

*An Investigation of the Alkaline Phosphatases
for
use in Organic Synthesis*

A Thesis submitted for the Degree of
Doctor of Philosophy
in
The Department of Chemistry
Faculty of Science
University of Leicester

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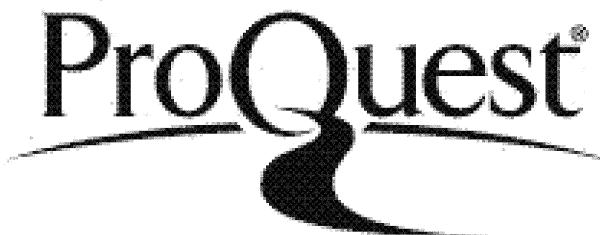


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Abstract:

An Investigation of the Alkaline Phosphatases for Use in Organic Synthesis.
by David Baker

The synthesis of a series of 1-phenylalkyl-1-phosphate analogues is described as well as the preparation of 2-phenylpropyl-1-phosphate, 2,3-dimethylbutyl-1-phosphate, 3-methyl-2-butyl-2-phosphate, 1-(*para*-methoxyphenyl)ethyl-1-phosphate and 1-(*para*-nitrophenyl)ethyl-1-phosphate. The enantioselectivities of bovine, rabbit and *E.coli* alkaline phosphatases with these various substrates were investigated. Bovine alkaline phosphatase showed the best enantioselectivity with 1-phenylethyl-1-phosphate showing an enantiomeric excess of 56% favouring the turnover of (R)-1-phenylethyl-1-phosphate enantiomer.

Attempts to improve the enantioselectivity of bovine alkaline phosphatase with 1-phenylethyl-1-phosphate by varying the reaction conditions was undertaken. These included changes in pH and sodium chloride concentration. The addition of organic solvents to the buffer solutions as well as changes in the zinc and magnesium ion concentrations were studied. Detailed analyses of the kinetic parameters of the individual enantiomers (1R)-phenylethyl-1-phosphate and (1S)-phenylethyl-1-phosphate with bovine and *E.coli* alkaline phosphatase were carried out. The results of these investigations were used to try to enhance the enantioselectivities of the bovine and *E.coli* phosphatases with 1-phenylethyl-1-phosphate.

Synthesis of a series of 2-alkylpropyl-1,3-bisphosphate from their 2-alkyl-1,3-propanediol derivatives is described. The prochiral selectivities of this series of 2-alkylpropyl-1,3-bisphosphates were evaluated with bovine alkaline phosphatase. Failure to isolate the desired monophosphate products prevented further use of the alkaline phosphatases to effect enantiotopic discrimination of the prochiral 1,3-bisphosphates. Inhibition studies of these materials with bovine and *E.coli* phosphatases were therefore carried out.

STATEMENT

The accompanying thesis submitted for the degree of Doctor of Philosophy in the Faculty of Science, entitled "An Investigation of the Alkaline Phosphatases for use in Organic Synthesis" is based on work conducted by the author in the Department of Chemistry at the University of Leicester in the period October 1990 to September 1993.

All the work recorded in this thesis is original unless otherwise acknowledged in the text or by references. None of the work has been submitted for another degree at this or any other University.

Signed D. Baker Date 16TH December 1993

DEDICATION

TONTINE

TO MY PARENTS

This thesis and all the research work described within is dedicated to my parents. For their love and understanding it stands as an acknowledgement of their continual help and commitment to my education and life. Through their hard work and support I have been able to enjoy immensely the pleasures of an education. May they share in the rewards of my education as the surviving subscribers.

The trips to Shevington seemed so long and tedious. I guess they proved their worth.

"Smile and Laugh Loud"

"The most wasted day is that in which one has not laughed"
Nicholas Chamfort

"Leave it my capable hands"
Ronald R. Baker

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ABBREVIATIONS

Ac	acetyl
AK	acetate kinase
ADP	adenosine diphosphate
ATP	adenosine triphosphate
bp	boiling point
Bn	benzyl
Bu ⁿ	butyl
Bu ^t	<i>tert</i> -butyl
Bz	benzoyl
Cy	cyclohexyl
°C	centigrade
DCC	dicyclohexylcarbodiimide
de	diastereomeric excess
DMAP	dimethylaminopyridine
DMS	dimethyl sulphide
DMSO	dimethyl sulfoxide
ee	enantiomeric excess
EI	electron impact
ES	electrospray
Et ₂ O	diethyl ether
Et	ethyl
EtOAc	ethyl acetate
EtOH	ethanol
FAB	fast atom bombardment
GK	glycerol kinase
HK	hexokinase
hr	hours
Hz	hertz
HLADH	horse liver alcohol dehydrogenase
IR	infra-red
Ipc ₂ BH	(+)- α -diisopinocampheylborane
KJ·mol ⁻¹	kilojoules per mole
Kcal·mol ⁻¹	kilocalorie per mole
LAH	lithium aluminium hydride
lit	literature
M ⁺	molecular ion
MCPBA	<i>meta</i> -chloroperbenzoic acid
Me	methyl
MeCN	acetonitrile
MeOH	methanol
MHz	megahertz
min	minute
mM	millimolar
mmol	millimole
mm Hg	millimetres of mercury

mp	melting point
NADH	nicotinamide adenine dinucleotide
NMR	nuclear magnetic resonance
NOBA	nitrobenzyl alcohol
NPP	<i>para</i> -nitrophenyl phosphate
PEP	phosphoenol pyruvate
pet ether	petroleum ether (40-60°C)
PK	pyruvate kinase
PLE	pig liver esterase
PLC	preparative layer chromatography
PPL	porcine pancreatic lipase
ppm	parts per million
PRPP	phosphoribosyl pyrophosphate
Pyr	pyruvate
MTPA	(R)-(-)- α -methoxy- α -(trifluoro methyl)-phenyl acetic acid
MTPA-Cl	(R)-(-)- α -methoxy- α -(trifluoro methyl)-phenylacetic acid chloride
Ph	phenyl
Pr	propyl
Pr ⁱ	<i>iso</i> -propyl
TBPP	tetrabenzylpyrophosphate
TEAB	triethylammonium bicarbonate
THF	tetrahydrofuran
TLC	thin layer chromatography
TMS	tetramethylsilane
TRIS	tris(hydroxymethyl)amino methane
TEBA-Cl	triethyl benzylammonium chloride

Chapter 1

Introduction

1.1 General Introduction:

The use of enzymatic catalysis in organic synthesis is an emerging field of chemistry that holds great potential due to the high *regio*-, *stereo*-, and *chemoselectivity* of enzymes, the mild reaction conditions and the ability to conduct reactions with the minimum number of protection steps. As pointed out by Knowles,¹ "Enzymes are not different, just better". Enzymes now offer an alternative route to synthetically useful products, especially carbohydrates, chiral synthons and food metabolites. This can be seen by the many reviews² and numerous articles³⁻⁹ that have appeared in the literature over the last ten years. Many hundreds of enzymes are readily available, from biochemical suppliers such as Sigma, Pharmacia and Gibco BRL, some in immobilized forms which can offer considerable practical advantages.

