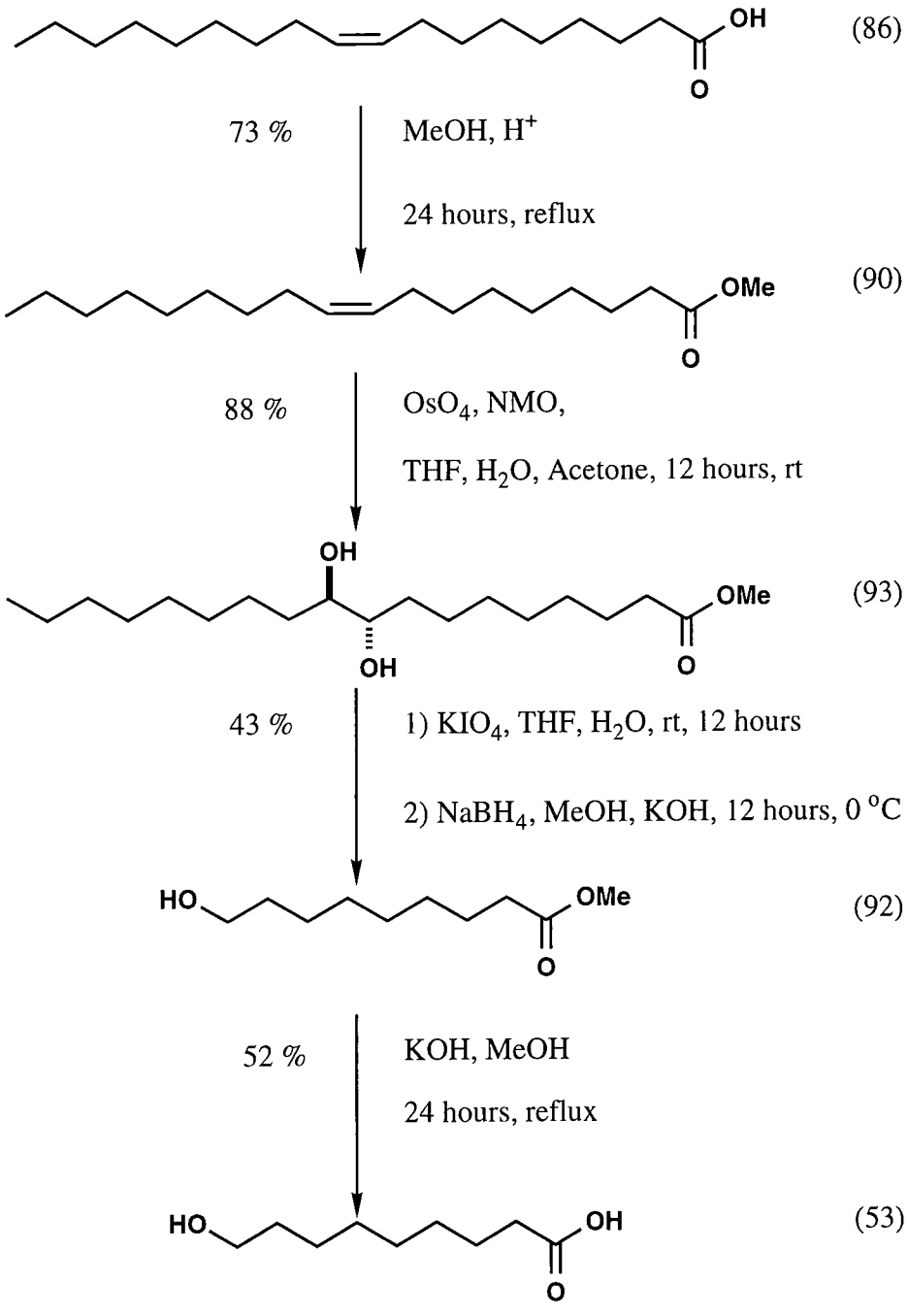


Figure 5.5. *9-HNA (53) synthesis from oleic acid (86) using OsO4[92].*

The resultant diol (93) was then cleaved to the aldehyde (91) using periodate, and then reduced *in situ* by the careful addition of an excess of sodium borohydride, affording the hydroxy ester (92) in low yield (43 %). The hydroxy acid (53) was then generated after basic hydrolysis of the ester in moderate yield (52 %).

5.3.4: In order to improve the yield of 9-HNA (53) a new synthetic route was addressed. A shorter synthesis was devised involving nonanediol (94) as the starting material. Initially the diol (94) was mono-protected as a THP ether (95)[93]. The problem with this step is the statistical distribution that inevitably occurs between the di-, mono- and un-protected diol. The yield can be improved here by collecting the residual starting material and the diprotected material and recycling them. The free hydroxyl group of (95) was then oxidised to the acid (96) using Sharpless' conditions with ruthenium trichloride and sodium periodate[94].

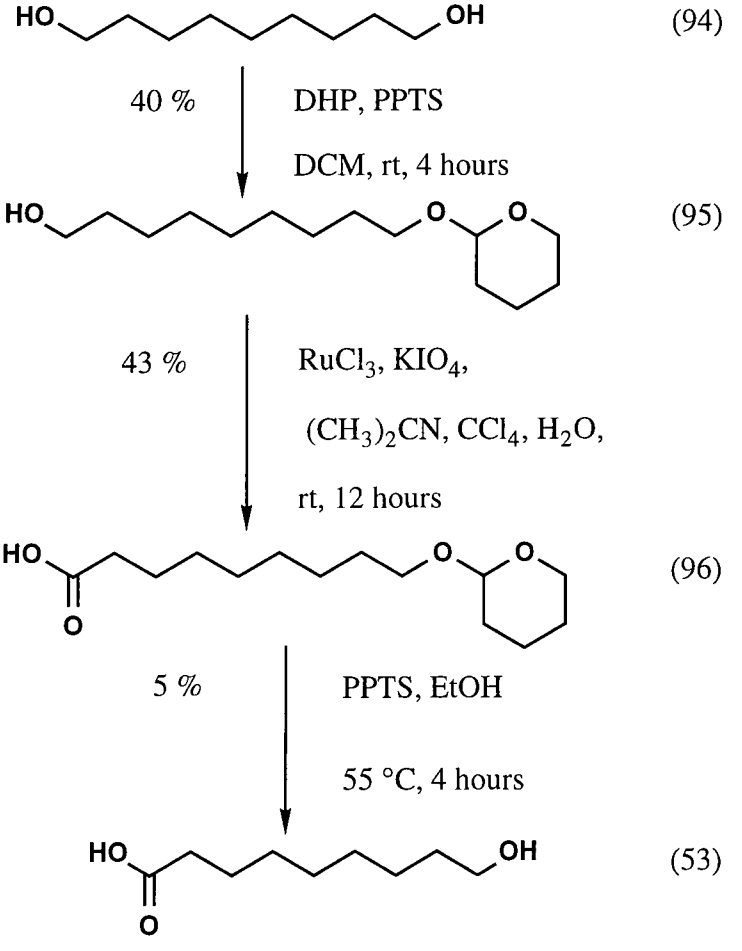


Figure 5.6. *9-HNA (53) synthesis from nonanediol (94)[93,94].*

The free 9-HNA (53) was isolated, however again in poor yield, after hydrolysis of the THP ether in acidic ethanol[93]. Again this synthesis was inefficient and the total mass of the product which could be prepared was low.

5.3.5: The synthetic approach was then altered to utilise cyanide as the latent carboxylate group, by extending an eight carbon chain to give the required 9-HNA (53).

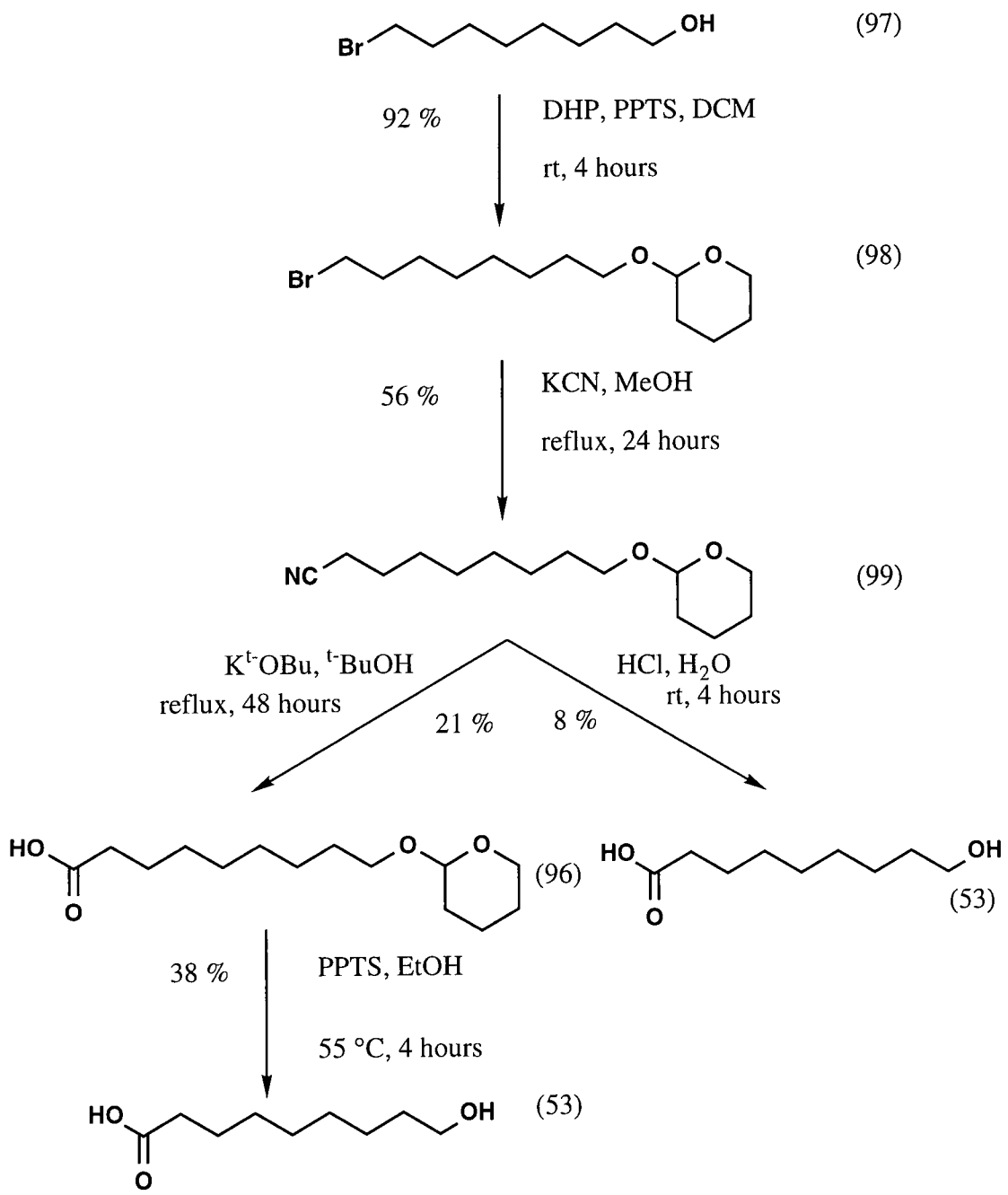


Figure 5.7. *Synthesis of 9-HNA (53) from 8-bromooctanol (97)*[93,95,96].

Accordingly 8-bromooctanol (97) was protected as its THP ether (98), a reaction which proceeded in good yield (90 %)[93]. The 1-bromo-2'-(tetrahydro-pyranyloxy) octane (98) was then subjected to nucleophilic substitution with potassium cyanide in methanol[95]. The resultant nitrile (99) was hydrolysed under basic conditions[96]. However, the reaction was poor, and proceeded in 21 % yield . The THP protecting group was then removed in acidic ethanol to generate 9-HNA (53) in 38 % yield for this final step[93].

The THP group is acid labile, and thus it appeared attractive to hydrolyse the nitrile (99) under acid conditions, and therefore these two steps could be combined. Unfortunately, however, when this was attempted the yield of the recovered hydroxy acid (53) was very low (8 %). In later attempts at the hydrolysis of the protecting group, aqueous HCl was used, but this led to only a minor improvement in the yield.

5.3.6: The final route to 9-HNA (53) involved a synthesis from propargylic alcohol (87) and diiodohexane (88). Propargylic alcohol (87) was protected using THP, as described earlier (95 %)[93]. This THP protected propargylic alcohol (100) was then cooled to -78 °C, and BuLi was added. After formation of the propargylic anion, diiodohexane (88) was added to the solution, and the material allowed to warm to ambient temperature[97]. The resultant 1-iodo-9-(tetrahydropyranyloxy)non-7-yne (101), isolated in 49 % yield, was then heated in DMSO to 130 °C to generate the aldehyde (102) (44 %)[98]. The aldehydic function was oxidised to an acid using PDC in DMF, which afforded the protected acetylenic hydroxy acid (104) in moderate yield (45 %)[99]. The THP group was then hydrolysed under acidic conditions, using PPTS in warm ethanol, to generate the acetylenic hydroxy acid (60)[93]. This acetylenic ω-hydroxy acid was employed as a monomer for the polymerisations reactions in its own right (see section 3.4.1), as well as being synthesised as the precursor to 9-HNA (53). Finally, the acetylenic bond was subjected to palladium catalysed hydrogenation to yield 9-hydroxynonanoic acid (53) in 34 % yield for this final step[100].

Of the eight routes attempted for the synthesis of 9-HNA (53), the route

employing the propargylic alcohol proved the best in terms of the quantity of material which was synthesised, and the ease of synthesis and purification of the products. The route employing OsO_4 and NMO was also performed with good yields of 9-HNA (53). One of the problems of many of these routes was the use of the THP protecting group. This group is attached in a straight forward manner in high yield[93]. However, when it is removed in acidic ethanol the yield of the deprotection step is lower than that expected from literature discussions[93].

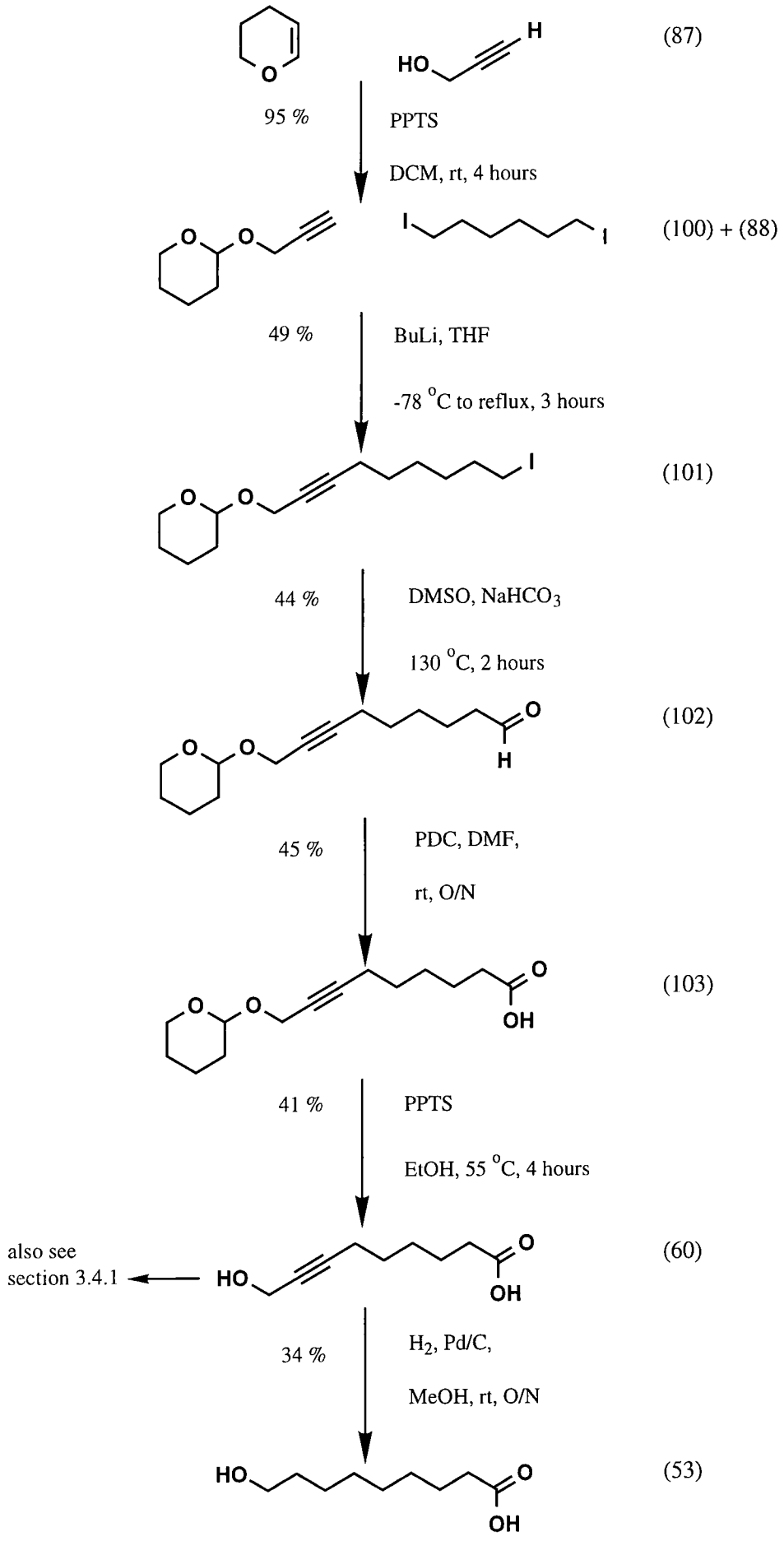


Figure 5.8. *9-HNA (53) synthesis from propargylic alcohol (87) and diiodohexane (88)*[93,97,98,99,100].

5.4: Synthesis for Chapter 3.

In Chapter 3 the monomers 12-HDDA (54) and 16-HHDA (55) were employed in the polymerisation reactions. These were commercially available. However, the isotopically labelled monomers 8-[$^2H_1$]-8-hydroxyoctanoic acid (58) and 9-[$^2H_1$]-9-hydroxynonanoic acid (57) had to be prepared. Also the functionalised monomers methyl 8-hydroxyoctanoate (59), 9-hydroxy-7-nonynoic acid (60), 10-hydroxy-undecanoic acid (61), and methyl 11-aminoundecanoate (62) were synthesised (see section 3.4.1). The monomers used in the transesterification expriments, discussed in section 3.6.1 and 3.6.2, were also synthesised. These included methyl 11-hydroxyundecanoate (64) and enantiomerically pure and racemic methyl 12-hydroxystearate (65).

5.5.1: Synthesis of [9-$^2H_1$]- 9 - hydroxynonanoic acid (57).

The mild oxidising agent, pyridinium dichromate (PDC)[99], was used in dichloromethane at room temperature to oxidise 9-HNA (53) to the semialdehyde (104) in low yield (34 %). There was no apparent over-oxidation to the diacid, as judged by ^1H NMR analysis.

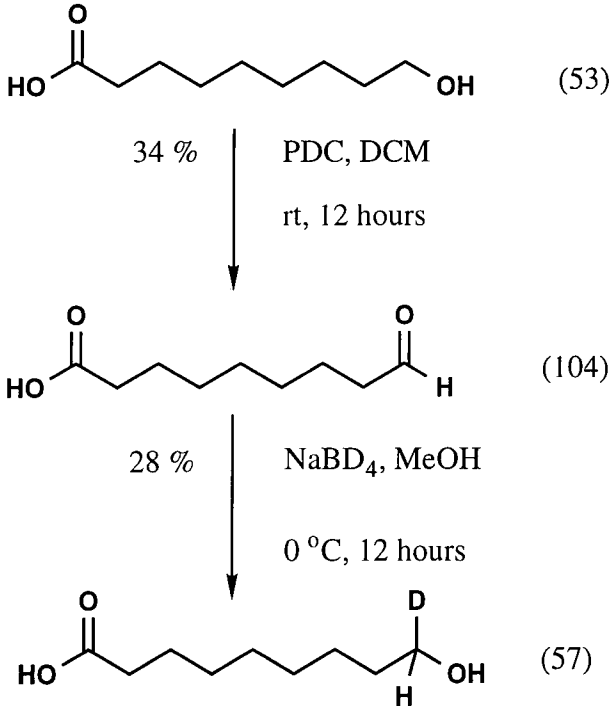


Figure 5.9. *Synthesis of [9-$^2H_1$]-9-hydroxynonanoic acid (57).*

The aldehyde (104) was then reduced with sodium borodeuteride (NaB^2H_4) in methanol at 0°C, as a cost effective means of introducing the isotopic label into 9-hydroxynonanoic acid (57), albeit in low yield (28 %). This position is distinguishable in terms of the alcoholic and ester end group signals in ^1H and ^2H NMR (3.6 ppm and 4.0 ppm respectively). The isotope content was judged to be 99 % after analysis by mass spectroscopy.

5.5.2: Synthesis of [8-$^2H_1$]- 8 - hydroxyoctanoic acid (58).

The route was altered a little for the synthesis of [8-$^2H_1$]- 8 - hydroxyoctanoic acid (58), due to the problems of polymerisation of the ω-hydroxy acid which had been encountered during the synthesis of [9-$^2H_1$]-9-HNA (57). The acid was less likely to undergo a polymerisation reaction if it was protected as the methyl ester, since the methyl ester is less reactive than the free acid species. Thus, 8-HOA (52) was treated with acidic methanol to generate methyl 8-hydroxyoctanoate (59), which was then subjected to oxidation using PDC to generate the aldehyde (105)[111], and then reduction using NaB^2H_4 gave the isotopically labelled hydroxy ester (106). Finally, hydrolysis of the ester using KOH in water, and acidic work up allowed recovery of the desired deuterium labelled ω-hydroxy acid (58). The isotope content was judged to be 99 % after analysis by mass spectroscopy.

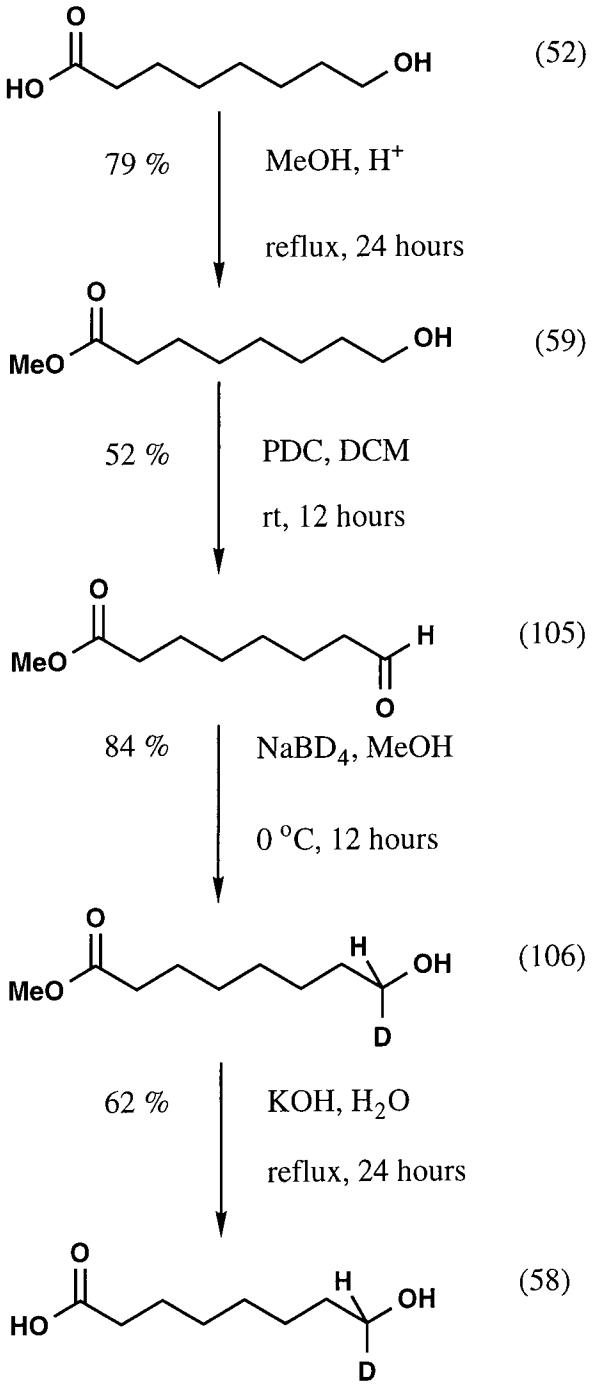


Figure 5.10. *Synthesis of [8-$^2H_1$]-8-hydroxyoctanoic acid (58)*[99].

5.6.1: Synthesis of 10-hydroxyundecylenic acid (61) and methyl 10-hydroxy-undecylenate (84).

Methyl 10-undecylenate (84) was prepared by refluxing undecylenic acid (107) in acidic methanol, as described earlier (see section 5.3.2). The resultant ester (79) was

isolated in very high yield (95 %). Methyl 10-hydroxyundecylenate (84) (see section 4.4.2) was then generated by adding methyl 10-undecylenate (79) to a stirred solution of mercuric acetate in a THF / water mix (1:1)[101]. After allowing 30 mins for the mercuration, sodium borohydride in sodium hydroxide was added to the solution, to achieve the demercuration, yielding methyl 10-hydroxyundecylenate (84) in moderate yield (69 %). 10-hydroxyundecanoic acid (61) (see section 3.4.1 and 4.4.2) could be prepared directly from 10-undecylenic acid (107) by mercuric acetate treatment in the manner described above (48 % yield)[101]. Mercuric acetate was employed in this system to exclusively allow the synthesis of the secondary alcohols (61 and 84). This $Hg(OAc)_2$ mediated hydration follows Markovnikov regiochemistry, and is well documented in the literature[101].

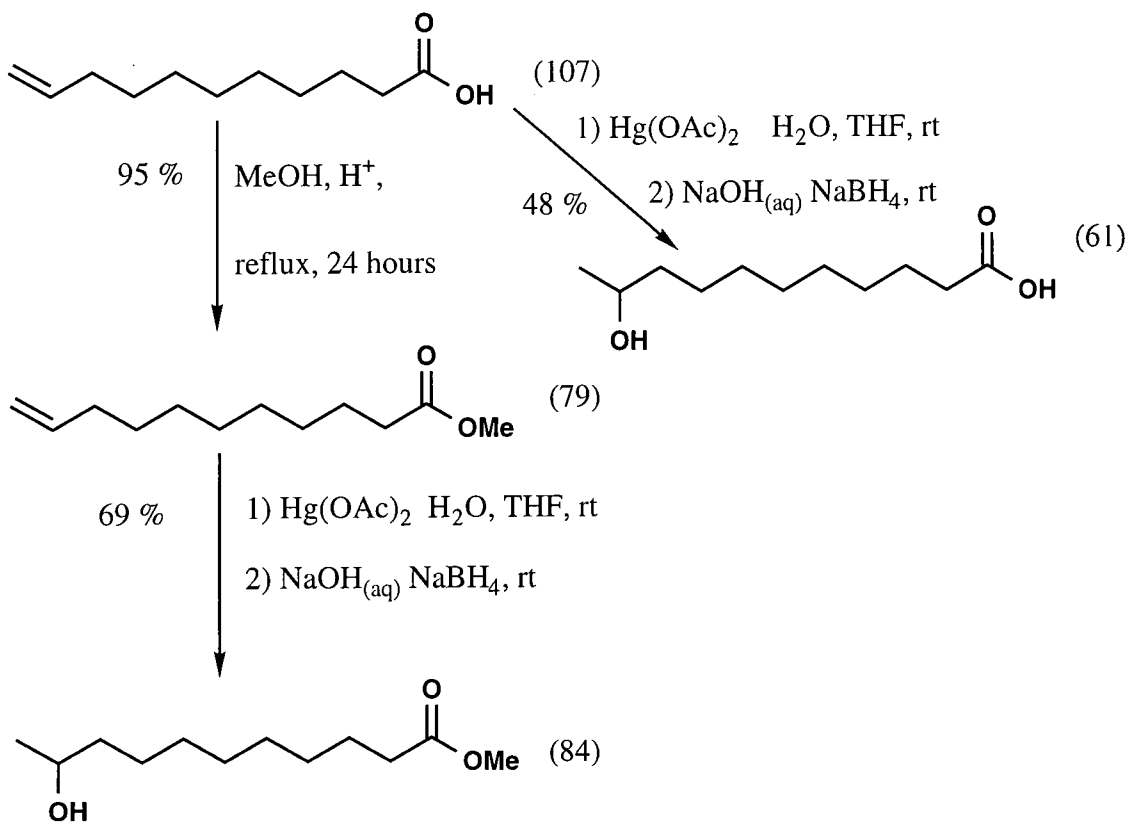


Figure 5.11. *Synthesis of 10-hydroxyundecanoic acid (61) and methyl 10-hydroxyundecylenate (84)[101].*

5.7.1: Methyl 11-hydroxyundecanoate (64) and (R) and (R,S) methyl 12-hydroxy-stearate (65).

11-Hydroxyundecanoic acid (43) was esterified in a straightforward manner by refluxing in acidic methanol (see Figure 5.12). This ester was employed in polymerisations using $Ti(OBu)_4$ as a catalyst (see section 3.6.1). Commercially available 12-(*R*)-hydroxystearate (108), derived from natural ricinoleic acid (109), was used as the starting material to prepare methyl 12-(*R*)-hydroxystearate (65) in good yield (75 %). The secondary alcohol was readily oxidised to the ketone (110) using PDC[99], and was subsequently reduced with borohydride to generate racemic methyl 12-hydroxy stearate (65), and this was also used in polymerisation reactions (see section 3.6.2). The synthesis is shown in Figure 5.13.

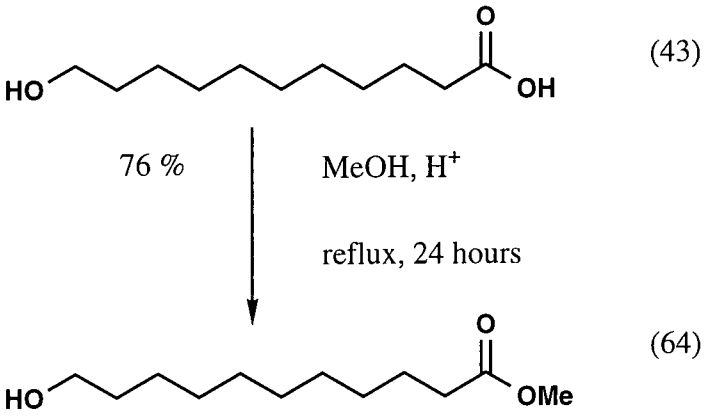


Figure 5.12. *Synthesis of methyl 11-hydroxyundecanoate (64).*

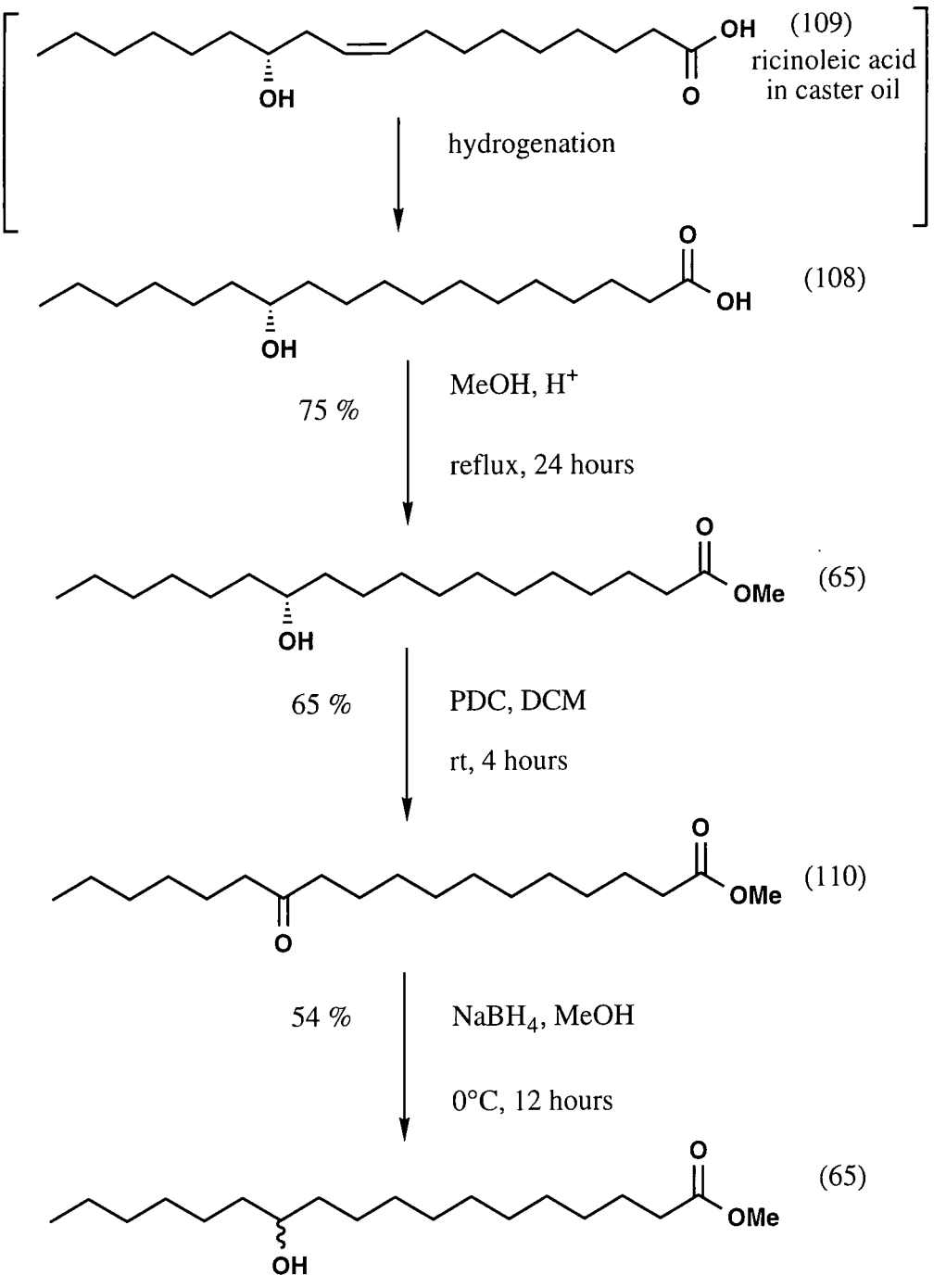


Figure 5.13. *Synthesis of* (R) *and* (R,S) *methyl 12-hydroxystearate (65) for the transesterification reaction.*

5.8: Synthesis for Chapter 4.

The synthesis for Chapter 4 involved the generation of trichloroethyl butyrate (74)[28], and the various candidate molecules for the affinity labelling experiments.

5.9.1: Synthesis of trichloroethyl butyrate (74).

Trichloroethyl butyrate (74) was generated as previously reported[28,84], in the reaction between 2,2,2-trichloroethanol (111) and butyryl chloride (112), in refluxing pyridine. Trichloroethyl butyrate (74) was purified by distillation and used in the investigations into the candidate affinity labels in section 4.3.2.

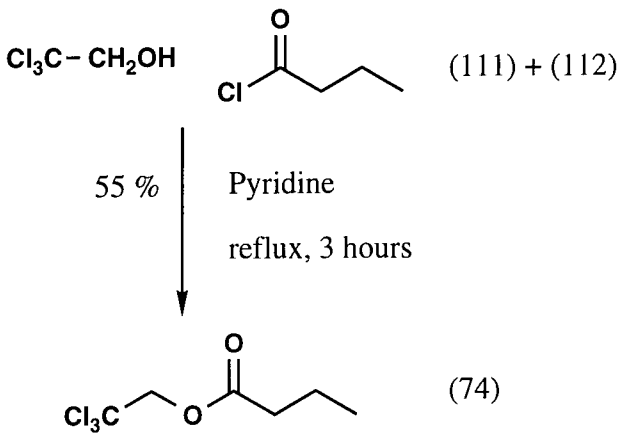


Figure 5.14. *Trichloroethyl butyrate (74) synthesis[28,84].*

5.10.1: Candidate affinity labels.