As with all catalysts it is generally accepted that the increased rate of reaction is due to the enzyme lowering the energy of activation for the process, by stabilising the transition state and/or destabilising the ground state. In addition, enzymes have several important characteristics which make them exceptional catalysts. They are highly efficient and can accelerate rates of reactions by factors of up to 10^{10} as compared to the corresponding uncatalysed reactions. Unlike some of the more traditional catalytic methods, enzyme reactions take place under extremely mild conditions, close to neutral pH and ambient temperatures. They usually require the use of simple apparatus and a simple work up procedure. The major synthetic value of enzymes lies in their high degree of selectivity. Enzymatic selectivity can be seen in a number of ways: *chemoselective reactions* differentiating between different functional groups within a molecule; *regioselective reactions* of one of the same or similar groups within a molecule; *enantioselective reactions* of one enantiomer of a racemate or enantiotopic reactions of

a group or face in a prochiral molecule. These characteristic properties are a consequence of binding of the substrate to the enzyme's active site in a defined manner with a conformation that ensures that only one orientation is presented to the catalytic site. The conformation of the enzyme substrate complex is determined by the many non-covalent interactions between the protein and the substrate. The much sought after specificity of enzymatic reactions therefore, depend on the stereospecificity exhibited in the formation of the enzyme-substrate complex. It is the degree to which enzymes discriminate between alternatively bound conformations that determine their utility in asymmetric synthesis.

Enzymes are extremely versatile and capable of catalysing a broad spectrum of reactions which can be divided into six main classes as shown below.

1. Oxidoreductases- catalyse oxidation-reduction reactions in which either oxygen is added or removed e.g. C-H to C-OH or the addition or removal of hydrogen atoms e.g. CH-CH to C=C.
2. Transferases- catalyse the transfer of groups e.g. acyl, phosphoryl or sugar, from one moiety to another.
3. Hydrolases- catalyse hydrolysis of a range of groups e.g. anhydrides, esters, glycosides and amides.
4. Lyases- catalyse addition of HX groups across double bonds e.g. C=C, C=N, C=O, or catalyse their removal.
5. Isomerases- catalyse isomerisation such as racemisations and C=C bond migrations.
6. Ligases- catalyse the formation of C-O, C-S, C-N and R-O-P bonds.

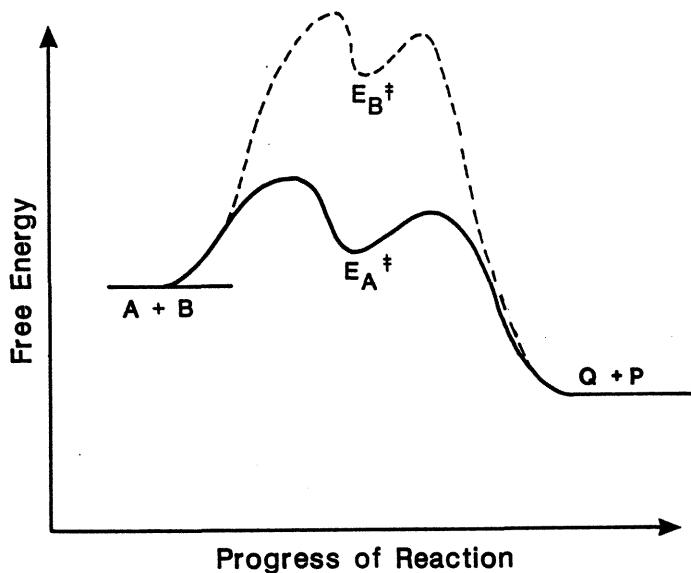
Quite clearly the most useful enzymes with applications in organic synthesis are those which can accept a wide variety of structural substrates while still retaining their stereospecificity. There is thus a paradox in that *low* substrate specificity is desirable and yet *high* stereo- and regiospecificity in subsequent reactions is sought. These two features are unfortunately not independent.

1.1.1. Reaction Rates:

The rate of any reaction is controlled by the activation energy, that is the energy required to traverse the highest point in the free energy profile for each of the individual steps. The reaction rate can be increased by either increasing the energy of the reactants or intermediates or lowering the energy of the transition state.

Figure 1.1:

The Effect of Catalysis on the Energy of Activation of a Reaction.



Enzymes are able to lower the energy of activation by employing several methods.

These include substrate strains, orientation and proximity effects, acid-base catalysis and covalent catalysis. Substrate strain involves distorting the substrate molecule through binding to the enzyme and the subsequent weakening of bonds that will be broken during catalysis. In bimolecular processes, the orientation of the substrates are controlled through binding to the enzyme and are brought into close proximity of one another. Thus the

enzyme speed up the collisions that lead to product formation. Covalent catalysis involves the substrate becoming covalently attached to the enzyme, facilitating further attack by other external nucleophiles. Finally, enzymes are catalysts that use general acid-base catalysis; requiring essential active site residues such as, histidine, aspartate, glutamate, serine and cysteine.