11-Hydroxyundecanoic acid (43) was esterified in refluxing acidic methanol in good yield (65 %). The hydroxy methyl ester (64) was then bromoacylated using bromoacetyl bromide (2 equivalents), administered dropwise, with a catalytic amount of DMAP and triethylamine (one equivalent), which proceeded in 60 % yield. Methyl 8-bromoacetoxyoctanoate (73) was then synthesised in the same manner, forming the hydroxymethyl ester (59) in 62 % yield, and the bromoester (73) in good yield (76 %). Both of these affinity labels were used in sections 4.3.1 and 4.3.2.

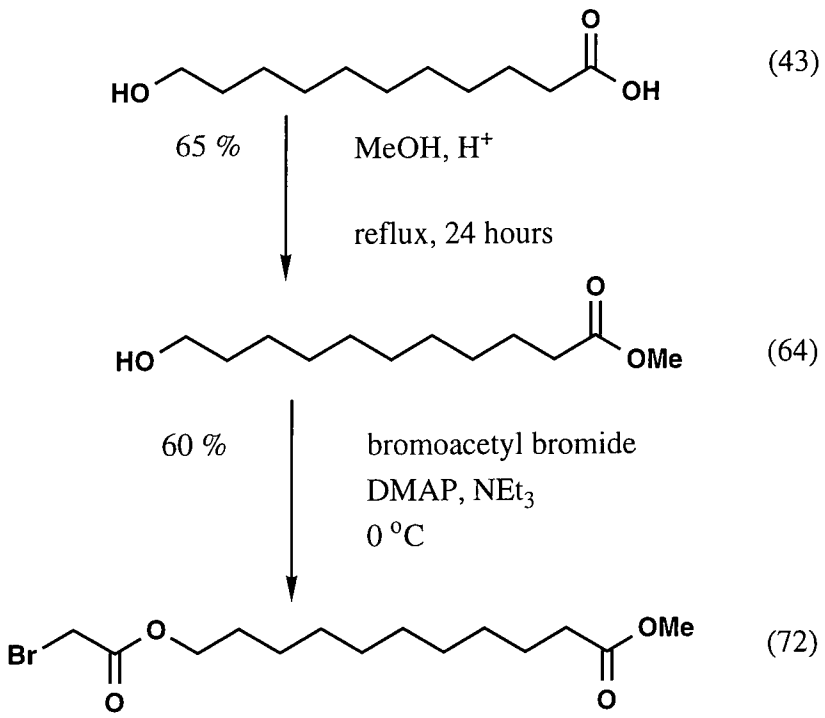


Figure 5.15. *Synthesis of methyl 11-bromoacetoxyundecanoate (72).*

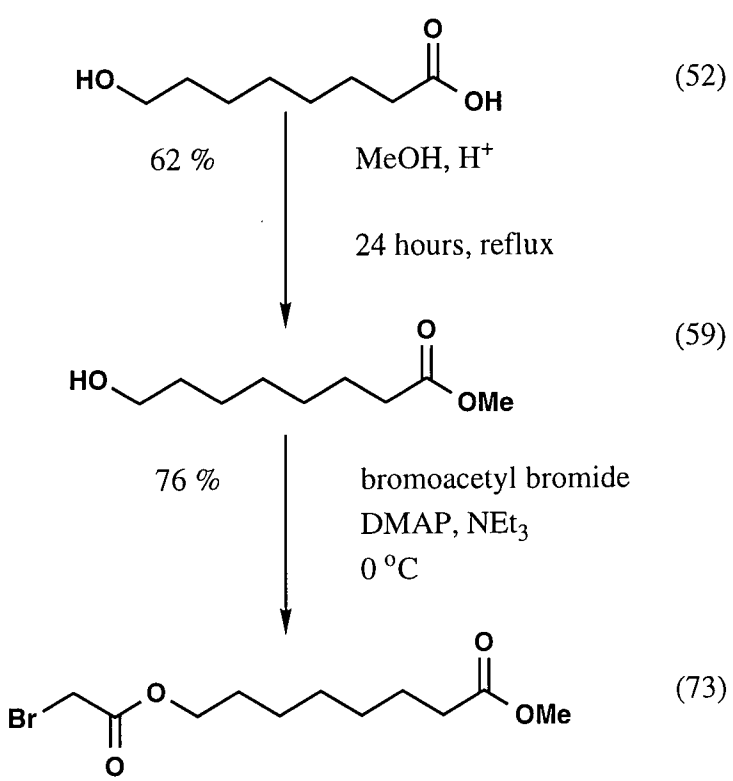


Figure 5.16. *Synthesis of methyl 8-bromoacetoxyoctanoate (73).*

CHAPTER SIX

EXPERIMENTAL

6.0: EXPERIMENTAL.

6.1: Instrumentation

NMR spectra were recorded on Varian-400S VXR, Varian-250 MHz Gemini and Varian-200 VXR spectrometers (^{1}H and ^{2}H at 400 MHz, 250 MHz and 200 MHz respectively, ^{13}C at 100.5 MHz and 62.5 MHz respectively). Infra red spectroscopy was run on either a Perkin Elmer 1600 series FTIR spectrometer or a Perkin Elmer Paragon 1000 FTIR spectrometer using NaCl disks. Mass spectrometry was run on a VG analytical 7070e mass spectrometer, in EI and CI [(M + H) or (M + NH_4)] modes. High resolution mass spectrometry was run on a VG ZAB-E mass spectrometer, in EI and CI (M + NH_4) modes. Matrix assisted laser desorption ionisation time of flight mass spectrometry (MALDITOF) was run on a Kompact Maldi iv machine, using a nitrogen laser (λ = 337 nm, with a 3 ns pulse). Samples were run in either a linear or reflectron mode, using a dihydroxybenzoic acid matrix in a saturated solution of 60 % acetonitrile and 40 % water. Elemental analysis was run on a CE-440 elemental analyzer. Gas chromatography was run on a Hewlett Packard 5890 Series 2 gas chromatograph, from 100 °C to 270 °C, with a flow rate of 10 $mmin.^{-1}$ on a 25 m silicone elastomer (SE 30) column. GPC was performed on Waters 590 Series chromatographic equipment, with three columns arranged in series and with dimensions 300 x 7.7 mm (Polymer Laboratories Ltd), packed with polystyrene PL gel (particle size 5 μm and pore sizes 100, 10^3, and 10^5 Å respectively). A Waters differential refractometer (model 401) was used as a detector, and polyester samples were dissolved in chloroform. The molecular mass averages were computed using Polymer Lab software, and calibrated against seven commercial polystyrene standards, molecular weights: 1 030 000; 777 000; 435 500; 127 000; 47 000; 3 250 and 162 Da. Hexane was dried over CaH_2 and stored over molecular sieves, all other solvents were distilled before use. All reations were carried out under N_2 unless otherwise stated. Ozone was generated using a Fischer Ozone Generator, model 500. Column chromatography was carried out on Kieselgel 60. TLCs were run on Kieselgel 60 F_{254} sheets.

6.2: Experimental for Chapter 2.

6.2.1: Enzymatic polymerisation, standard method.

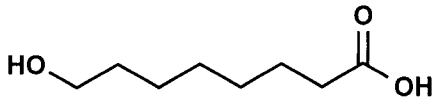
The *Candida rugosa* lipase (2.0 g, Sigma Chem. Co. Type VII) was added to a conical flask (50 ml), containing a suspension of 9-hydroxynonanoic acid (53) (200 mg, 1.1 mmol) in dry hexane (50 ml). (Initially 3 Å molecular sieves were employed to remove water formed through the esterification process. In the later experiments the molecular sieves were removed from the standard protocol). The flask was stoppered (septum seal) and placed on a shaker (200 rpm) at 55 °C for the required length of time (15 minutes to 10 days). The mixture was then filtered under suction, and washed with dichloromethane. Removal of the solvent under reduced pressure gave a residue containing both polymer and oligomer (80 to 150 mg, 40 to 75 % respectively)[61,62]. This material was analysed directly by ^{1}H NMR and GPC.

($CDCl_3$, 200 MHz) δ_H 4.0, 3.6 (2H, t, $J_{H\text{-}H}$ 6.6 Hz, $CH_2COOC\underline{H}_2$ (polymer), $C\underline{H}_2OH$ (monomer)), 2.3 (2H, t, $J_{H\text{-}H}$ 7.2 Hz, $C\underline{H}_2COOCH_2$), 1.6 (4H, m, $C\underline{H}_2CH_2COO$ and $OCOCH_2C\underline{H}_2$), 1.3 (4H to 20H, m, $C\underline{H}_2$'s). GPC analysis: M_n ranged from 600 to 10 000; M_W ranged from 1000 to 16 000.

6.2.2: Polymer precipitations in hexane.

A suspension of poly(11-hydroxyundecanoic acid) (43) (250 mg, 1.2 mmol) in hexane (40 ml) was shaken (200 rpm, at 55 °C) for 1 hour. The solution was filtered warm, at a temperature of 55 °C, and the solvent removed under reduced pressure, to yield a white amorphous residue (60 mg, 24 %). Both the filtrate and the residue were analysed by GPC: the low mass material remained predominantly in the hexane solution, whereas the higher mass material precipitated from solution. A typical run using polymer of M_W = 3000 yielded reprecipitated material with M_W = 16 000, and polydispersity of 1.6; the hexane-soluble material had M_W = 1500, with a polydispersity of 1.2.

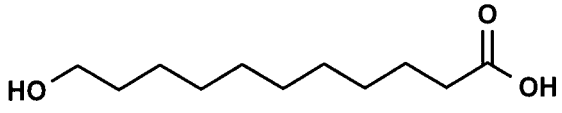
6.3.1: Preparation of 8-hydroxyoctanoic acid (52).



8-Bromooctanoic acid (89) (8.5 g, 0.04 mol) was heated under reflux for 7 days in a solution of KOH (10.8 g, 18.0 mol) in water (150 ml). The reaction mixture was cooled, filtered, and the filtrate evaporated under reduced pressure. Residual material was recrystallised three times from methanol, and washed with acetone. The resultant white solid was dissolved in water (100 ml), and acidified to pH 1 using 6N HCl, and the aqueous solution extracted into diethyl ether (2 x 50 ml). The combined organic extracts were washed with water and then dried ($MgSO_4$). Evaporation of the solvent under reduced pressure and purification by column chromatography (DCM:acetone, 6:1) afforded the title compound (4.0 g, 65 %) as a white amorphous solid, mp 56-57 °C (lit 58-58.5 °C)[89,90].

($CDCl_3$, 250 MHz) δ_H 3.66 (2H, t, J_{H-H} 6.6 Hz, C$\underline{H}_2$OH), 2.37 (2H, t, J_{H-H} 7.4 Hz, C$\underline{H}_2$COOH), 1.59 (4H, m, C$\underline{H}_2$CH$_2$OH and C$\underline{H}_2$CH$_2$COOH), 1.34 (6H, m, C$\underline{H}_2$s). ($CDCl_3$) δ_C 180.0 (COOH), 63.4 (CH_2OH), 34.5 (CH_2), 33.0 (CH_2), 29.5 (CH_2), 29.5 (CH_2), 26.0 (CH_2), 25.1 (CH_2). IR, (neat), υ_{max}/cm^{-1}: 3221 (OH), 2895 (CH), 2840 (CH), 1673 (CO), 975 (CH), 722 (CH). MS: m/z (EI) 161 [$(M + H)^+$ 3 %], 143 (54 %), 73 (60 %), 55 (100 %).

6.3.2: Preparation of 11- hydroxyundecanoic acid (43).

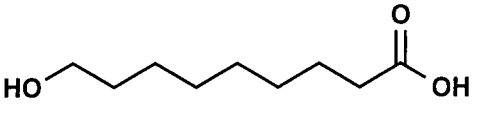


11-Bromoundecanoic acid (71) (15.0 g, 6.0 mmol) was heated under reflux for 7 days in a solution of KOH (10.8 g, 18.0 mol) in water (150 ml). The reaction mixture was cooled, filtered, and the filtrate evaporated under reduced pressure. Residual material

was recrystallised three times from methanol, and washed with acetone. The resultant white solid was dissolved in water (100 ml), and acidified to pH 1 using 6N HCl, and the aqueous solution extracted into diethyl ether (2 x 50 ml). The combined organic extracts were washed with water and then dried ($MgSO_4$). Evaporation of the solvent under reduced pressure and purification by column chromatography (DCM:acetone, 8:1) afforded the title compound (7.1 g, 62 %) as a white amorphous solid, mp 67-69 °C (lit 65-67 °C)[89].

($CDCl_3$, 250 MHz) δ_H 3.64 (2H, t, J_{H-H} 6.5 Hz, C<u>H</u>$_2$OH), 2.34 (2H, t, J_{H-H} 7.6 Hz, C<u>H</u>$_2$COOH), 1.65 (4H, m, C<u>H</u>$_2$CH$_2$COOH and HOCH$_2$C<u>H</u>$_2$), 1.29 (12H, m, C<u>H</u>$_2$s). ($CDCl_3$) δ_C 179.5 (COOH), 63.0 (CH_2OH), 34.0 (CH_2), 32.6 (CH_2), 29.4 (CH_2), 29.3 (CH_2), 29.2 (CH_2), 29.1 (CH_2), 29.0 (CH_2), 25.6 (CH_2), 24.6 (CH_2). IR, (neat), υ_{max}/cm^{-1}, 2917 (CH), 2848 (CH), 1686(CO), 1275 (CH), 1060 (CH), 915 (CH), 722 (CH). MS: m/z (CI) 220 [$(M + NH_4)^+$ 100 %], 202 (26 %), 185 (7 %).

6.4.1: Preparation of 9-hydroxynonanoic acid (53).

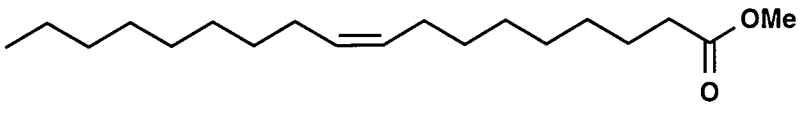


$NaBH_4$ (0.6 g, 15.0 mmol) and LiCl (0.6 g, 15.0 mmol) were added directly to a stirred solution of nonanedioic acid monomethyl ester (85) (1.0 g, 5.0 mmol) at 0 °C in monoglyme (20 ml). The reaction mixture was allowed to warm overnight, and then quenched with water and acidified to pH 1 using 6N HCl, which generated a white precipitate. The product was extracted into diethyl ether (3 x 30 ml), washed with water (30 ml), and the combined organic extracts dried ($MgSO_4$). The solvent was removed under reduced pressure, and purification by column chromatography (DCM:acetone, 6:1) afforded the product as a white waxy solid, (101 mg, 11 %), mp 50 °C (lit 51-51.5 °C)[90,102].

($CDCl_3$, 250 MHz) δ_H 3.63 (2H, t, J_{H-H} 7.2 Hz, C<u>H</u>$_2$OH), 2.31 (2H, t, J_{H-H} 7.6 Hz, C<u>H</u>$_2$COOH), 1.62 (4H, m, C<u>H</u>$_2$CH$_2$COOH and HOCH$_2$C<u>H</u>$_2$), 1.30 (8H, m C<u>H</u>$_2$s).

($CDCl_3$) δ_C 179.6 (COOH), 63.4 (CH_2OH), 34.5 (CH_2), 33.0 (CH_2), 29.6 (CH_2), 29.6 (CH_2), 29.4 (CH_2), 26.1 (CH_2), 25.1 (CH_2). IR, (neat), υ_{max}/cm^{-1}, 3439 (OH), 2931 (CH), 1733 (CO), 1214 (CH), 732 (CH). MS: m/z (CI) 192 [$(M + NH_4)^+$ 100 %], 174 (5 %), 157 (2 %). HRMS: (CI) $(M + NH_4)^+$ found 192.1600, $C_9H_{22}NO_3$ requires 192.1600.

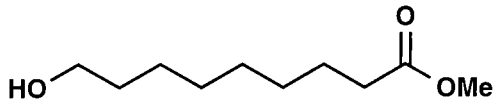
6.5.1: Preparation of methyl oleate (90).



A solution of oleic acid (86) (2.0 g, 7.0 mmol) in methanol (50 ml) containing conc. H_2SO_4 (2 ml) was heated under reflux for 24 hours. The solution was allowed to cool to ambient temperature before the greater part of the methanol was removed under reduced pressure, and the solution diluted with water (50 ml). The organic material was extracted into diethyl ether (3 x 20 ml), and the combined organic layers were washed with water (1 x 20 ml), and dried ($MgSO_4$). The solvent was removed under reduced pressure to afford the title compound as a colourless oil (1.6 g, 76 %) after purification by column chromatography.

($CDCl_3$, 250 MHz) δ_H 5.42 (2H, dt, J_{H-H} 6.7, 2.4 Hz, C<u>H</u>=C<u>H</u>), 3.66 (3H, s, COOC<u>H</u>$_3$), 2.26 (2H, t, J_{H-H} 7.4 Hz, C<u>H</u>$_2$COOMe), 1.99 (4H, dt, J_{H-H} 1.2 and 7.0 Hz, C<u>H</u>$_2$CH=CHC<u>H</u>$_2$), 1.58 (2H, m, C<u>H</u>$_2$CH$_2$COOMe), 1.27 (20H, m, C<u>H</u>$_2$s), 0.84 (3H, t, J_{H-H} 7.6 Hz, CH_3). ($CDCl_3$) δ_C 174.6 (<u>C</u>OOMe), 130.4, 130.1 (<u>C</u>=<u>C</u>), 51.8 (COO<u>C</u>H$_3$), 34.5 (CH_2), 32.4 (CH_2), 30.2 (CH_2), 30.1 (CH_2), 30.0 (CH_2), 29.8 (CH_2), 29.6 (CH_2), 29.6 (CH_2), 29.5 (CH_2), 29.5 (CH_2), 27.7 (CH_2), 27.6 (CH_2), 25.4 (CH_2), 23.1 (CH_2), 14.5 (CH_3). IR, (neat) υ_{max}/cm^{-1}, 2921 (CH), 2851 (CH), 1740 (CO), 1168 (CH), 722 (CH). MS: m/z (CI) 315 [$(M + NH_4)^+$, 100 %], 297 [$(M + H)^+$ 14 %]. Anal: found C (76.79), H (12.17), $C_{19}H_{36}O_2$ requires C (77.03), H (12.16).

6.5.2: Preparation of methyl 9-hydroxynonanoate (92).

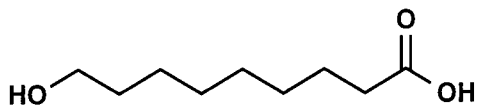


Ozone was passed through a solution of methyl oleate (90) (1.6 g, 5.4 mmol) in dichloromethane (20 ml) at -78°C. When the reaction mixture turned blue, dimethyl sulphide (670 mg, 10.8 mmol) was added. The solvent was then removed under reduced pressure, and the residue suspended in methanol (20 ml) at 0 °C[91].

A solution of $NaBH_4$ (0.4 g, 10.8 mmol) in methanol (20 ml) was added directly at 0 °C, and the solution allowed to warm to ambient temperature overnight. Excess solvent was removed under reduced pressure, the solution diluted with water (50 ml) and the organic product extracted into diethyl ether (3 x 20 ml). The combined organic extracts were washed with water (1 x 20 ml), dried ($MgSO_4$), and the solvent removed under reduced pressure. Purification by column chromatography (DCM:acetone, 10:1) afforded the title compound as a colourless oil (51 mg, 5 %).

($CDCl_3$, 200 MHz) δ_H 3.66 (3H, s, COOC<u>H</u>$_3$), 3.65 (2H, t, J_{H-H} 6.8 Hz, C<u>H</u>$_2$OH), 2.30 (2H, t, J_{H-H} 7.2 Hz, C<u>H</u>$_2$COOMe), 1.61 (4H, m, C<u>H</u>$_2$CH$_2$OH and C<u>H</u>$_2$CH$_2$COOMe), 1.31 (8H, m, CH_2s). ($CDCl_3$) δ_C 176.4 (<u>C</u>OOMe), 65.3 (COH), 53.3 (COO<u>C</u>H$_3$), 36.4 (CH_2), 33.3 (CH_2), 31.5 (CH_2), 31.4 (CH_2), 31.0 (CH_2), 27.7 (CH_2), 26.9 (CH_2). IR, (neat) υ_{max}/cm^{-1}: 2935 (CH), 2846 (CH), 1736 (CO), 1175 (CH), 1061 (CH). MS: m/z (CI) 206 [$(M + NH_4)^+$ 100 %] 189 (8 %), 173 (4 %). Anal: found C (63.69), H (10.74), $C_{10}H_{20}O_3$ requires C (63.83), H (10.64).

6.5.3: Preparation of 9-hydroxynonanoic acid (53).

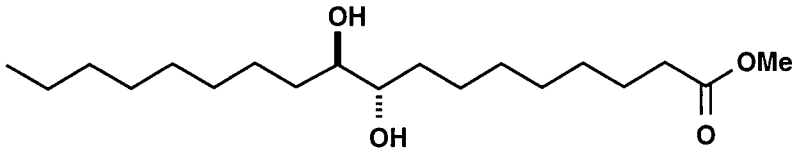


To a stirred solution of methyl 9-hydroxynonanoate (92) (51 mg, 0.3 mmol) in

methanol (10 ml) was added a solution of KOH (0.7 g) in water (2 ml), and the mixture heated under reflux for 24 hours. The solution was allowed to cool to ambient temperature before the greater part of the methanol was removed under reduced pressure, and the residue diluted with water (20 ml). The organic material was extracted into diethyl ether (3 x 20 ml), and the combined organic extracts were washed with water (20 ml) and dried ($MgSO_4$). The solvent was removed under reduced pressure to afford the title compound as a white waxy solid (24 mg, 51 %) after purification by column chromatography, mp 51 °C (lit 51-51.5 °C)[89,102].

This material was spectroscopically identical with previously prepared material (see section 6.3.1).

6.6.1: Preparation of methyl erythro-*9,10-dihydroxyoleate (93).*

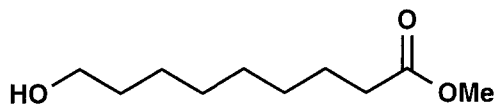


To a stirred solution of methyl oleate (90) (1.0 g, 3.4 mmol) in THF:acetone:water (30 ml of solvent in a 9:1:1 ratio respectively) at room temperature was added a solution of OsO_4 (1 ml, 0.39 molar solution in toluene), and N-methylmorpholin-N-oxide (NMO, 1.41 g, 12.0 mmol). The reaction was quenched after 24 hours by dilution with water (30 ml) and the solution acidified. The organic products were extracted into diethyl ether (3 x 30 ml) and the combined organic layers washed with water (1 x 30 ml), dried ($MgSO_4$), and the solvent removed under reduced pressure. The title compound was obtained after purification by column chromatography (eluent DCM:acetone, 4:1) as a colourless oil (1.0 g, 88 %)[92].

($CDCl_3$, 250 MHz) δ_H 6.32 (2H, s, 2 x OH), 3.66 (3H, s, $COOC\underline{H}_3$), 3.60 (2H, m, 2 x $C\underline{H}OH$), 2.30 (2H, t, J_{H-H} 7.2 Hz, $C\underline{H}_2COOMe$), 1.78 (4H, m, 2 x $C\underline{H}_2CHOH$), 1.44 (2H, m, $C\underline{H}_2CH_2COOMe$), 1.30 (20H, m, $C\underline{H}_2s$), 0.87 (3H, t, J_{H-H} 7.6 Hz, $C\underline{H}_3$). ($CDCl_3$) δ_C 176.3 ($\underline{C}OOCH_3$), 155.8 (CH_2OH diol), 154.5 (CH_2OH diol), 53.5

(COO<u>C</u>H3), 36.1 (CH_2), 33.9 (CH_2), 33.2 (CH_2), 33.2 (CH_2), 31.7 (CH_2), 31.7 (CH_2), 31.4 (CH_2), 31.3 (CH_2), 31.2 (CH_2), 31.1 (CH_2), 29.0 (CH_2), 27.9 (CH_2), 26.9 (CH_2), 24.7 (CH_2), 16.1 (CH_3). IR, (neat), υ_{max}/cm^{-1} 3265 (OH), 2914 (CH), 2847 (CH), 1734 (CO), 1172 (CH), 1069 (CH), 720 (CH). MS: m/z (EI), sample decomposed on mass analysis. Significant peaks were found at: 298 (4 %), 143 (25 %), 75 (100 %). Anal: found C (68.72), H (11.55), $C_{19}H_{38}O_4$ requires C (69.09), H (11.52).

6.6.2: Preparation of methyl 9-hydroxynonanoate (92).

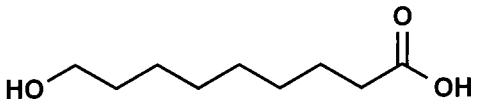


To a solution of methyl *erythro*-9,10-dihydroxyoleate (93) (1.0 g, 3.0 mmol) in THF:water (30 ml, 1:1 ratio) was added $NaIO_4$ (1.5 g, 6.8 mmol), and the reaction monitored by working up aloquots and recording ^1H NMR spectra until the aldehydic proton was present. The solvent was removed after 24 hours under reduced pressure, and the product stirred in a solution of methanol (30 ml) at 0 °C.

To this cooled solution was added $NaBH_4$ (260 mg, 6.8 mmol) and the solution was allowed to warm to ambient temperature overnight, after which most of the methanol was removed under reduced pressure. The residual solution was diluted with water (30 ml), acidified to pH 1 with 6N HCl, and the organic material extracted into diethyl ether (3 x 30 ml). The combined organic layers were washed with water (1 x 30 ml), dried ($MgSO_4$), and the solvent removed under reduced pressure, to afford the title compound after purification by column chromatography (DCM:acetone, 10:1) as a colourless oil (245 mg, 43 %).

This material was spectroscopically identical with material previously prepared (see section 6.4.2).

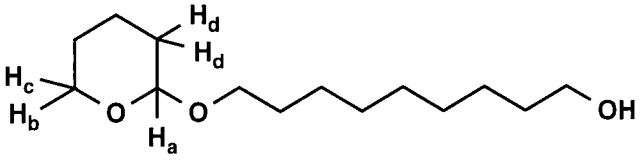
6.6.3: Preparation of 9-hydroxynonanoic acid (53).



Methyl 9-hydroxynonanoate (92) (245 mg, 1.3 mmol) was stirred at room temperature for 48 hours in an aqueous methanolic solution of KOH (0.7 g KOH in 10 ml MeOH). After complete hydrolysis (followed by TLC), the methanol was removed under reduced pressure, the residue was suspended in water (30 ml), and acidified to pH 1 using 6N HCl. The white solid was filtered, washed with water (1 x 30 ml), and dried ($MgSO_4$) to yield the title compound (118 mg, 52 %) after purification by column chromatography (DCM:acetone 8:1), mp 49 °C (lit 51-51.5 °C)[89,102].

This material was specroscopically identical with previously prepared material (see section 6.3.1).

6.7.1: Preparation of 9-(tetrahydropyran-2-yloxy)nonan-1-ol. (95)

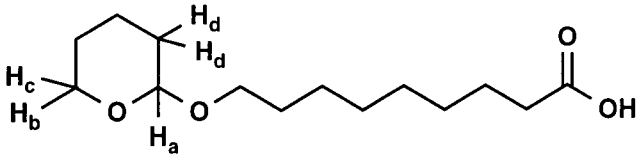


Nonane-1,9-diol (94) (12.0 g, 0.08 mol) was stirred in dry dichloromethane (60 ml) containing dihydropyran (9.5 g, 0.11 mol) and pyridinium *p*-toluene sulfonate (1.6 g, 8.0 mmol) at room temperature for 24 hours. The reaction was quenched by dilution with diethyl ether (50 ml) and the reaction mixture was washed with brine (50 ml). The organic extract was then dried ($MgSO_4$), and the solvent removed under reduced pressure. Purification by column chromatography (DCM:acetone, 10:1) afforded the title compound as a colouress oil (7.3 g, 40 %)[93].

($CDCl_3$, 400 MHz) δ_H 4.56 (1H, t, J_{H-H} 2.9 Hz, $\underline{H}a$), 3.81 (1H, td, J_{H-H} 10.2 and 2.4 Hz, $\underline{H}b$), 3.64 (1H, dt, J_{H-H} 9.6 and 2.4 Hz, $\underline{H}c$), 3.63 (4H, t, J_{H-H} 6.8 Hz, 2 x $OC\underline{H}_2$),

1.62 (2H, m, $\underline{H}_d$), 1.54 (4H, m, 2 x $C\underline{H}_2CH_2O$), 1.29 (14H, m, $C\underline{H}_2$'s). ($CDCl_3$) δ_C 98.2 (3°C), 67.3 (CH_2O), 63.5 (CH_2OH), 61.7 (CH_2O), 33.9 (CH_2), 32.9 (CH_2), 30.0 (CH_2), 28.8 (CH_2), 28.8 (CH_2), 28.7 (CH_2), 26.7 (CH_2), 25.3 (CH_2), 25.0 (CH_2), 24.4 (CH_2). IR, (neat), υ_{max}/cm^{-1}, 2916 (CH), 2855 (CH), 1450 (CH), 1116 (CH), 1013 (CH). MS: m/z (EI) 243 [$(M - H)^+$ 2 %], 85 (100 %). Anal: found C (68.05), H (11.30), $C_{14}H_{28}O_3$ requires C (68.85), H (11.48).

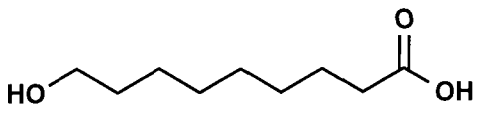
6.7.2: Preparation of 9-(tetrahydropyran-2-yloxy)nonanoic acid (96).



To a vigourously stirred solution of racemic 9-(tetrahydropyran-2-yloxy)nonan-1-ol (95) (7.3 g, 0.03 mmol) in carbon tetrachloride (30 ml), acetonitrile (30 ml) and water (40 ml) were added $RuCl_3.6H_2O$ (115 mg, 0.022 mol equivalents) and KIO_4 (18.0 g, 0.08 mol). The reaction was quenched after 12 hours by dilution with dichloromethane (50 ml), the layers were separated, and the organic material was extracted into diethyl ether (2 x 30 ml). The combined organic extracts were dried ($MgSO_4$), the solvent removed under reduced pressure and the residue diluted with diethyl ether (30 ml). This solution was filtered through celite, the filtrate collected, and the solvent evaporated under reduced pressure to afford the title compound (3.3 g, 43 %) as a colourless oil, after purification by column chromatography (DCM:acetone, 10:1)[94]. ($CDCl_3$, 400 MHz) δ_H 4.56 (1H, t, J_{H-H} 3.2 Hz, $\underline{H}_a$), 3.81 (1H, td, J_{H-H} 10.0 and 2.6 Hz, $\underline{H}_b$), 3.64 (1H, dt, J_{H-H} 9.3 and 2.6 Hz, $\underline{H}_c$), 3.63 (2H, t, J_{H-H} 6.8 Hz, $OC\underline{H}_2$), 2.34 (2H, t, J_{H-H} 7.6 Hz, $C\underline{H}_2COOH$), 1.62 (2H, m, $\underline{H}_d$), 1.54 (4H, m, $C\underline{H}_2CH_2O$), 1.29 (12H, m, $C\underline{H}_2$'s). ($CDCl_3$) δ_C 178.5 (COOH), 99.3 (3°C), 68.0 (CH_2O), 62.8 (CH_2O), 33.7 (CH_2), 31.2 (CH_2), 29.3 (CH_2), 29.0 (CH_2), 28.9 (CH_2), 28.7 (CH_2), 26.5 (CH_2), 25.8 (CH_2), 25.1 (CH_2), 24.4 (CH_2). IR, (neat), υ_{max}/cm^{-1}, 2930 (CH), 2856 (CH), 1733 (CO), 1708 (CO), 1462 (CH), 1179 (CH), 733 (CH). MS: m/z (EI) 257 [$(M - H)^+$

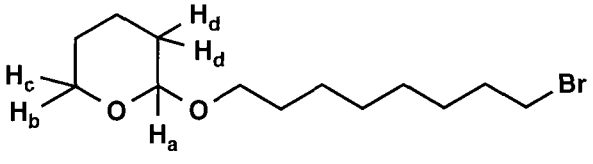
6 %], 85 (100 %). HRMS: (EI) $(M - H)^+$ found 257.1753, $C_{14}H_{25}O_4$ requires 257.1753.

6.7.3: Preparation of 9-hydroxynonanoic acid (53).



A solution of 9-(tetrahydropyran-2-yloxy)nonanoic acid (96) (3.3 g, 0.01 mol) and pyridinium *p*-toluenesulphonate (216 mg, 0.86 mmol) in ethanol (50 ml) was stirred for 5 hours at 55 °C. The solvent was removed under reduced pressure, and the reaction mixture purified by column chromatography (DCM:acetone, 8:1) to afford the title compound as a waxy white solid (112 mg, 5 %), mp 49 °C (lit 51-51.5 °C)[93,102]. This material was spectroscopically identical with previously prepared material (see section 6.3.1).

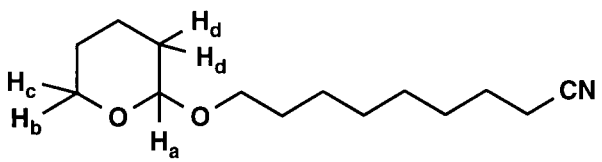
6.8.1: Preparation of 1-bromo-8-(tetrahydropyran-2-yloxy)octane (98)



To a stirred solution 8-bromooctanol (97) (5.0 g, 24.0 mmol) at room temperature in dry dichloromethane (30 ml) were added dihydropyran (3.0 g, 36.0 mmol) and pyridinium *p*-toluene sulfonate (600 mg, 2.4 mmol). After four hours the solution was diluted with diethyl ether (30 ml), washed with brine (20 ml), dried ($MgSO_4$), and the solvent removed under reduced pressure. After purification by column chromatography (DCM:acetone, 12:1) the title compound was recovered (6.3 g, 92 %) as a colourless oil[93].

($CDCl_3$, 400 MHz) δ_H 4.52 (1H, t, J_{H-H} 2.4 Hz, $\underline{H}_a$), 3.80 (1H, td, J_{H-H} 10.4 and 2.6 Hz, $\underline{H}_b$), 3.67 (1H, dt, J_{H-H} 9.4 and 2.6 Hz, $\underline{H}_c$), 3.62 (2H, t, J_{H-H} 6.8 Hz, $C\underline{H}_2O$), 3.30 (2H, t, J_{H-H} 6.9 Hz, $C\underline{H}_2Br$), 1.60 (2H, m, $\underline{H}_d$), 1.49 (4H, m, $C\underline{H}_2CH_2O$), 1.24 (12H, m, $C\underline{H}_2$'s). ($CDCl_3$) δ_C 98.7 (3°C), 67.5 (CH_2O), 62.2 (CH_2O), 33.8 (CH_2Br), 32.7 (CH_2), 30.7 (CH_2), 29.6 (CH_2), 29.2 (CH_2), 28.6 (CH_2), 28.0 (CH_2), 26.0 (CH_2), 25.4 (CH_2), 19.6 (CH_2). IR, (neat), υ_{max}/cm^{-1}, 2927 (CH), 1118 (CH), 1075 (CH), 1021 (CH), 867 (CH), 812 (CH), 736 (CH), 642 (CBr). MS: m/z (EI) 293 [$(M - H)^+$ 2 %], 291 [$(M - H)^+$ 3 %)] 85 (100 %). HRMS: (EI) $(M-1)^+$ 291.0960, $C_{13}H_{24}O_2Br$ requires 291.0960.

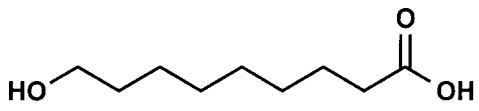
6.8.2: Preparation of 9-(tetrahydropyran-2-yloxy)nonanonitrile (99).