1.1.2. Two Point Attachment:

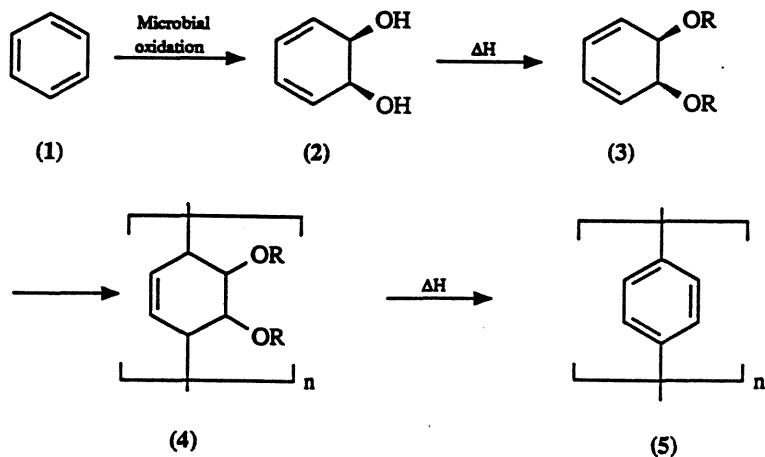
In order for stereospecific enzyme catalysis to be observed, Vennesland and Westheimer have shown that two points of attachment between the substrate and the active site of the enzyme are required.³ The asymmetric environment of the active site discriminates between the two chemically identical ligands of a prochiral centre resulting in stereospecificity. To do so, the enzyme must possess at least two different binding regions which are fixed with respect to one another. The interaction of these two distinct sites with a similar number of groups of a prochiral substrate, in a well defined orientation, will be seen under optimum circumstances. Consequently, one configuration of the substrate binds to the enzyme in the desired orientation and undergoes a stereospecific reaction. The other is poorly bound or at worst unbound and so remains unreacted.

1.2. Specificity:

One of the most outstanding characteristics of enzymes is their specificity for substrates and their specificity to catalyse defined chemical reactions. Each enzyme is able to catalyse only one type of reaction (phosphate transfer, oxidation-reduction) for a number of structurally related substrates. The structural specificities of enzymes can be used to great advantage to carry out selective transformations on numerous substrates. Many of these reactions have proved difficult to reproduce chemically except through complicated multi-step procedures.

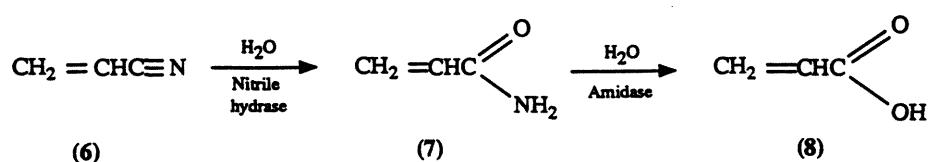
The specificity of oxidative enzymes is illustrated by the microbial conversion of benzene to *cis*-1,2-dihydroxycyclohexa-3,5-diene (**2**) effected by *Pseudomonas putida*, a benzene dioxygenase enzyme.⁴ This cyclohexa-3,5-diene derivative plays a pivotal role in the production of the versatile polymer poly-(1,4-phenylene) (**5**) shown in **Scheme 1.1**. (**2**) Is also a key intermediate in the synthesis of (+)- and (-)-pinitol.⁵ A range of 3-substituted *cis*-cyclohexa-3,5-diene-1,2-diol derivatives have also been prepared from the respective benzene derivatives by microbial oxidation, and in these, the symmetry of the product is destroyed allowing the enantiospecificity of the process to be demonstrated. These have been widely used in the stereoselective synthesis of natural products such as zeylена,^{6a} conduritol C,^{6b} dihydroconduritol^{6c} and terpenes^{6d} as well as the synthesis of carbohydrate derivatives.^{6e,f}

Scheme 1.1:



Hydrolytic enzymes have also been widely exploited in this way to catalyse selective reactions. An example of this is the hydrolysis of acrylonitrile to its amide (**7**)⁷ and subsequently to the acrylic acid (**8**),⁸ both of which are achieved under mild conditions, shown in **Scheme 1.2**.

Scheme 1.2:



Many other enzyme systems have been used to effect other transformations, for example, chloroperoxidases have been used to achieve halogenation of vinylic hydrogens,⁹ to form mono- or bis-chlorinated barbiturates. Sulphatases have been exploited to selectively hydrolyse sulphates of α and β naphthol mixtures,¹⁰ while the access to lactones has been greatly assisted by enzymatic Baeyer-Villiger reactions using microbial oxidations.¹¹

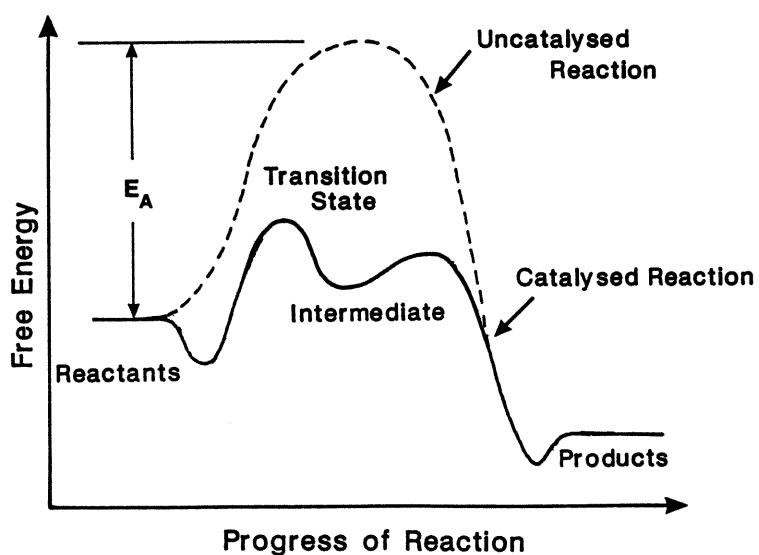
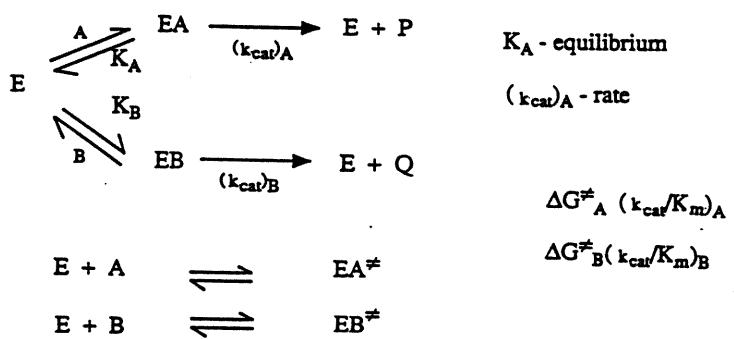
1.3. Enantioselectivity:

All stereoselective reactions are the result of differences in the energies of the enzyme-transition-state complexes of the two reactants or different conformations or orientations of a single reactant. Enzyme catalysis is an excellent example of diastereoselectivity and kinetic resolution. The chiral environment at the active site enables the enzyme to resolve racemates and differentiate between enantiotopic and diastereotopic atoms, groups or faces leading to selectivity.