1-Bromo-8-(tetrahydropyran-2-yloxy) octane (98) (6.3 g, 21.5 mmol) was heated under reflux in dry methanol (50 ml) containing KCN (1.4 g, 21.0 mmol) for 48 hours. The solution was allowed to cool to ambient temperature before the solvent was removed under reduced pressure, and the residue extracted into diethyl ether (3 x 30 ml). The combined organic extracts were washed with water (30 ml), dried ($MgSO_4$), and the solvent removed under reduced pressure. Purification by column chromatography (DCM:acetone, 10:1) afforded the title compound as a colourless oil (2.88 g, 56 %)[95]. ($CDCl_3$, 400 MHz) δ_H 4.59 (1H, t, J_{H-H} 3.0 Hz, $\underline{H}_a$), 3.83 (1H, td, J_{H-H} 10.2 and 2.8 Hz, $\underline{H}_b$), 3.65 (1H, dt, J_{H-H} 9.6 and 2.4 Hz, $\underline{H}_c$), 3.63 (2H, t, J_{H-H} 6.8 Hz, $C\underline{H}_2O$), 3.39 (2H, t, J_{H-H} 6.9 Hz, $C\underline{H}_2Br$), 2.21 (2H, t, J_{H-H} 6.5 Hz, $C\underline{H}_2CN$), 1.63 (2H, m, $\underline{H}_d$), 1.56 (4H, m, J_{H-H} 6.2 Hz, $C\underline{H}_2CH_2O$), 1.29 (10H, m, $C\underline{H}_2$'s). ($CDCl_3$) δ_C 119.5 (CN), 98.6 (3°C), 67.5 (CH_2O), 62.1 (CH_2O), 30.5 (CH_2), 29.5 (CH_2), 28.9, (CH_2) 28.4 (CH_2), 28.3 (CH_2), 25.9 (CH_2), 25.2 (CH_2), 25.1 (CH_2), 19.4 (CH_2), 16.8 (CH_2). IR, (neat), υ_{max}/cm^{-1}, 3467 (OH), 2933 (CH), 2857 (CH), 2246 (CN), 1120 (CH), 1032 (CH),

733 (CH). MS: m/z (EI) 239 (M^+, 15 %), 210 (37 %), 166 (100 %), 156 (89 %). HRMS: (EI) $(M-H)^+$ found 238.1807, $C_{14}H_{24}NO_2$ requires 238.1807.

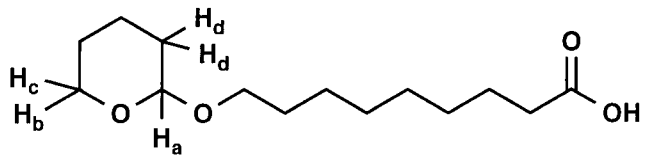
6.8.3: Preparation of 9-hydroxynonanoic acid (53).



9-(Tetrahydropyran-2-yloxy)nonanonitrile (99) (2.88 g, 12.0 mmol) was heated under reflux in conc. HCl (50 ml) and water (50 ml) for 48 hours. The organic products were extracted into diethyl ether (3 x 30 ml), and the combined organic layers were washed with water (1 x 30 ml) and dried ($MgSO_4$). The solvent was then removed under reduced pressure to afford the title product after purification by column chromatography (DCM:acetone, 6:1) as a white waxy solid (168 mg, 8 %) (mp 50 °C, lit 51-51.5 °C)[102].

This material was spectroscopically identical with previously prepared material (see section 6.3.1).

6.8.4: Preparation of 9-(tetrahydropyran-2-yloxy)nonanoic acid (96).

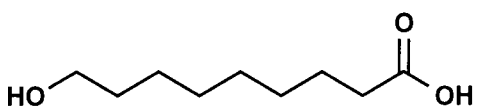


9-(Tetrahydropyran-2-yloxy)nonanonitrile (99) (1.0 g, 4.2 mmol) was heated under reflux in K^{t}OBu in ^{t}BuOH (30 ml, containing 10 % H_2O_2 v/v) for 48 hours. The solution was acidified to pH 1 with 6N HCl, the organic products were extracted into diethyl ether (3 x 30 ml), and the combined organic extracts washed once with water (30 ml), and dried ($MgSO_4$). The solvent was then removed under reduced pressure,

and the title product was obtained after column chromatography (DCM:acetone, 8:1) as a colourless oil (227 mg, 21 %)[96].

($CDCl_3$, 400 MHz) δ_H 4.55 (1H, t, J_{H-H} 2.9 Hz, $\underline{H}_a$), 3.79 (1H, td, J_{H-H} 10.2 and 2.6 Hz, $\underline{H}_b$), 3.66 (1H, dt, J_{H-H} 9.6 and 2.4 Hz, $\underline{H}_c$), 3.60 (2H, t, J_{H-H} 6.6 Hz, $OC\underline{H}_2$), 2.31 (2H, t, J_{H-H} 7.4 Hz, $C\underline{H}_2COOH$), 1.60 (2H, m, $\underline{H}_d$), 1.53 (4H, m, $C\underline{H}_2CH_2O$), 1.27 (12H, m, $C\underline{H}_2$'s). ($CDCl_3$) δ_C 178.6 (COOH), 98.3 (3°C), 68.3 (CH_2O), 62.7 (CH_2O), 34.4 (CH_2), 33.9 (CH_2), 29.9 (CH_2), 29.5 (CH_2), 28.9 (CH_2), 28.6 (CH_2), 26.6 (CH_2), 25.7 (CH_2), 25.4 (CH_2), 24.7 (CH_2). IR, (neat), υ_{max}/cm^{-1}, 2927 (CH), 2851 (CH), 1729 (CO), 1700 (CO), 1459, 1184 (CH). MS: m/z (EI) 257 [$(M - H)^+$ 6 %], 85 (100 %). HRMS: (EI) $(M - H)^+$ found 257.1753, $C_{14}H_{25}O_4$ requires 257.1753.

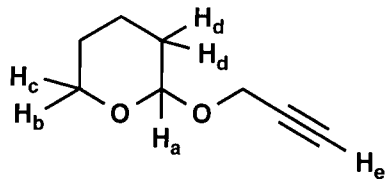
6.8.5: Preparation of 9-hydroxynonanoic acid (53).



9-(Tetrahydropyran-2-yloxy)nonanoic acid (96) (212 mg, 0.8 mmol) was dissolved in ethanol (20 ml) containing pyridinium *p*-toluenesulphonate (22 mg, 0.08 mmol), and the solution was stirred for 5 hours at 55 °C. The solution was allowed to cool to ambient temperature before the solvent was removed under reduced pressure. The reaction mixture was then purified by column chromatography (DCM:acetone, 6:1) to afford the title compound as a waxy white solid (78 mg, 39 %), mp 51 °C (lit 51-51.5 °C)[93,102].

This material was spectroscopically identical with previously prepared material (see section 6.3.1).

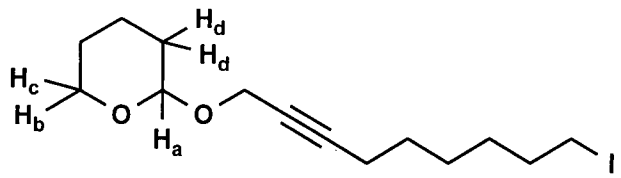
6.9.1: Preparation of 2-(prop-2'-ynyloxy)tetrahydropyran (100).



To a stirred solution of propargylic alcohol (5.0 g, 89.3 mmol) and PPTS in DCM (15 ml) at room temperature was added dihydropyran (8.3 g, 98.2 mmol). The reaction mixture was stirred for four hours, diluted with diethyl ether (15 ml), washed once with brine, dried ($MgSO_4$), and the solvent removed under reduced pressure. After distillation (bp 45 °C, 10 mm Hg) the title product was obtained as a colourless oil (11.9 g, 95 %)[93].

($CDCl_3$, 400 MHz) δ_H 4.71 (1H, t, J_{H-H} 3.2 Hz, Ha), 4.14 [2H, 2 x dd (AB), J_{H-H} 9.6, 2.4 and 0.8 Hz, CCCH_2O], 3.72 (1H, dt, J_{H-H} 10.2 and 2.4 Hz, Hb), 3.45 (1H, dt, J_{H-H} 9.6 and 2.4 Hz, Hc), 2.35 (1H, t, J_{H-H} 2.4 Hz, He), 1.62 (2H, m, Hd), 1.45 (4H, m, CH_2's). ($CDCl_3$) δ_C 98.7 (3°C), 81.7 (CH_2CC), 76.0 (CH_2CC), 63.8 (CH_2CC), 55.9 (CH_2O), 32.1 (CH_2), 27.2 (CH_2), 20.0 (CH_2). IR, (neat), υ_{max}/cm^{-1}, 3262 (OH), 2941 (CH), 2047 (CC), 1117 (CH), 1022 (CH), 869 (CH), 626 (CH). MS: m/z (EI) 139 [$(M - H)^+$ 12 %], 85 [$(MH - C_3H_3O)^+$ 100 %], 56 [$(C_3H_3O)^+$ 82 %]. HRMS: (EI) $(M - H)^+$ found 139.0758, $C_8H_{11}O_2$ requires 139.0758.

6.9.2: Preparation of 1-iodo-9-(tetrahydropyran-2-yloxy)non-7-yne (101).

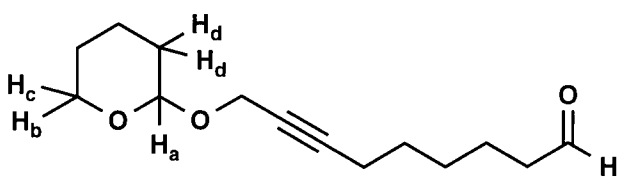


To a stirred solution of 2-(prop-2-ynyloxy)tetrahydropyran (100) (5.0 g, 35.7 mmol) in THF (30 ml) at -78 °C was added nBuLi (24.5 g, 39.3 mmol). After 30 minutes 1,6-diiodohexane (18.0 g, 53.3 mmol) was added to this solution, which was then heated

under reflux for one hour. The solvent was removed under reduced pressure, and the product purified by column chromatography (DCM:acetone, 14:1) to afford the title product as a colourless oil (6.1 g, 49 %)[97].

($CDCl_3$, 400 MHz) δ_H 4.70 (1H, t, J_{H-H} 2.9 Hz, $\underline{H}_a$), 4.14 (2H, 2 x td, J_{H-H} 12.8 and 2.0 Hz, CCC$\underline{H}_2$O), 3.73 (1H, td, J_{H-H} 9.8 and 2.6 Hz, $\underline{H}_b$), 3.43 (1H, dt, J_{H-H} 9.2 and 2.8 Hz, $\underline{H}_c$), 3.09 (2H, t, J_{H-H} 7.2 Hz, C$\underline{H}_2$I), 2.13 (2H, tt, J_{H-H} 6.8 and 2.0 Hz, CCC$\underline{H}_2$), 1.62 (2H, m, $\underline{H}_d$), 1.45 (4H, m, C$\underline{H}_2$'s), 1.36 (8H, m, C$\underline{H}_2$'s). ($CDCl_3$) δ_C 96.2 (3°C), 85.9 ($CH_2\underline{C}C$), 75.7 ($OCH_2C\underline{C}$), 61.6 ($O\underline{C}H_2CC$), 54.2 (CH_2O), 33.0 (CH_2), 30.1 (CH_2), 29.7 (CH_2), 28.0 (CH_2), 27.4 (CH_2), 25.1 (CH_2), 18.8 (CH_2), 18.4 (CH_2), 6.7 (CH_2). IR, (neat), υ_{max}/cm^{-1}, 2935 (CH), 2855 (CH), 2222 (CC), 1201 (CH), 1117 (CH), 1023 (CH), 903 (CH), 815 (CH), 668 (C-I). MS: m/z 351 [$(M + H)^+$ 1 %], 249 (6 %), 85 [$(C_5H_9O)^+$ 100 %). Anal: found C (48.15), H (6.48), $C_{14}H_{23}O_2I$ requires C (48.00), H (6.57).

6.9.3: Preparation of 9-(tetrahydropyran-2-yloxy)non-7-ynal (102).

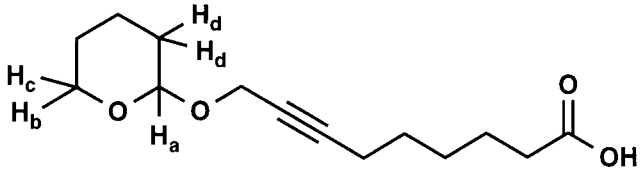


To a solution of 1-iodo-9-(tetrahydropyran-2-yloxy)non-7-yne (101) (5.0 g, 14.3 mmol) in DMSO (25 ml) at room temperature was added $NaHCO_3$ (2.5 g, 29.6 mmol). The solution was then heated at 130 °C for 2 hours, when it was poured onto ice (30 ml), and the organic material extracted into diethyl ether (3 x 20 ml). The combined organic extracts were dried ($MgSO_4$), and the solvent removed under reduced pressure. Purification by column chromatography (DCM:acetone, 10:1) afforded the title compound as a colourless oil (1.5 g, 44 %)[98].

($CDCl_3$, 400 MHz) δ_H 9.70 (1H, t, J_{H-H} 1.6 Hz, C$\underline{H}$O), 4.73 (1H, t, J_{H-H} 3.6 Hz, $\underline{H}_a$), 4.18 (2H, 2 x td, J_{H-H} 12.8 and 2.4 Hz, CCC$\underline{H}_2$O), 3.76 (1H, td, J_{H-H} 10.2 and 2.6 Hz, $\underline{H}_b$), 3.46 (1H, dt, J_{H-H} 9.2 and 2.6 Hz, $\underline{H}_c$), 2.39 (2H, td, J_{H-H} 7.6 and 1.6 Hz,

$C\underline{H}_2CHO$), 2.16 (2H, tt, $J_{H\text{-}H}$ 6.8 and 2.4 Hz, $CCC\underline{H}_2$), 1.62 (2H, m, $\underline{H}_d$), 1.45 (4H, m, $C\underline{H}_2$'s), 1.36 (6H, m, $C\underline{H}_2$'s). ($CDCl_3$) δ_C 202.1 (CHO), 96.3, (3°C), 85.8 ($OCH_2\underline{C}C$), 75.8 ($OCH_2C\underline{C}$), 61.6 ($O\underline{C}H_2CC$), 54.2 (CH_2O), 43.4 ($\underline{C}H_2CHO$), 30.0 (CH_2), 28.0 (CH_2), 28.0 (CH_2), 25.1 (CH_2), 21.3 (CH_2), 18.8 (CH_2), 18.3 (CH_2). IR, (neat), υ_{max}/cm^{-1}, 2941 (CH), 2861 (CH), 2721 (CH), 2249 (CC), 1724 (CO), 1201 (CH), 1117 (CH), 1022 (CH), 909 (CH), 815 (CH). MS: m/z, (EI) 239 [$(M + H)^+$ 37 %], 155 (5 %), 85 (100 %). HRMS: (EI) $(M + H)^+$ found 239.1647, $C_{14}H_{23}O_3$ requires 239.1647.

6.9.4: Preparation of 9-(tetrahydropyran-2-yloxy)non-7-ynoic acid (103).

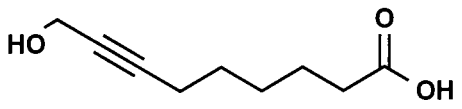


9-(Tetrahydropyran-2-yloxy)non-7-ynal (102) (1.2 g, 5.0 mmol) was stirred at 20 °C in DMF (25 ml) containing pyridinium dochromate (10.0 g, 25.0 mmol) for 18 hours. The solution was filtered through celite, and the solvent removed under reduced pressure. Purification by column chromatography (DCM:acetone 9:1) afforded the title product as a colourless oil (572 mg, 45 %)[99].

($CDCl_3$, 400 MHz) δ_H 4.71 (1H, t, $J_{H\text{-}H}$ 3.2 Hz, $\underline{H}_a$), 4.14 (2H, 2 x td, $J_{H\text{-}H}$ 12.8 and 2.0 Hz, $CCC\underline{H}_2O$), 3.72 (1H, td, $J_{H\text{-}H}$ 10.2 and 2.4 Hz, $\underline{H}_b$), 3.45 (1H, dt, $J_{H\text{-}H}$ 9.6 and 4.0 Hz, $\underline{H}_c$), 2.34 (2H, t, $J_{H\text{-}H}$ 7.6 Hz, $C\underline{H}_2COOH$), 2.1 (2H, tt, $J_{H\text{-}H}$ 6.8 and 2.0 Hz $CC\text{-}C\underline{H}_2$), 1.62 (2H, m, $\underline{H}_d$), 1.45 (4H, m, $C\underline{H}_2$'s), 1.36 (6H, m, $C\underline{H}_2$'s). ($CDCl_3$) δ_C 179.1 (COOH), 96.5 (3°C), 86.2 ($OCH_2\underline{C}C$), 75.8 ($OCH_2C\underline{C}$), 61.8 ($O\underline{C}H_2CC$), 61.4 (CH_2COOH), 54.9 (CH_2O), 33.7 (CH_2), 30.1 (CH_2), 28.0 (CH_2), 27.0 (CH_2), 2.52 (CH_2), 18.9 (CH_2), 18.5 (CH_2). IR, (neat), υ_{max}/cm^{-1}, 2943 (CH), 2864 (CH), 2253 (CC), 1709 (CO), 1667 (CO), 1277 (CH), 1134 (CH), 1021 (CH), 908 (CH), 734 (CH), 650 (CH). MS: m/z (CI) 272 [$(M + NH_4)^+$ 100 %]. HRMS: (CI) $(M + NH_4)^+$ found

272.1862, $C_{14}H_{26}NO_4$ requires 272.1862.

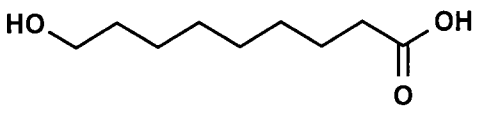
6.9.5: Preparation of 9-hydroxy-7-nonynoic acid (60).



9-(Tetrahydropyran-2-yloxy)non-7-ynoic acid (103) (100 mg, 0.4 mmol) was stirred at room temperature in 6N HCl (10 ml) for 12 hours. The organic products were extracted into diethyl ether (3 x 20 ml), the combined organic extracts washed with water (20 ml), dried ($MgSO_4$), and the solvent was removed under reduced pressure. Purification by column chromatography (DCM:acetone, 6:1) afforded the title product as an off-white amorphous solid (28 mg, 45 %), mp 37 °C (lit 43 °C)[103].

($CDCl_3$, 400 MHz) δ_H 4.18 (2H, t, J_{H-H} 2.0 Hz, CCC$\underline{H}_2$OH), 2.30 (2H, t, J_{H-H} 7.2 Hz, C$\underline{H}_2$COOH), 2.17 (2H, tt, J_{H-H} 6.8 and 2.0 Hz, CCC$\underline{H}_2$CH$_2$), 1.59 (2H, m, HOOCCH$_2$C$\underline{H}_2$), 1.45 (4H, m, C$\underline{H}_2$'s). ($CDCl_3$) δ_C 178.8 (COOH), 86.1 (CH$_2\underline{C}$C), 78.6 (CH$_2$C$\underline{C}$), 51.3 (CH_2OH), 33.7 (CH_2), 28.0 (CH_2), 27.9 (CH_2), 24.1 (CH_2), 18.5 (CH_2). IR, (neat), υ_{max}/cm^{-1}, 3441 (OH), 2938 (CH), 2864, (CH) 2253 (CC), 1708 (CO), 1009 (CH), 908 (CH), 734 (CH), 651 (CH). MS: m/z (CI) 188 [$(M + NH_4)^+$ 2 %] 88 (100 %). HRMS: (CI) $(M + NH_4)^+$ found 188.1287, $C_9H_{18}NO_3$ requires 188.1287.

6.9.6: Preparation of 9-hydroxynonanoic acid (53).



To a stirred solution of the 9-hydroxy-7-nonynoic acid (60) (500 mg, 2.9 mmol) in methanol (5 ml) was added palladium on activated charcoal (250 mg). Nitrogen gas

was flushed through the flask, and this was replaced by hydrogen, under which atmosphere the reaction mixture was left for 12 hours. The solution was filtered over celite, and the solvent removed under reduced pressure. Purification by column chromatography (DCM:acetone, 6:1) afforded the title compound as a white amorphous solid (221 mg, 44 %), mp 45 °C (*lit* 51-51.5 °C)[100, 102].

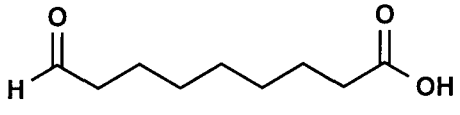
This material was spectroscopically identical with previously prepared material (see section 6.3.1).

6.10: Experimental for Chapter 3.

6.10.1: Polymerisation reaction pulsing in fresh monomer.

The experiment was set up as described in 6.2.1. Aliquots were taken and monitored by ^1H NMR until all of the monomer was consumed (there was an absence of signal at 3.6 ppm for the monomer). Additional 9-hydroxynonanoic acid (53) (100 mg, 0.65 mmol) was then added, and the loss of monomer against time was monitored by ^1H NMR. ($CDCl_3$, 250 MHz) δ_H 4.0 and 3.6 [2H, t, J_{H-H} 6.8 Hz, $CH_2COOC\underline{H}_2$ (ester) and $C\underline{H}_2OH$ (monomer) respectively], 2.3 (2H, t, J_{H-H} 7.2 Hz, $C\underline{H}_2COOCH_2$), 1.6 (4H, m, $C\underline{H}_2CH_2COO$ and $OCOCH_2C\underline{H}_2$), 1.3 (4H to 20H, bs, $C\underline{H}_2$'s). When [9-$^2H_1$]-9-hydroxynonanoic acid (56) and [8-$^2H_1$]-8-hydroxyoctanoic acid (57) were used the products were also analysed by ^2H NMR ($CHCl_3$, 400 MHz) δ_{2H} 4.0 and 3.6 [1D, bs, $CH_2COOCHD$ (ester) and CHDOH (monomer) respectively].

6.11.1: Preparation of 9-oxanonanoic acid (104).

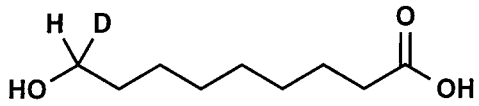


To a stirred solution of 9-hydroxynonanoic acid (53) (500 mg, 2.9 mmol) in dichloromethane at 20 °C was added pyridinium dichromate (3.7 g, 9.9 mmol). After 12 hours the reaction mixture was filtered through silica gel GF_{254} in a sintered glass funnel and was eluted with ethyl acetate until the chromium layer reached the bottom of the silica (*i.e.* until all the organic layer had been washed through). The solvent was removed under reduced pressure to give the title compound (168 mg, 34 %) as a white amorphous solid, mp 35-36 °C (lit 38 °C)[91,99].

($CDCl_3$, 250 MHz) δ_H 9.81 (1H, t, J_{H-H} 1.6 Hz, C<u>H</u>O), 2.35 (4H, t, J_{H-H} 7.2 Hz, $C\underline{H}_2COO$, and tt, JH-H 1.6 and 7.6 Hz $C\underline{H}_2CHO$), 1.61 (4H, m, $C\underline{H}_2CH_2COOH$ and $C\underline{H}_2CH_2CHO$), 1.34 (12H, m, $C\underline{H}_2$'s). ($CDCl_3$) δ_C 203.4 (CHO), 178.4 (<u>C</u>OOH), 44.3 (<u>C</u>H_2CHO), 34.6 (<u>C</u>H_2COOH), 30.4 (CH_2), 29.8 (CH_2), 28.4 (CH_2), 28.1 (CH_2), 26.8 (CH_2). IR, (neat), υ_{max}/cm^{-1}: 2925 (CH), 2856 (CH), 1729 (CO), 1423 (CH), 1179

(CH), 776 (CH). MS: m/z (EI) 172 (M^+, 20 %), 43 (100 %).

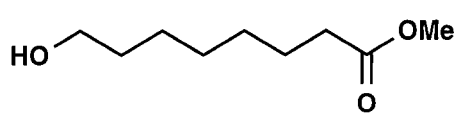
6.11.2: Preparation of [9-$^2H_1$]-9-hydroxynonanoic acid (57).



To a solution of 9-oxanonanoic acid (104) (170 mg, 1.0 mmol) in methanol (5 ml) at 0°C was added a solution of $NaBD_4$ (165 mg, 4 mmol) in methanol (10 ml) at a rate of 0.5 ml per minute. The reaction vessel was kept cool in an ice bath. When addition of the $NaBD_4$ was complete, most of the methanol was removed under reduced pressure, the residue added to water (50 ml), and acidifed to pH 1 with 6N HCl. The organic product was extracted into diethyl ether (3 x 20 ml), washed with water (20 ml), and dried ($MgSO_4$). The solvent was removed under reduced pressure, and purification by column chromatography (DCM:acetone, 8:1) afforded the title compound (131 mg, 74 %) as a white amorphous solid, mp 50 °C (lit 52 °C)[102].

($CDCl_3$, 250 MHz) δ_H 3.64 (1H, s, C<u>H</u>DOH), 2.33 (2H, t, J_{H-H} 7.6 Hz, C<u>H</u>$_2$COOH), 1.65 (4H, m, C<u>H</u>$_2$CH$_2$COOH and C<u>H</u>$_2$CHDOH), 1.34 (8H, m, C<u>H</u>$_2$s). ($CHCl_3$) $\delta 2_H$ 3.6 (1D, s, CH<u>D</u>OH) ($CDCl_3$) δ_C 180.1 (COOH), 63.2 (CHDOH), 34.5 (CH_2), 32.7 (CH_2), 29.6 (CH_2), 29.4 (CH_2), 29.3 (CH_2), 26.0 (CH_2), 25.1 (CH_2). IR, (neat), υ_{max}/cm^{-1}: 2929 (CH), 2851 (CH), 1693 (CO), 1409 (CH), 1192 (CH), 1071 (CH). MS: m/z (CI) 193 [$(M + NH_4)^+$ 100 %], 58 (52 %), 52 (49 %), 44 (56 %). HRMS: (CI) $(M + NH4)^+$ found 193.1662, $C_9H_{21}DNO_3$ requires 193.1661.

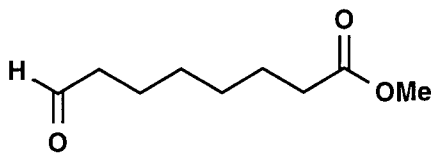
6.12.1: Preparation of methyl 8-hydroxyoctanoate (59).



A solution of 8-hydroxyoctanoic acid (52) (1.0 g, 6.3 mmol) in methanol (50 ml) containing conc. H_2SO_4 (2 ml) was heated under reflux for 24 hours. On cooling to ambient temperature, most of the methanol was removed under reduced pressure and the solution diluted with water (100 ml), to afford a fine white precipitate. The product was extracted into diethyl ether (3 x 30 ml), washed with water (20 ml), dried ($MgSO_4$), and the solvent removed under reduced pressure. Purificiation by column chromatography (DCM:acetone 8:1) afforded the title product as a colourless oil (859 mg, 79 %).

($CDCl_3$, 200 MHz) δ_H 3.66 (3H, s, C<u>H</u>$_3$), 3.63 (2H, t, J_{H-H} 6.5 Hz, C<u>H</u>$_2$OH), 2.34 (2H, t, J_{H-H} 7.6 Hz, C<u>H</u>$_2$COOMe), 1.61 (4H, m C<u>H</u>$_2$CH$_2$OH and C<u>H</u>$_2$CH$_2$COOMe), 1.29 (6H, m, C<u>H</u>$_2$'s). ($CDCl_3$) δ_C 174.8 (<u>C</u>OOCH$_3$), 63.1 (CH$_2$OH), 51.8 (COO<u>C</u>H$_3$), 34.4 (CH$_2$), 33.1 (CH$_2$), 29.8 (CH$_2$), 29.5 (CH$_2$), 26.1 (CH$_2$), 25.3 (CH$_2$). IR, (neat), υ_{max}/cm^{-1}, 2923 (CH), 2852 (CH), 1736 (CO), 1195 (CH), 1170 (CH),1054 (CH). MS: m/z (CI) 175 [$(M + H)^+$ 100%], 157 (9%), 58 (3%). HRMS: (CI) $(M + H)^+$ found 175.1334, $C_9H_{19}O_3$ requires 175.1334.

<u>6.12.2: Preparation of methyl 8-oxaoctanoate (105).</u>

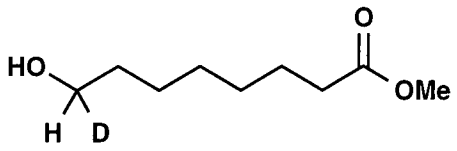


To a stirred solution of methyl 8-hydroxyoctanoate (59) (500 mg, 2.9 mmol) in dichloromethane (15 ml) at 20 °C was added pyridinium dichromate (2.8 g, 6.6 mmol). After 12 hours the reaction mixture was filtered through silica gel GF_{254} in a sintered glass funnel and was eluted with ethyl acetate until the chromium layer reached the bottom of the silica (*i.e.* until all the organic layer had been washed through). The solvent was removed under reduced pressure. Purification by column chromatography (DCM:acetone 9:1) afforded the title product as a colourless oil (257 mg, 52 %)[99].

($CDCl_3$, 200 MHz) δ_H 9.74 (1H, t, J_{H-H} 1.6 Hz, C<u>H</u>O), 3.65 (3H, s, OC<u>H</u>$_3$), 2.41 (2H,

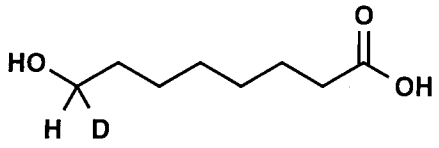
tt, J_{H-H} 1.6 and 7.6 Hz, C<u>H</u>$_2$CHO), 2.32 (2H, t, J_{H-H} 7.6 Hz, C<u>H</u>$_2$COOMe), 1.61 (4H, m, C<u>H</u>$_2$CH$_2$COOMe and C<u>H</u>$_2$CH$_2$CHO), 1.32 (4H, m C<u>H</u>$_2$'s). ($CDCl_3$) δ_C 204.6 (<u>C</u>HO), 176.1 (<u>C</u>OOMe), 53.5 (COO<u>C</u>H$_3$), 45.8 (CHO<u>C</u>H$_2$), 35.9 (<u>C</u>H$_2$COOMe), 31.0 (CH_2), 30.8 (CH_2), 30.8 (CH_2), 26.7 (CH_2). IR, (neat), υ_{max}/cm^{-1}, 2936 (CH), 2858 (CH), 1732 (CO), 1168 (CH), 668 (CH). MS: m/z (CI) 190 [$(M + NH_4)^+$ 100 %]. HRMS: (CI) $(M + NH_4)^+$ found 190.1443, $C_9H_{16}O_3$ requires 190.1443.

<u>6.12.3: Preparation of methyl 8-[^{2}H]-8-hydroxyoctanoate (106).</u>



To a stirred solution of methyl 8-oxaoctanoate (105) (250 mg, 1.5 mmol) in methanol (5 ml) at 0 °C was added a solution of $NaBD_4$ (165 mg, 4 mmol) in methanol (10 ml) at a rate of 0.5 ml per minute. The reaction vessel was kept cool in an ice bath. When addition of the $NaBD_4$ was complete, most of the methanol was removed under reduced pressure, and the residue added to water (50 ml). The organic product was extracted into diethyl ether (3 x 20 ml), washed with water (20 ml), and dried ($MgSO_4$). The solvent was removed under reduced pressure and purification by column chromatography (DCM:acetone, 8:1) afforded the title compound (214 mg, 84 %) as a colourless oil. ^{2}H NMR also indicated the presence of the dimeric species.
($CDCl_3$, 250 MHz) δ_H 3.70 (3H, s, C<u>H</u>$_3$), 3.65 (1H, bs, C<u>H</u>DOH), 2.36 (2H, t, J_{H-H} 7.6 Hz, C<u>H</u>$_2$COOMe), 1.65 (4H, m, C<u>H</u>$_2$CH$_2$COOMe and C<u>H</u>$_2$CHDOH), 1.37 (6H, m, C<u>H</u>$_2$'s). δ^2H ($CHCl_3$) 4.0 (s, CH$_2$COOCH<u>D</u>, from the dimer), 3.6 (s, CH<u>D</u>OH, from the monomer). ($CDCl_3$) δ_C 180.1 (COOMe), 63.2 (d, CHDOH), 34.5 (CH_2), 32.7 (CH_2), 29.6 (CH_2), 29.4 (CH_2), 29.3 (CH_2), 26.0 (CH_2), 25.1 (CH_2). IR, (neat), υ_{max}/cm^{-1}: 2933 (CH), 2850 (CH), 1691 (CO), 1172 (CH), 725 (CH). MS: m/z (CI) 175 [$(M + H)^+$ 5.3 %], 105 (100 %).

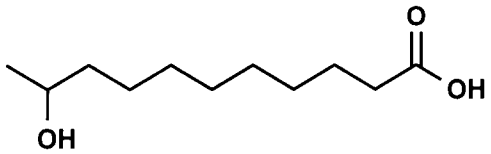
6.12.4: Preparation of 8-[$^2H_1$]-8-hydroxyoctanoic acid (58).



To a stirred solution of methyl 8-[$^2H_1$]-8-hydroxyoctanoate (106) (150 mg, 0.9 mmol) in water was added KOH (250 mg, 4.5 mmol), and the solution was heated under reflux for 24 hours. The reaction mixture was then cooled, filtered, and the filtrate evaporated under reduced pressure. The resultant white solid was dissolved in water (100 ml), and acidified to pH 1 using 6N HCl. The organic product was extracted into diethyl ether (2 x 50 ml), and the combined organic extracts were washed with water and dried ($MgSO_4$). Evaporation of the solvent under reduced pressure and purification by column chromatography (DCM:acetone, 6:1) afforded the title compound (86 mg, 62 %) as a white amorphous solid mp 55-56 °C (lit 58-58.5 °C)[89].

($CDCl_3$, 250 MHz) δ_H 3.66 (1H, bs, C<u>H</u>DOH), 2.37 (2H, t, J_{H-H} 7.4 Hz, C<u>H</u>$_2$COOH), 1.59 (4H, m, C<u>H</u>$_2$CH$_2$OH and C<u>H</u>$_2$CH$_2$COOH), 1.34 (6H, m, C<u>H</u>$_2$s). ($CHCl_3$) δ_{2H} 4.0 (s, CH$_2$COOCH<u>D</u>), 3.6 (s, CH<u>D</u>OH). ($CDCl_3$) δ_C 180.0 (COOH), 63.4 (t, J_{C-D} 159.6 Hz, CHDOH), 34.5 (CH_2), 33.0 (CH_2), 29.5 (CH_2), 29.5 (CH_2), 26.0 (CH_2), 25.1 (CH_2). IR, (neat), υ_{max}/cm^{-1}, 3186 (CH), 2933 (CH), 2850 (CH), 1727 (CO), 1172 (CH), 928 (CH), 725 (CH). MS: m/z (EI) 179 [$(M + NH_4)^+$ 100 %]. HRMS: (CI) $(M + NH_4)^+$ found 179.1506, $C_8H_{19}DNO_3$ requires 179.1506.

6.13.1: Preparation of 10-hydroxyundecanoic acid (61).



To a stirred solution of mercuric acetate (6.4 g, 20.0 mmol) in water (20 ml) and THF (20 ml) was added undecylenic acid (107) (3.7 g, 20.0 mmol). After 30 minutes NaOH

(1.0 g in 10 ml water) and $NaBH_4$ (0.5 g in 10 ml of 10 % NaOH solution) were added. The layers were allowed to separate, and the organic phase removed. Purification by column chromatography (DCM:acetone, 8:1) afforded the title product as a white amorphous solid (1.9 g, 48 %), mp 32.5 °C (lit 34 °C)[101,104].

($CDCl_3$, 400 MHz) δ_H 3.79 (1H, m, $CH_3C\underline{H}OH$), 2.34 (2H, t, J_{H-H} 7.6 Hz, $C\underline{H}_2COOH$), 1.61 (2H, m, $C\underline{H}_2CH_2COOH$), 1.34 (12H, m, $C\underline{H}_2$'s), 1.18 (3H, d, J_{H-H} 6.4 Hz, $C\underline{H}_3$). ($CDCl_3$) δ_C 179.2 (COOH), 68.2 (CHOH), 39.1 (CH_3), 34.0 (CH_2), 29.4 (CH_2), 29.3 (CH_2), 29.1 (CH_2), 28.9 (CH_2), 25.6 (CH_2), 24.6 (CH_2), 23.2 (CH_2). IR, (neat), υ_{max}/cm^{-1}: 3365 (OH), 2926 (CH), 2850 (CH), 1697 (CO), 1219 (CH), 1109 (CH), 940 (CH), 723 (CH), 681 (CH). MS: m/z (EI) 203 [$(M + 1)^+$ 87 %], 185 (100 %), 149 (57 %). Anal: found C (65.21), H (11.12), $C_{11}H_{22}O_3$ requires C (65.35), H (10.89).