In the enantioselective transformation of the two enantiomeric substrates A and B, they are both competing for the active site of the enzyme (E). The reaction profile in Figure 1.2 shows that the energy of activation of the EA-transition state is lower than that of EB. The result of this is that A reacts much faster than B, leading to selectivity in which substrate A is converted to product while B remains unreacted or only "turned over" slowly. The selectivity of substrate A can arise from its enhanced complementary binding

to the active site (K_A), or a more reactive EA complex (k_{cat}). On the other hand B has a configuration about its chiral centre which hinders its interactions with the enzyme's active site, or is bound in a manner in which catalysis is inherently slower. Since the enzyme is a chiral "reagent", the transition states EA^+ and EB^+ are diastereotopic and therefore, can be considerably different in free energy.

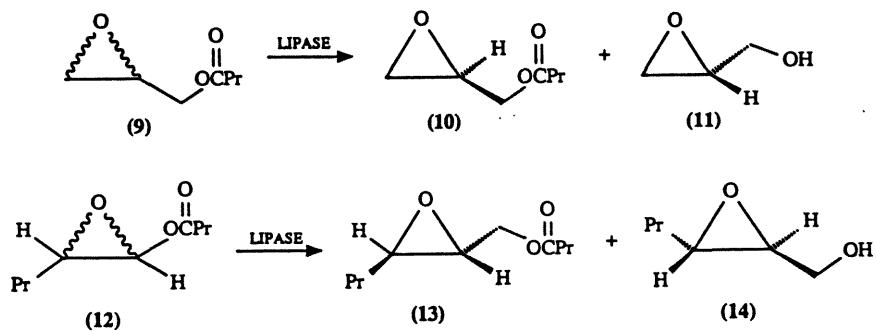
Figure 1.2:



Enantioselective reactions that yield only one enantiomer mediated by enzymes are well documented.¹² The ability of enzymes to discriminate between enantiomers is very important for the purposes of resolution and asymmetric synthesis. The essential requirement for the kinetic resolution of a racemic mixture is that there are differences in the rate constants for the individual enantiomers reacting in the *enzyme-catalysed* process. This type of enzymatic reaction suffers from the disadvantage that as with all kinetic resolutions at best only 50% of the starting material is converted to the desired chiral product, although for some examples, racemisation of the unreacted starting material and a second round of enzyme catalysis can improve on this.

Ester hydrolases are widely used in the enantioselective resolution of enantiomers of esters and alcohols. This is illustrated in **Scheme 1.3**, by the lipase catalysed hydrolysis of esters of epoxy alcohols (**9**) and (**12**).¹³ These hydrolysis reactions are seen to yield the chiral epoxy alcohols (**11**) and (**14**) with low ees of between 30-60%, while the unreacted chiral esters (**10**) and (**13**) are isolated with high enantiomeric purity of 80-95%ee. It is interesting to note that this enzymatic hydrolysis is an alternative to the Sharpless procedure¹⁴ in providing a practical route to a group of important synthetic intermediates.¹⁵

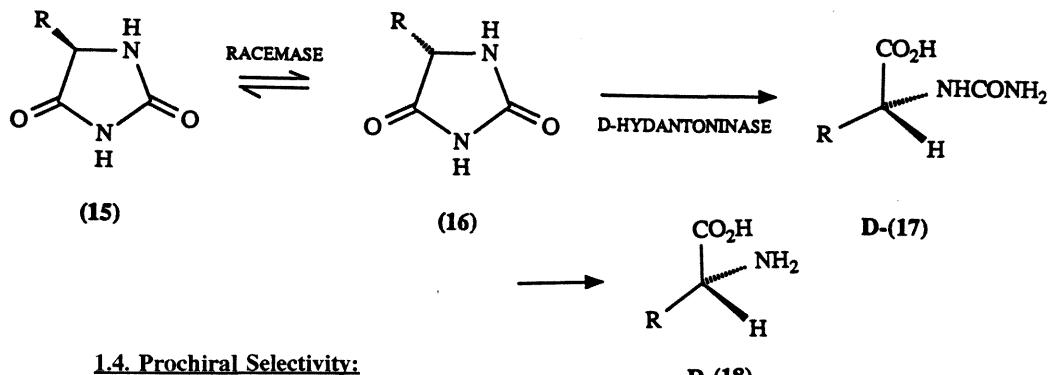
Scheme 1.3:



Shown in **Scheme 1.4**, the preparation of chiral amino acids can be achieved using hydantoinase which catalyses the hydrolysis of racemic hydantoins (**15**) and (**16**) to produce the enantiomerically pure (*R*)-carbamates (**17**) which in turn can be readily converted to the corresponding amino acid (**18**).¹⁶ The attraction of this synthesis is that the unreacted hydantoin enantiomer (**15**) is converted by racemase to (**16**). This enables 100% conversion of a racemic substrate to a single chiral product.

Hog kidney acylase has also been used in the preparation of chiral amino acids by selective hydrolysis of the corresponding N-acyl amino acids.¹⁷ Recent attention has focused on the broad synthetic utility of pig liver esterase and chymotrypsin in the stereospecific hydrolysis of esters to their chiral acids.¹⁸

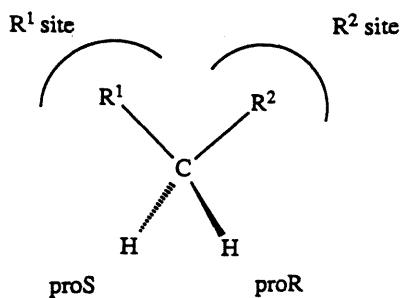
Scheme 1.4:



1.4. Prochiral Selectivity:

The stereospecificity of enzyme catalysis extends beyond molecules containing asymmetric centres to the differentiation between two identical protons (H_A and H_B) on a carbon atom which is also substituted with two other different groups (R¹ and R²). The asymmetric enzyme is seen to confer asymmetry on a symmetric substrate by distinguishing between its identical (prochiral) groups.

Figure 1.3:



Although the two hydrogen atoms in the prochiral molecule $\text{CH}_2\text{R}^1\text{R}^2$ are equivalent in simple chemical reactions, this equivalence is lost when bound to the asymmetric active site of the enzyme. The binding of R^1 and R^2 to specific binding sites of the enzyme, as shown in **Figure 1.3**, allows for only one orientation of the molecule, such that the pro-R hydrogen is always above the plane and the pro-S hydrogen is below. If the catalytic groups in the active site are on the top face, clearly, the pro-R hydrogen will be abstracted stereospecifically. So, it is possible to exploit the prochiral specificities of enzymes, whereby the prochiral substrate can be converted into a chiral product. This approach has been applied with very impressive results using pig liver esterases and lipases on various *meso*-diester and -diol substrates.¹⁹

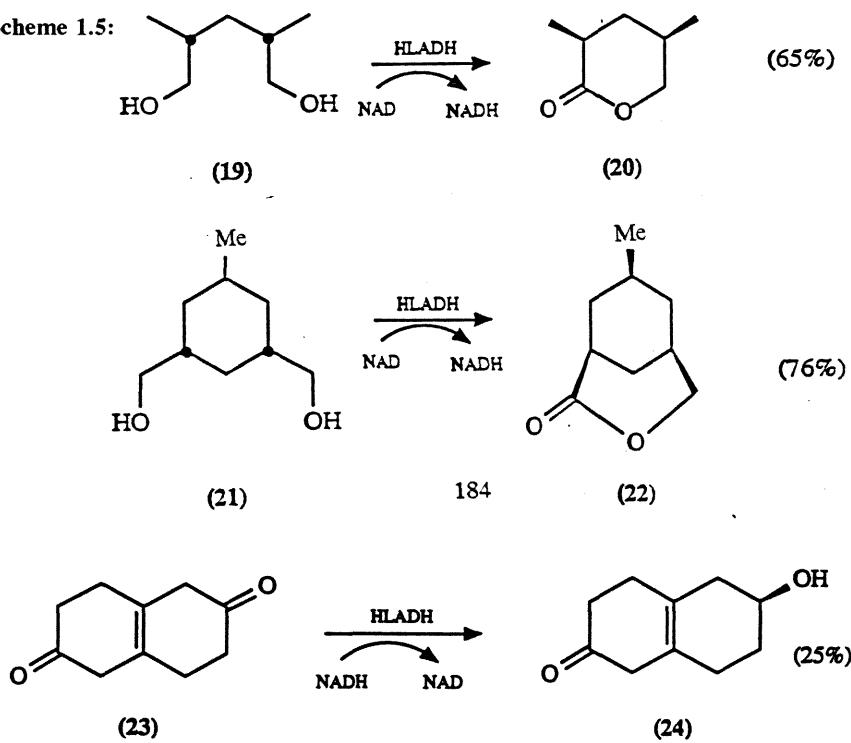
1.4.1. Enantiotopic Selectivity:

The ability of enzymes to discriminate between enantiotopic (prochiral) atoms or groups is an important aspect of enzyme specificity which can be used in asymmetric reactions of *meso* substrates.

Jones *et.al.*²⁰ have shown that a broad range of structural *meso*-1,3-disubstituted diols (**19**) and (**21**), can be stereospecifically oxidised using horse liver alcohol dehydrogenase (HLADH). The products of which are attractive intermediates for macrolide and prostaglandin synthesis. HLADH has also been used to promote

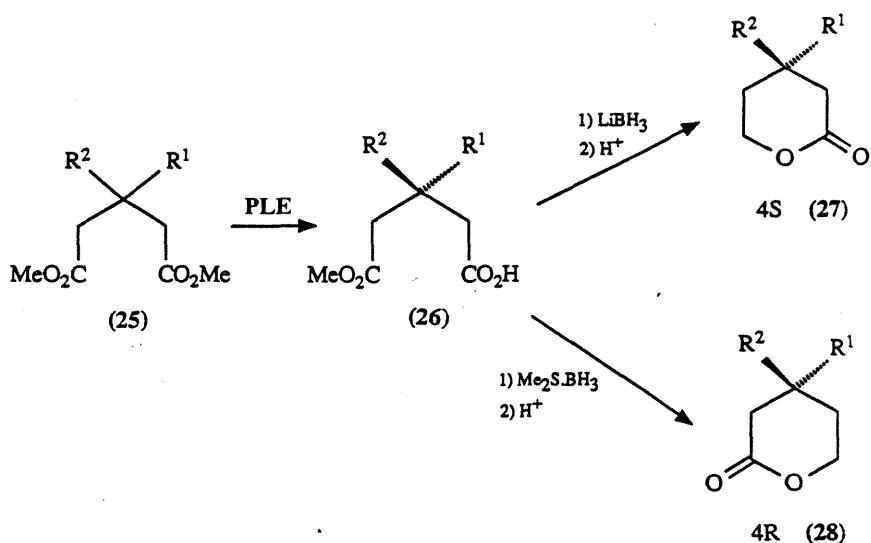
enantiotopic-group-specific reductions of decalin diones (**23**) to yield enantiomerically pure ketoalcohols (**24**).²¹ See **Scheme 1.5** below.