6.14: Experimental for Chapter 4.

6.14.1: Preparation of poly (9-hydroxynonanoic acid) (53) with a) 11-bromo-undecanoic acid (70) or b) 1-bromoundecane (69) employed as a potential inhibitor.

Candida rugosa lipase (2.0 g) was added to a suspension of 9-hydroxynonanoic acid (53) (200 mg, 1.1 mmol) and the potential affinity label (50 mg, 0.19 mmol) in dry hexane (30 ml). The flasks were sealed (septum seal), and shaken for 24 hours (200 rpm) at 55 °C. Standard work up afforded a) with 11-bromoundecanoic acid (71), ($CDCl_3$, 200 MHz) δ_H 4.03 (4/3H, t, J_{H-H} 6.5 Hz, $CH_2COOC\underline{H}_2$, polymer), 3.63 (2/3H, t, J_{H-H} 6.6 Hz, $C\underline{H}_2OH$, monomer), 2.32 (2H, t, J_{H-H} 7.1 Hz, $C\underline{H}_2COOCH_2$), 1.62 (4H, m, CH_2), 1.28 (6H, m, CH_2).

b) with 11-bromoundecane (70), ($CDCl_3$, 200 MHz) δ_H 4.02 (2H, t, J_{H-H} 6.6 Hz, $CH_2COOC\underline{H}_2$), 2.31 (2H, t, J_{H-H} 7.2 Hz, $C\underline{H}_2COOCH_2$), 1.63 (4H, m, CH_2), 1.30 (6H, m, CH_2).

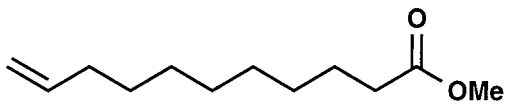
6.14.2:: Lipase inhibition studies.

Candida rugosa lipase (250 mg) was added to a solution of hexan-1-ol (75) (64 mg, 0.63 mmol), trichloroethyl butyrate (74) (139 mg, 0.63 mmol) and ^{t}butylbenzene (46 mg, 0.34 mmol) in diethyl ether (3 ml), and the suspension shaken at 200 rpm at room temperature. Rates of transesterification with enzyme were monitored by GC, employing the different potential inhibitors: 1-Bromoundecane (70); 11-bromoundecanoic acid (71); methyl 11-(bromoacetoxy) undecanoate (72) or methyl 8-(bromoacetoxy) octanoate (73) [either 5 mg (0.02, 0.02, 0.01, 0.01mmol respectively) or 50 mg (0.19, 0.21, 0.15, 0.16mmol respectively)]. Inhibitors were either incubated with the enzyme in hexane (20 ml) for up to 24 hours prior to the addition of the reagents, or were added with the reagents without preincubation. Aliquots were taken every 30 minutes from 0-5 hours, in 100 µl samples of the supernatent, and stored at -78 °C until they could be assayed by GC.

GC Retention times: diethyl ether 2.6 mins; hexan-1-ol (75) 3.5 mins; trichloroethanol 3.5 mins; ^{t}butyl benzene 4.4 mins; trichloroethyl butyrate (74) 6.1

mins; hexyl butyrate (76) 6.3 mins.

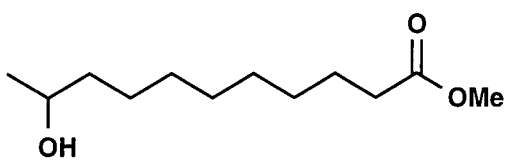
6.15.1: Preparation of methyl 10-undecylenate (79).



A solution of 10-undecylenic acid (107) (10.0 g, 53.1 mmol) in methanol (150 ml) containing conc. H_2SO_4 (3 ml) was heated under reflux for 12 hours. The solvent was removed under reduced pressure, and the residual oil was dissolved in water (50 ml). The organic product was extracted into diethyl ether (3 x 30 ml), and the organic layers were combined and dried ($MgSO_4$). The solvent was removed under reduced pressure, and the residue purified by column chromatography (DCM:acetone, 10:1) to afford the title compound as a colourless oil (10.0 g, 95 %).

($CDCl_3$, 400 MHz) δ_H 5.71 (1H, ddt, J_{H-H} 20.4, 10.4 and 1.2 Hz, CH_2=C<u>H</u>), 4.88 and 4.82 (1H each, 2 x dd, J_{H-H} 16.8 and 1.2 Hz, C<u>H</u>$_2$=CH), 2.21 (2H, t, J_{H-H} 7.2 Hz, C<u>H</u>$_2$COOMe), 1.96 (2H, m, CH_2=CHC<u>H</u>$_2$), 1.55 (2H, m, C<u>H</u>$_2$$CH_2$COOMe), 1.29 (10H, m, C<u>H</u>$_2$'s). ($CDCl_3$) δ_C 173.9 (<u>C</u>OOMe), 138.8 (H<u>C</u>=CH_2), 114.0 (HC=<u>C</u>H_2), 51.1 (COO<u>C</u>H_3), 33.8 (CH_2), 33.6 (CH_2), 29.1 (CH_2), 29.0 (CH_2), 28.9 (CH_2), 28.9 (CH_2), 28.7 (CH_2), 24.7 (CH_2). IR, (neat), υ_{max}/cm^{-1}: 2924 (CH), 2854 (CH), 1738 (CO), 1169 (CH), 991 (CH), 909 (CH). MS: m/z (CI) 199 [$(M + H)^+$ 9 %], 167 (52 %), 83 (100 %). HRMS: (CI) $(M + H)^+$ found 199.1698, $C_{12}H_{22}O_2$ requires 199.1698.

6.15.2: Preparation of methyl 10 -hydroxyundecanoate (84).

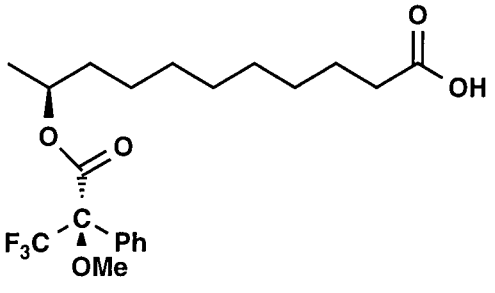


To a stirred solution of mercuric acetate (8.0 g, 25.0 mmol) in water (20 ml) and THF

(20 ml) was added methyl undecylenate (79) (5.0 g, 25.0 mmol). After 30 minutes NaOH (1.0 g in 10 ml water) and $NaBH_4$ (0.5 g in 10 ml of 10 % NaOH solution) were added. The layers were allowed to separate, and the organic phase removed. Purification by column chromatography (DCM:acetone, 10:1) afforded the title product as a white amorphous solid (3.7 g, 69 %), mp 19-20 °C (lit 21.5 °C)[90,101].

($CDCl_3$, 400 MHz) δ_H 3.79 (1H, m, $CH_3C\underline{H}OH$), 3.65 (3H, s, $C\underline{H}_3$), 2.28 (2H, t, J_{H-H} 7.6 Hz, $C\underline{H}_2COOMe$), 1.61 (2H, m, $C\underline{H}_2CH_2COOMe$), 1.40 (12H, m, $C\underline{H}_2$'s), 1.17 (3H, d, J_{H-H} 6.4 Hz, $C\underline{H}_3$). ($CDCl_3$) δ_C 174.3 ($\underline{C}OOMe$), 68.1 (CHOH), 51.4 ($COO\underline{C}H_3$), 39.1 (CH_3), 34.0 (CH_2), 29.3 (CH_2), 29.2 (CH_2), 29.1 (CH_2), 29.1 (CH_2), 25.7 (CH_2), 24.9 (CH_2), 23.4 (CH_2). IR, (neat), υ_{max}/cm^{-1}, 3338 (OH), 2913 (CH), 2847 (CH), 1729 (CO), 1172 (CH), 1126 (CH), 998 (CH), 930 (CH), 837 (CH). MS: m/z (CI) molecule decomposed on analysis. Main fragments at: 201 (1 %), 97 (17 %), 71 (100 %). Anal: found C (66.02), H (11.32), $C_{12}H_{24}O_3$ requires C (66.67), H (11.11).

6.15.3: Preparation of 10-(S)-[2'-(R)-2'-methoxy-2'-phenyl-2'-(trifluoromethyl)acetoxy] undecanoate (80).



Initially the enantiomers of 10-hydroxyundecanoic acid (61) were separated enzymatically. *Candida rugosa* lipase (2.0 g) was added to a suspension of 10-hydroxyundecanoic acid (61) (200 mg, 1.0 mmol) in hexane (25 ml). The flask was stoppered (septum seal) and placed on a shaker. At the halfway point in the reaction, as judged by ^1H NMR from the formation of the ester peak, the reaction was worked up. The polyester of 10-hydroxyundecanoic acid (61) was separated from the residual 10-

hydroxyundecanoic acid (61) by column chromatography (DCM:acetone, 7:1), and the two fractions were collected. ^{1}H NMR analysis showed the 10-hydroxyundecanoic acid (61) to be spectroscopically identical to material previously prepared (see section 4.4.2). ^{1}H NMR analysis of the polyester fraction is given below.

($CDCl_3$, 400 MHz) δ_H 4.75 (1H, m, $CH_3C\underline{H}(CH_2)OCOCH_2$), 3.66 (3H, s, $COOC\underline{H}_3$), 2.28 (2H, t, J_{H-H} 7.5 Hz, $C\underline{H}_2COOMe$), 1.62 (2H, m, $C\underline{H}_2CH_2COOMe$), 1.42 (12H, m, $C\underline{H}_2$'s), 1.15 (3H, d, J_{H-H} 6.5 Hz, $C\underline{H}_3$).

(*R*)-Mosher's acid (140 mg, 0.6 mmol) was heated under reflux in thionyl chloride (107 mg, 0.9 mmol) for 12 hours. After this time excess thionyl chloride was removed under reduced pressure[105].

To a solution of the (*S*)-acid chloride (33 mg, 0.1 mmol) was added 10-hydroxyundecanoic acid (61) (22 mg, 0.1 mmol), DMAP (15 mg), pyridine (1.5 ml) and DCM (1 ml). The reaction mixture was stirred at room temperature for three hours, after which it was diluted with DCM (3 ml), washed with dilute HCl (3 ml) and with water (3 ml). Purification by column chromatography (DCM:acetone, 20:1) afforded the title compound as a colourless oil (41 mg, 86 %)[105].

($CDCl_3$, 400 MHz) δ_H 7.51 (5H, m, Ph), 5.12 (1H, m, $CH_3C\underline{H}O$), 3.65 (3H, s, $OC\underline{H}_3$), 2.34 (2H, t, J_{H-H} 7.4 Hz, $C\underline{H}_2COOH$), 1.62 (2H, m, $C\underline{H}_2CH_2COOH$), 1.31 (12H, m, 6 $C\underline{H}_2$'s), 1.27 (3H, 2 x d, J_{H-H} 7.0 Hz, $C\underline{H}_3$). ($CDCl_3$) δ_C 174.3 ($\underline{C}OOMe$), 166.1 and 166.1 (PhC-$\underline{C}OO$), 132.6 and 132.4 [$\underline{C}Ph(OMe)(CF_3)$], 129.5 (2 C in Ph), 128.3 and 128.3 (2 C in Ph), 127.3 and 127.2 (2 C in Ph), 122.0 [qt, J_{C-F} 300Hz, $CPh(OMe)(\underline{C}F_3)$], 74.1 and 74.0 (CHOH), 55.4 [$CPh(O\underline{C}H_3)(CF_3)$], 51.4 ($COO\underline{C}H_3$), 35.5 and 35.5 ($\underline{C}H_3CHO$), 34.0 ($\underline{C}H_2COOMe$), 29.2 and 29.2 ($CHO\underline{C}H_2$), 29.2 ($\underline{C}H_2COOMe$), 29.1 and 29.0 ($CHOCH_2\underline{C}H_2$), 25.3 ($CH_2$), 24.9 ($CH_2$), 19.8 ($CH_2$), 19.5 ($CH_2$). ($CDCl_3$) δ_F -71.9 (s). IR, (neat), υ_{max}/cm^{-1}, 2931 (CH), 2859 (CH), 1736 (CO), 1166 (CH), 1017 (CH), 713 (CF). MS: m/z (CI) 450 [$(M + NH_4)^+$ 100 %], 433 [$(M + 1)^+$ 13 %]. HRMS: (CI) $(M + NH_4)^+$ found 450.2467, $C_{22}H_{35}NO_5F_3$ requires 450.2467.

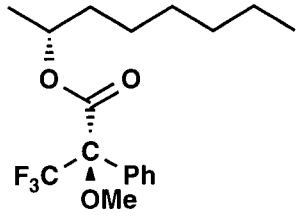
The peak in the ^{1}H NMR spectrum at 1.27 ppm was split into two doublets, as a result of the diastereomeric nature of the material. However, the ratio was not 1:1. The

doublet to lower frequency was approximately three times larger than that to higher frequency (see section 4.4.2).

10-Hydroxyundecanoic acid (polyester fraction) (100 mg, 0.5 mmol) was heated under reflux in an aqueous solution of KOH (1.0 g, 17.2 mmol, in 15 ml water) for 24 hours. The solution was allowed to cool to ambient temperatures and acidified to pH 1 with 6N HCl. The organic material was extracted into diethyl ether (3 x 10 ml), and the combined organic layers were washed with water (10 ml). Solvent was removed under reduced pressure to afford the monomeric material after purification by column chromatography (DCM:acetone, 6:1) as a white waxy solid (62 mg, 62 %).
This material was spectroscopically identical to material previously prepared.

The procedure for synthesizing the Mosher's acid derivative of the 10-hydroxyundecanoic acid was repeated as described above[105]. The product was spectroscopically identical to the material previously prepared, except for the ratio of the two doublets in the ^{1}H NMR spectrum at 1.27 ppm. The larger doublet was now to higher frequency (see Figure 4.20).

6.15.4: Preparation of 2-(R)-[2'-(R)-methoxy-2'-phenyl-2'-(trifluoromethyl)acetoxy] octanol (83).



To a stirred solution of (*S*)-Mosher's acid chloride (33 mg, 0.1 mmol) was added (*R*)-2-octanol (*R*)-(82) (17 mg, 0.1 mmol), DMAP (15 mg), pyridine (1.5 ml) and DCM (1 ml). The reaction mixture was stirred at room temperature for three hours, after which time it was diluted with DCM (3 ml), washed with dilute acid (3 ml) and then water (3 ml). Purification by column chromatography (DCM:PE, 1:1) afforded the title

compound as a colourless oil (28 mg, 80 %)[105].

($CDCl_3$, 400 MHz) δ_H 7.43 (5H, m, Ph), 5.11 (1H, m, $CH_3C\underline{H}O$), 3.56 (3H, s, $OC\underline{H}_3$), 1.59 (2H, m, $CHC\underline{H}_2$), 1.29 (8H, m, 4 $C\underline{H}_2$'s), 1.26 (3H, d, J_{H-H} 6.4 Hz, $C\underline{H}_3$), 0.88 (3H, t, J_{H-H} 7.0 Hz, $C\underline{H}_3$). ($CDCl_3$) δ_C 166.2 and 166.1 (PhC-$\underline{C}OO$), 132.6 and 132.5 [$\underline{C}Ph(OMe)(CF_3)$], 129.7 (2 C in Ph), 128.4 and 128.3 (2 C in Ph), 127.3 and 127.3 (2 C in Ph), 120.0 [q, J_{C-F} 300Hz, $CPh(OMe)(\underline{C}F_3)$], 74.1 and 74.0 (CHOH), 51.4 ($COO\underline{C}H_3$), 35.5 and 35.5 ($\underline{C}H_3CHO$), 29.2 and 29.2 ($CHO\underline{C}H_2$), 29.1 and 29.0 ($CHOCH_2\underline{C}H_2$), 25.3 (CH_2), 19.8 (CH_2), 19.5 (CH_2), 16.3 (CH_3). ($CDCl_3$) δ_F -71.7 (s). IR, (neat), υ_{max}/cm^{-1}, 2930 (CH), 1741 (CO), 1165 (CH), 1017 (CH), 714 (CH). MS: m/z (CI) 364 [$(M + NH_4)^+$ 100 %], 347 [$(M + H)^+$ 29 %]. HRMS: (CI) $(M + H)^+$ found 347.1834, $C_{17}H_{25}O_3F_3$ requires 347.1834.

This procedure was repeated for (*R,S*)-2-octanol, and the product was spectroscopically identical to the material previously prepared, except that there were two doublets at 1.26 and 1.19 ppm in the ^1H NMR spectrum, both with J_{H-H} of 6.4 Hz (see Figure 4.21).

6.15.5: Preparation of 10-(S)-[2'-(R)-2'-methoxy-2'-phenyl-2'-(trifluoromethyl) acetoxy] undecanoate (80) involving enzymatic hydrolysis of methyl 10-hydroxyundecanoate (84).

Methyl 10-hydroxyundecanoate (84) was prepared as described earlier (see section 6.12.3). Methyl 10-hydroxyundecanoate (84) (500 mg, 2.3 mmol) was added to a stirred solution of water (27 ml) and methanol (3 ml) containing CRL (5.0 g), NaCl (0.5 g) and tris(hydroxymethyl)aminomethane hydrochloride (0.03 g). After 15 minutes the reaction had proceeded to approximately 50 % conversion. The solution was then acidifed to pH 1 with 6 N HCl, and the organic products were extracted into diethyl ether (3 x 20 ml). This afforded a mixture of 10-hydroxyundecanoic acid (61) and methyl 10-hydroxyundecanoate (84), which were separated by column chromatography (DCM:acetone, 7:1), to afford 10-hydroxyundecanoic acid (61) (189 mg, 41 %) and methyl 10-hydroxyundecanoate (84) (178 mg, 36 %).

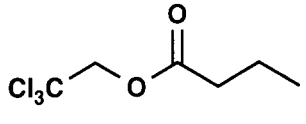
These materials were spectroscopically identical to materials previously prepared (see section 6.15.3).