Scheme 1.5:



Similar enantiotopic group distinctions of secondary alcohols have been observed by Whitesides using glycerol dehydrogenase.²² An important application of enantiotopic group discrimination has also been observed with pig liver esterase.^{19a,b} **Scheme 1.6** shows the hydrolysis of C-3 monosubstituted glutaric acid diesters to the useful chiral intermediate (**26**), which can be readily be converted to either enantiomer of the corresponding 3-substituted valerolactones (**27** and **28**) with 100% ee. Pig liver esterase (PLE) has also been used in a similar fashion to HLADH to bring about enantiotopic distinctions of *meso*-diester substrates.^{19a-f,23}

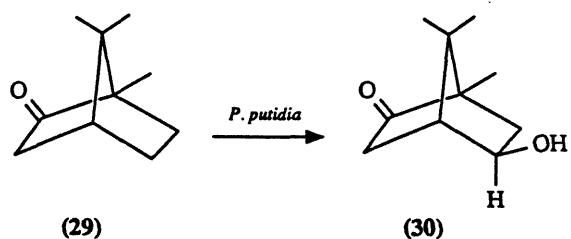
Scheme 1.6:



1.4.2. Diastereotopic Selectivity:

In addition to enantiotopic selectivity, enzymes are also able to distinguish between diastereotopic atoms or groups. Much of this work involves the specific substitution of hydroxyl groups for methylene protons over a broad range of substrate structures. In fact, these stereospecific substitutions on steroids, terpenes and alkaloids can now be achieved very readily with many of the reactions being catalysed by cytochrome P-450 enzymes. This is illustrated in Scheme 1.7 in which the conversion of camphor (29) to *exo*-5-hydroxycamphor (30) is achieved using *Pseudomonas putida*.²⁴ The particular appeal of this reaction is the fact that the otherwise unactivated methylene group is functionalised in a reaction that would be difficult to achieve chemically.

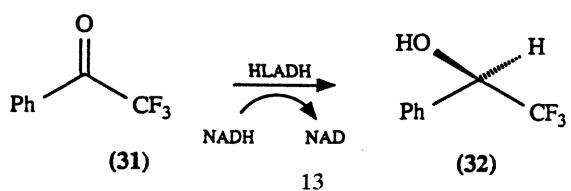
Scheme 1.7:



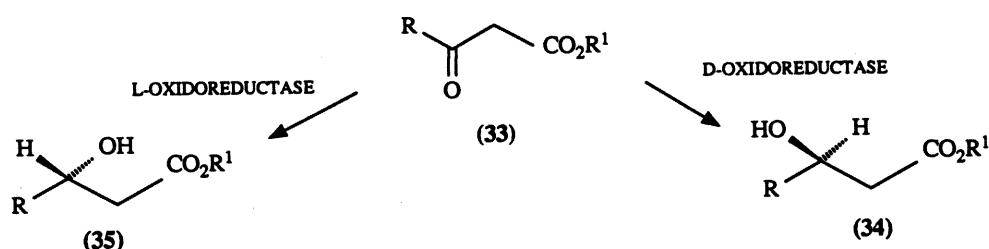
1.4.3. Stereoheterotopic Selectivity:

In these reactions enzymes show enantiotopic and diastereotopic face specificity of planar groups such as carbonyl, alkenes and imines. An optically active compound is formed if a reagent attacks just one face of a planar trigonal carbon centre. Stereospecific reductions of these types are well known and can readily be achieved both enzymatically and microbially. One of the obvious transformations is the stereospecific reduction of aldehydes or ketones to their respective alcohols using alcohol dehydrogenase enzymes (HLADH). In the reduction of 2,2,2-trifluoroacetophenone (**31**) shown in **Scheme 1.8a**, we see that attack is from the *re* face (front of the page) leading to the production of the (*R*)-enantiomeric alcohol (**32**). Many of the chiral alcohols produced are proving to be key intermediates in the synthesis of important alkaloids such as griseoviridin.²⁵ It is also possible to synthesis either enantiomer of a chiral alcohol by selecting enzymes with opposite facial specificities, using the same carbonyl substrate. An example of this is shown in **Scheme 1.8b** in which the β -keto ester (**33**) is reduced to the *R*-(**34**) and *S*-(**35**) chiral alcohols using the respective D and L oxidoreductases.²⁶

Scheme 1.8a:

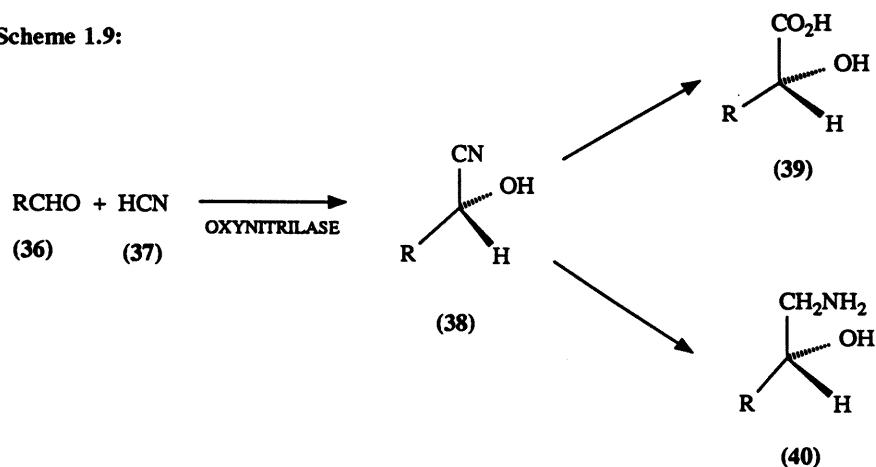


Scheme 1.8b:



Another stereospecific reaction of importance within this class of transformations is shown in **Scheme 1.9**. The oxynitrilases mediate the addition of HCN to aldehydes to form important cyanohydrins (38), which in turn are easily converted to the α -hydroxy acid (39) and amino alcohol (40) derivatives.

Scheme 1.9:



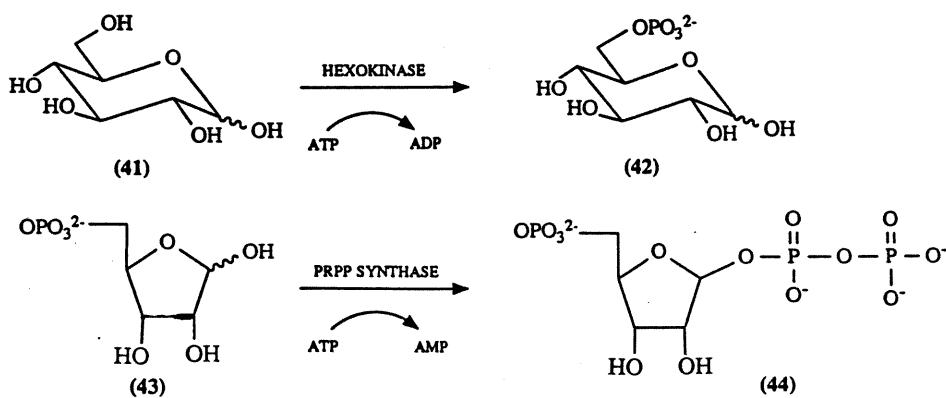
Organic Solvents: Enzyme reactions are generally conducted in aqueous media, since it is assumed that this is the ideal environment for optimal catalytic activity as well as maintaining the correct protein binding and folding interactions. However many of the substrates used have low solubilities in water and this has led to the use of biphasic

organic-aqueous systems to avoid some of the solubility problems. It has been shown by Kilbanov,²⁷ Sih^{28a} and other groups^{28b,c} that many enzymes show improved catalytic activity in apolar solvents (toluene, octane, hexane) in particular the lipases and esterases.¹⁹⁻²¹ It is now possible to use compounds that are unstable in water (anhydrides, halides) as substrates for enzymes using nonaqueous media. It is clear that carrying out biocatalytic transformations in organic media is a rapidly developing field.

1.5. Phosphoryl Transfer Reactions:

Of particular relevance to this thesis are the phosphotransferases. Many substrate specific phosphate hydrolysis enzymes are known. Some of these have been exploited by organic chemists for example, in Scheme 1.10 the phosphorylation of glucose (41) at the C-6 position using hexokinase to give the phosphorylated glucose derivative (42).²⁹ The synthesis of the important nucleotide intermediate phosphoribosyl pyrophosphate (44) from phosphoribose (43) using phosphoribosyl pyrophosphate synthase (PRPP) is an example of an enzyme used to catalyse a displacement reaction at a phosphorus centre. The ability of such enzymes to form selective phosphate esters without the need for protecting groups makes them potentially useful synthetic tools.³⁰

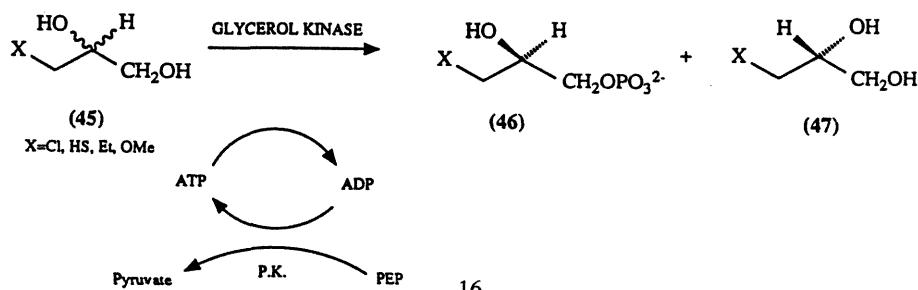
Scheme 1.10:



One of the key features of these enzyme reactions are their compatabilities, especially with the kinases which Wong and Whitesides have managed to incorporate into multi-enzyme systems, which tackle the important problems of regenerating cofactors. This has enabled them to produce ethanol, ribose-5-phosphate and ribulose-5-phosphate from the simple starting material glucose.³¹ Though these systems are at present too complex to be synthetically useful, they are proving very useful in studying metabolic systems.

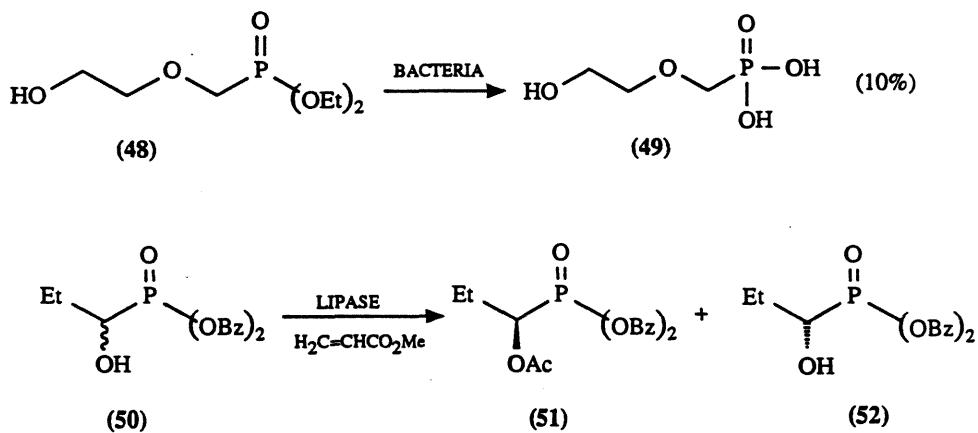
Many phosphorylation reactions consume ATP and it is the availability of this substrate that can often prove the limiting factor. Regeneration of ATP *in situ* can be achieved on a significant scale using either, phosphoenolpyruvate with pyruvate kinase or acetyl phosphate with acetate kinase.^{31,32} The application of glycerol kinase to selective bond formation has proved particularly interesting, as shown in **Scheme 1.11**. The enzyme is known to select between two prochiral hydroxymethyl groups in a highly stereoselective manner, converting the diol derivatives (**45**) to the chiral monophosphate esters (**46**). It has been shown that glycerol kinase can catalyse the phosphorylation of some 66 analogues of glycerol in which various alkyl, sulphur and nitrogen substituents were used in place of the terminal hydroxyl group.³³ The phosphorylation of racemic mixtures was shown to yield the chiral organic phosphates (**46**) with ees >90%, and the unphosphorylated enantiomers (**47**) with ees of between 80-90%.³³

Scheme 1.11:



Recently O'Toole³⁴ and coworkers have managed to isolate bacteria that display diethyl phosphonate-hydrolase activity, converting diethyl phosphonate (**48**) to the corresponding phosphonic acid (**49**) as shown in **Scheme 1.12**. These conversions were very poor with yields of <10% being attained, clearly more work with these isolated strains needs to be done. In the same article they were also able to demonstrate that racemic substrates containing phosphonates (**50**) are turned over by lipases in the presence of vinyl acetate to yield the enantiomerically pure acetate (**51**) in reasonable yield, and the unreacted enantiomer (**52**) with an ee of 60%.