To a stirred solution of (*S*)-Mosher's acid chloride (33 mg, 0.1 mmol) was added 10-hydroxyundecanoic acid (61) (20 mg, 0.1 mmol), DMAP (15 mg), pyridine (1.5 ml) and DCM (1 ml). The reaction mixture was stirred at room temperature for three hours, after which it was diluted with DCM (3 ml), washed with dilute acid (3 ml) and with water (3 ml). Purification by column chromatography (DCM:PE, 1:1) afforded the title compound as a colourless oil (37 mg, 83 %)[105]. This was spectroscopically identical to previously prepared materials (see section 6.15.3). A 1:1 diastereomeric ratio was observed in the ^{1}H NMR spectrum (see Figure 4.20).

To a stirred solution of (*S*)-Mosher's acid chloride (33 mg, 0.1 mmol) was added methyl 10-hydroxyundecanoate (84) (22 mg, 0.1 mmol), DMAP (15 mg), pyridine (1.5 ml) and DCM (1 ml). The reaction mixture was stirred at room temperature for three hours, after which it was diluted with DCM (3 ml), washed with dilute acid (3 ml) and with water (3 ml). Purification by column chromatography (DCM:PE, 1:1) afforded the title compound as a colourless oil (34 mg, 74 %)[105]. Characterisation was performed using ^{1}H NMR to determine the diastereomeric ratio present in the methyl 10-hydroxyundecanoate fraction after enzymatic hydrolysis. This was determined to be a 1:1 mixture of diastereomers.

($CDCl_3$, 400 MHz) δ_H 7.51 (5H, m, Ph), 5.12 (1H, m, $CH_3C\underline{H}O$), 3.65 (3H, s, $OC\underline{H}_3$), 2.34 (2H, t, $J_{H\text{-}H}$ 7.4 Hz, $C\underline{H}_2COOH$), 1.62 (2H, m, $C\underline{H}_2CH_2COOH$), 1.31 (12H, m, 6 $C\underline{H}_2$'s), 1.27, 1.19 (3H, 2 x d, $J_{H\text{-}H}$ 7.0 Hz, $C\underline{H}_3$).

6.16.1: Preparation of 2,2,2-trichloroethyl butyrate (74).

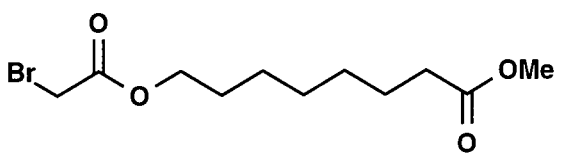


2,2,2-Trichloroethanol (6.7 g, 45.2 mmol) and pyridine (3.6 g, 45.2 mmol) were added to a stirred solution of butyryl chloride (4.8 g, 45.2 mmol) in CH_2Cl_2 (30 ml), and the

reaction heated under reflux for 3 hours. The reaction mixture was washed with brine (4 x 50 ml), dried ($MgSO_4$), and the solvent removed under reduced pressure. The residue was distilled (35 °C, 0.3 mm Hg) to afford the title compound as a colourless oil, (5.5 g, 55 %)[28,84].

($CDCl_3$, 250 MHz) δ_H 4.70 (2H, s, $CCl_3C\underline{H}_2$), 2.40 (2H, t, J_{H-H} 7.2 Hz, $O_2CC\underline{H}_2$), 1.67 (2H, m, $O_2CCH_2C\underline{H}_2$), 0.95 (3H, t, J_{H-H} 7.2 Hz, $C\underline{H}_3$). δ_C ($CDCl_3$) 173.6 (COOH), 97.1 (CCl_3), 75.7 ($Cl_3C\underline{C}H_2O$), 37.6 (CH_2), 20.2 (CH_2), 15.5 (CH_3). IR, (neat) υ_{max}/cm^{-1} 2974 (CH), 2882 (CH), 1754 (CO), 1156 (CH), 720 (CCl). MS: m/z (EI) 219 (M^+, 20 %), 43 (100%)

6.17.1: Preparation of methyl 8-(bromoacetoxy)octanoate (73).

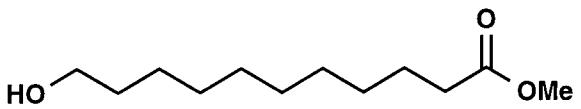


Bromoacetyl bromide (1.4 g, 6.4 mmol) was added dropwise to a stirred solution of methyl 8-hydroxyoctanoate (59) (1.1 g, 6.4 mmol) and dimethylaminopyridine (50 mg, 0.4 mmol) in triethylamine (750 mg) at 0 °C. The reaction was monitored by TLC until the starting material had been consumed, and then diluted with dichloromethane (50 ml), washed with 1M HCl (1 x 50 ml), 1M NaOH (1 x 50 ml), water (2 x 50 ml), and dried ($MgSO_4$). The solvent was removed under reduced pressure, and purification by column chromatography (DCM:pet ether 4:1) afforded the title compound as a pale yellow oil (1.2 g, 76 %).

($CDCl_3$, 200 MHz) δ_H 4.01 (2H, m, $C\underline{H}_2O_2CCH_2Br$), 3.94 (2H, s, $C\underline{H}_2Br$), 3.70 (3H, s, $CO_2C\underline{H}_3$), 2.32 (2H, t, J_{H-H} 7.4 Hz, $C\underline{H}_2CO_2Me$), 1.58 (4H, m, $C\underline{H}_2CH_2CO_2Me$), 1.24 (6H, m, $C\underline{H}_2$'s). ($CDCl_3$) δ_C 174.5 ($\underline{C}OOMe$), 167.7 ($\underline{C}OOCH_2Br$), 66.7 (CH_2Br), 51.9 ($COO\underline{C}H_3$), 34.4 (CH_2), 29.4 (CH_2), 29.2 (CH_2), 28.8 (CH_2), 26.4 (CH_2), 26.0 (CH_2), 25.2 (CH_2). IR, (neat), υ_{max}/cm^{-1}, 2935 (CH), 2857 (CH), 1735 (CO), 1170 (CH) 663 (CBr). MS: m/z (CI) 297 [$(M + H)^+$ 100 %, bromine isotope

peaks]. HRMS: (CI) $(M + H)^+$ found 295.0547 and 297.0440, $C_{11}H_{23}NO_4Br$ requires 295.0545 and 297.0438.

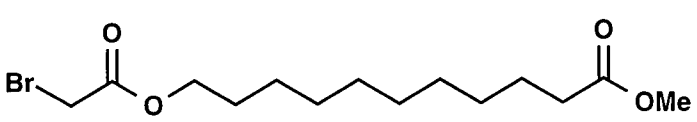
6.17.2: Preparation of methyl 11-hydroxyundecanoate (64)



A solution of 11-hydroxyundecanoic acid (43) (1.0 g, 5.0 mmol) in methanol (50 ml) containing conc. H_2SO_4 (1 ml) was heated under reflux for 24 hours. After cooling to ambient temperature, most of the methanol was removed under reduced pressure and the solution diluted into water (100 ml), to afford a fine white precipitate. The product was extracted into diethyl ether (3 x 30 ml), washed with water (20 ml), dried ($MgSO_4$), and the solvent removed under reduced pressure. Purification by column chromatography (DCM:acetone 8:1) afforded the title product as a colourless oil (760 mg, 76 %).

($CDCl_3$, 200 MHz) δ_H 3.66 (3H, s, C<u>H</u>$_3$), 3.63 (2H, t, J_{H-H} 6.5 Hz, C<u>H</u>$_2$OH), 2.34 (2H, t, J_{H-H} 7.6 Hz, C<u>H</u>$_2$COOMe), 1.61 (4H, m, C<u>H</u>$_2$CH$_2$OH and C<u>H</u>$_2$CH$_2$COOMe), 1.29 (12H, m, C<u>H</u>$_2$'s). ($CDCl_3$) δ_C 174.8 (<u>C</u>OOMe), 63.1 (CH_2OH), 51.8 (COO<u>C</u>H$_3$), 34.4 (CH_2), 33.1 (CH_2), 29.8 (CH_2), 29.8 (CH_2), 29.6 (CH_2), 29.5 (CH_2), 26.1 (CH_2), 25.3 (CH_2), 24.3 (CH_2). IR, (neat), υ_{max}/cm^{-1}, 2923 (CH), 2852 (CH), 1736 (CO), 1195 (CH), 1170 (CH), 1054 (CH). MS: m/z (EI) 217 [$(M + H)^+$ 100 %], 199 (76 %), 167 (94 %), 74 (65 %). HRMS: (EI) $(M + H)^+$ found 217.1804, $C_{12}H_{25}O_3$ requires 217.1804.

6.17.3: Preparation of methyl (11-bromoacetoxy)undecanoate (72).



Bromoacetyl bromide (1.4 g, 6.4 mmol) was added dropwise to a stirred solution of methyl 11-hydroxyundecanoate (64) (1.4 g, 6.4 mmol) and dimethylaminopyridine (50 mg, 0.4 mmol) in triethylamine (750 mg) at 0 °C. The reaction was monitored by TLC until the starting material had been consumed, and then diluted with dichloromethane (50 ml), washed with 1M HCl (1 x 50 ml), 1M NaOH (1 x 50 ml), water (2 x 50 ml), and dried ($MgSO_4$). The solvent was removed under reduced pressure, and purification by column chromatography (DCM:pet ether 4:1) afforded the title compound as a pale yellow oil (1.1 g, 60 %).

($CDCl_3$, 200 MHz) δ_H 4.01 (2H, m, $C\underline{H}_2O_2CCH_2Br$), 3.94 (2H, s, $C\underline{H}_2Br$), 3.70 (3H, s, $CO_2C\underline{H}_3$), 2.34 (2H, t, J_{H-H} 7.2 Hz, $C\underline{H}_2CO_2Me$), 1.56 (4H, m, $C\underline{H}_2CH_2CO_2Me$), 1.28 (12H, m, $C\underline{H}_2$'s). ($CDCl_3$) δ_C 174.0 ($\underline{C}OOMe$), 157.0 ($\underline{C}OOCH_2Br$), 66.3 (CH_2Br), 51.3 ($COO\underline{C}H_3$), 34.0 (CH_2), 29.1 (CH_2), 29.0 (CH_2), 29.0 (CH_2), 29.0 (CH_2), 28.3 (CH_2), 28.3 (CH_2), 25.8 (CH_2), 25.6 (CH_2), 24.8 (CH_2). IR, (neat), υ_{max}/cm^{-1}, 2928 (CH), 1738 (CO), 733 (CH) 668 (CBr). MS: m/z, (CI) 354 [$(M + NH_4)^+$ 100 %, bromine isotope peaks], 337 (20 %). HRMS: (CI) $(M + NH_4)^+$ found 354.1280, $C_{14}H_{29}NO_4Br$ requires 354.1280.

6.18.1: Polymerisations of ω-hydroxy esters using Ti(OBu)4.

The reaction vessel (apparatus shown in Figure 3.9) was lowered into a Woods metal bath, which had been preheated to 100 °C, whilst stirring the monomer (ethyl 6-hydroxyhexanoate (63), methyl 8-hydroxyoctanoate (59) or methyl 11-hydroxyundecanoate (64), 2.0 g, 12.5 mmol, 12.3 mmol or 11.9 mmol respectively) with $Ti(OBu)_4$ (0.05 mol%) under a flow of nitrogen. The bath temperature was increased at a rate of 10 °C/min to 150 °C with continued stirring, where it was held for one hour. At this stage the nitrogen flow was stopped and the pressure in the reaction vessel reduced (1 mmHg). Heating was continued for a further hour at 150 °C to remove residual methanol and drive the reaction to higher conversion. On completion of the reaction, the nitrogen flow was restored and the reaction mixture cooled to room temperature. The resultant oil solidified to give a brittle pale yellow polymeric

material, which was collected without purification (1.2-1.6 g)[69]. Analysis of the polymers was carried out using ^1H NMR and GPC.

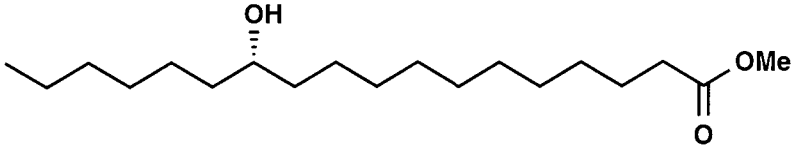
Polymer from ethyl 6-hydroxyhexanoate: ($CDCl_3$, 200 MHz) δ_H 4.06 (2H, t, J_{H-H} 6.1 Hz, OCH_2), 2.30 (2H, t, J_{H-H} 7.4 Hz, CH_2COO), 1.64 (4H, m, $C\underline{H}_2CH_2O$), 1.37 (2H, m, CH_2).

Polymer from methyl 8-hydroxyoctanoate: ($CDCl_3$, 200 MHz) δ_H 4.05 (2H, t, J_{H-H} 6.3 Hz, OCH_2), 2.29 (2H, t, J_{H-H} 7.0 Hz, CH_2COO), 1.61 (4H, m, $C\underline{H}_2CH_2O$), 1.33 (6H, m, CH_2's).

Polymer from methyl 11-hydroxyundecanoate: ($CDCl_3$, 200 MHz) δ_H 4.05 (2H, t, J_{H-H} 6.1 Hz, OCH_2), 2.29 (2H, t, J_{H-H} 7.2 Hz, CH_2COO), 1.61 (4H, m, $C\underline{H}_2CH_2O$), 1.29 (12H, m, CH_2's).

For GPC data and profiles see section 3.6.1.

6.18.2: Preparation of methyl (S)-12-hydroxystearate (S)-(65).

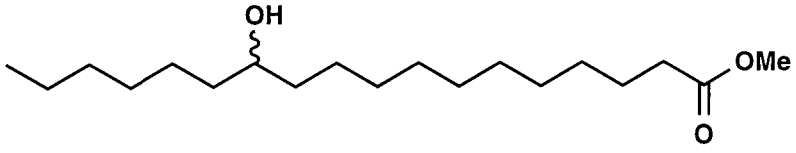


A solution of (*S*)-12-hydroxystearic acid (108) (9.93 g, 31.5 mmol) in methanol (50 ml) containing sulphuric acid (2 ml) was heated under reflux for 24 hours. On cooling to ambient temperature, most of the methanol was removed under reduced pressure and the solution diluted with water (100 ml), to afford a fine white precipitate. The product was extracted into diethyl ether (3 x 30 ml), washed with water (50 ml), dried ($MgSO_4$), and the solvent removed under reduced pressure. Purification by distillation (210 °C, 1 mmHg) afforded the title compound as a colourless oil (7.4 g, 73 %).

($CDCl_3$, 200 MHz) δ_H 3.65 (3H, s, $OC\underline{H}_3$), 3.50 (1H, m, $C\underline{H}OH$), 2.35 (2H, t, J_{H-H} 7.2 Hz, $C\underline{H}_2COOMe$), 1.59 (2H, m, $C\underline{H}_2CH_2COOMe$), 1.48 (4H, m, $C\underline{H}_2CHOHC\underline{H}_2$), 1.29 (24H, m, $C\underline{H}_2$'s), 0.76 (3H, t, J_{H-H} 6.6 Hz, $C\underline{H}_3$). ($CDCl_3$) δ_C 164.9 ($\underline{C}OOCH_3$), 74.0 (CHOH), 53.2 ($COO\underline{C}H_3$), 39.5 (CH_2), 37.1 (CH_2), 36.1 (CH_2), 33.9 (CH_2), 31.7

(CH_2), 31.6 (CH_2), 31.6 (CH_2), 31.5 (CH_2), 31.4 (CH_2), 31.4 (CH_2), 31.3 (CH_2), 31.2 (CH_2), 27.6 (CH_2), 27.0 (CH_2), 24.6 (CH_2), 16.1 (CH_3). IR, (neat), υ_{max}/cm^{-1}, 2914 (CH), 2847 (CH), 1737 (CO), 1199 (CH), 1170 (CH), 883 (CH), 722 (CH). MS: m/z (EI) 313 [$(M-H)^+$, 1%], 297 (100%), 197 (57%). Anal: found C (72.82), H (12.28), $C_{19}H_{37}O_3$ requires C (72.61), H (12.10).

6.18.3: Preparation of methyl (R,S)-12-hydroxystearate (R,S)-(65).



To a stirred solution of methyl (*S*)-12-hydroxystearate (108) (7.4 g, 22.9 mmol) in DCM (100 ml) at 20 °C was added pyridinium chlorochromate (7.96 g, 34.3 mmol). To this solution at 0 °C was added $NaBH_4$ (4.0 g, 91.6 mmol) in methanolic KOH (50 ml), and the solution was allowed to warm to ambient temperature over 12 hours. The greater part of the solvent was removed under reduced pressure, the solution diluted with water (100 ml), acidified, and the organic product extracted into diethyl ether (3 x 30 ml). The combined organic extracts were washed with water (30 ml), dried ($MgSO_4$), and the solvent removed under reduced pressure. Purification by column chromatography (DCM:acetone, 8:1) afforded the title compound as a colourless oil (2.6 g, 35 %).

($CDCl_3$, 200 MHz) δ_H 3.67 (3H, s, $OC\underline{H}_3$), 3.56 (1H, m, $C\underline{H}OH$), 2.32 (2H, t, J_{H-H} 7.2 Hz, $C\underline{H}_2COOMe$), 1.57 (2H, t, J_{H-H} 6.8 Hz, $C\underline{H}_2CH_2COOMe$), 1.45 (4H, m, $C\underline{H}_2CHOHC\underline{H}_2$), 1.26 (24H, m, $C\underline{H}_2$'s), 0.77 (3H, t, J_{H-H} 6.6 Hz, $C\underline{H}_3$). ($CDCl_3$) δ_C 165.0 ($\underline{C}OOCH_3$), 74.1 (CHOH), 52.3 ($COO\underline{C}H_3$), 39.4 (CH_2), 37.2 (CH_2), 36.1 (CH_2), 33.9 (CH_2), 32.7 (CH_2), 32.5 (CH_2), 31.7 (CH_2), 31.5 (CH_2), 31.4 (CH_2), 31.4 (CH_2), 31.3 (CH_2), 31.3 (CH_2), 27.5 (CH_2), 27.1 (CH_2), 24.5 (CH_2), 16.8 (CH_3), IR, (neat), υ_{max}/cm-1: 2913 (CH), 2846 (CH), 1734 (CO), 1190 (CH), 1170 (CH), 882 (CH), 723 (CH). MS: m/z (EI) 313 [$(M-H)^+$ 1%], 297 (100%) 197 (57%).

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