Scheme 1.12:



Finally, the enzyme system C-P lyase (phosphonatase) isolated from *E. coli* has been studied to determine the stereochemistry of the carbon-phosphorus bond cleavage. (R)-and (S)-[1-²H and 1-³H]ethylphosphonate were used as substrates and the cleavage of these to the corresponding (R)- and (S)-ethanes and inorganic phosphate was investigated for potential in the production of chiral synthons.³⁵ Phosphonatases are known to cleave functionalised phosphonates such as phosphonoacetaldehyde, but little work on this system has been done.³⁶

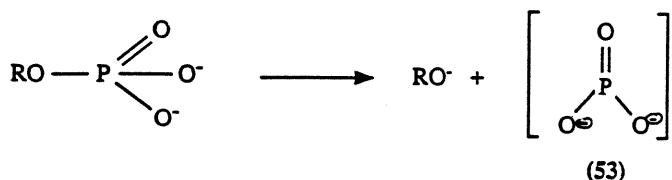
From this very brief outline it is clear that enzymes are attractive practical catalysts for a number of synthetic strategies. The capability of enzymes to produce chiral synthons containing esters, alcohols and amines is now well established. The major problem is identifying classes of enzymes with useful enantioselectivity, developing conditions for their use and identifying chiral products sufficiently valuable to warrant further investigation.

1.6. Fundamental Mechanisms:

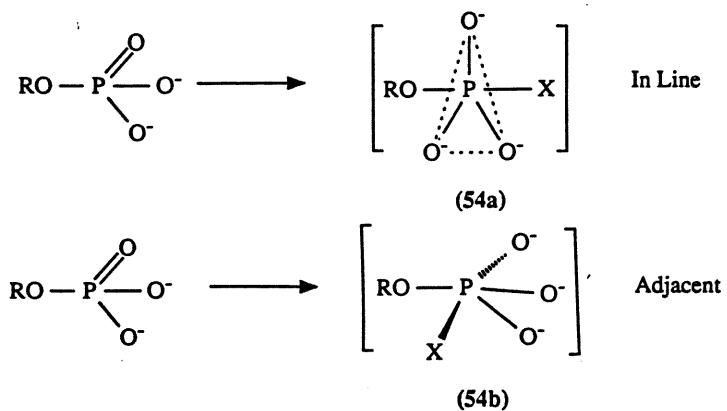
A considerable body of evidence suggests that hydrolysis of phosphate monoesters occurs *via* a dissociative mechanism.³⁷ However, there is considerable controversy surrounding the existence of a metaphosphate intermediate (53). Alternative mechanism involves associative reactions proceeding through isomeric pentacoordinate transition states (54a) and (54b), which may arise from adjacent or in-line addition of the nucleophile to the phosphate monoester, as shown in Scheme 1.13.³⁸ More information concerning these mechanisms can be found in several good reviews and the references therein.^{39,40d}

The phosphatases are of a subgroup of enzymes that catalyse phosphoryl transfer reactions. Other phosphotransferases include the phosphokinases, phosphomutases, phosphodiesterases and pyrophosphokinases. Phosphotransferases have received considerable attention in terms of their fundamental structure and mechanism and several good reviews have appeared.³⁹

Scheme 1.13: Dissociative Pathway

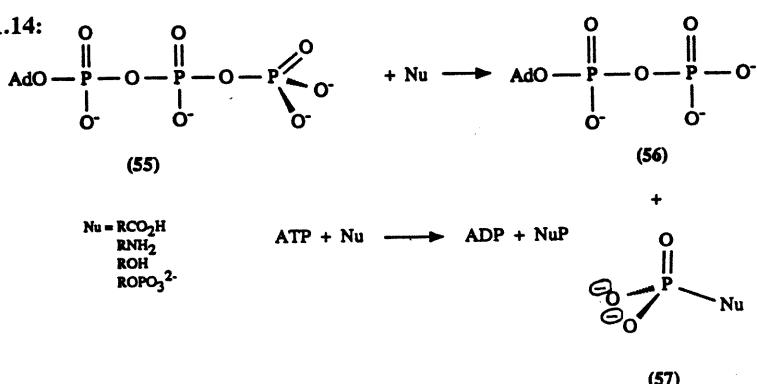


Associative Pathway



1.6.1. Phosphokinases: The kinases are a group of enzymes that catalyse the transfer of the gamma-phosphoryl of nucleoside triphosphates to acceptor nucleophiles. These acceptor functionalities include carboxyl (acetate kinase), hydroxyl (hexokinases), nitrogen (creatine and arginine kinases), and phosphate (adenylate kinase) derivatives. An example of this process is illustrated in Scheme 1.14 to yield (57) as the product.

Scheme 1.14:

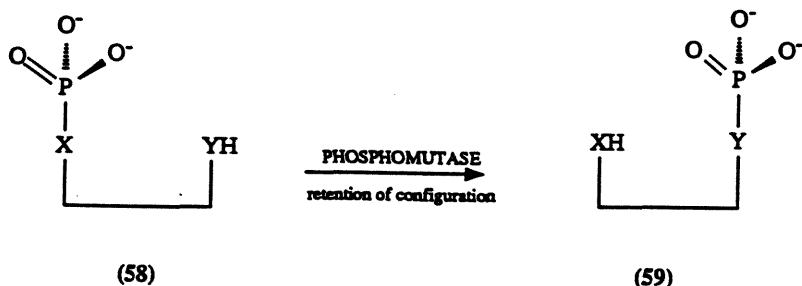


The phosphokinases are known to react *via* a sequential 5 step mechanism involving a ternary complex.⁴¹ The stereochemical course of these displacement reactions

has been investigated using isotopically chiral [^{16}O , ^{17}O , ^{18}O]phosphate esters.⁴⁰ The results show that the vast majority of the kinases proceed with inversion of configuration though a few exceptions are known to go with retention.

1.6.2. Phosphomutases: The phosphomutases catalyse the intramolecular phosphoryl group transfer. The mutases, such as the cofactor-dependent form of phosphoglycerate mutase and phosphoglucomutase, require the bisphosphorylated substrate as an essential cofactor. These cofactor-dependent enzymes proceed through a phosphoenzyme intermediate in which a phosphorylated serine^{42a} or histidine^{42b} residue is observed. Some of these enzymes have also been shown to catalyse intermolecular phosphoryl transfers between two monophosphorylated substrates.⁴³ The phosphomutases proceed with retention of configuration about the transferred phosphoryl group, and this is illustrated in **Scheme 1.15** in which (58) is converted to (59).

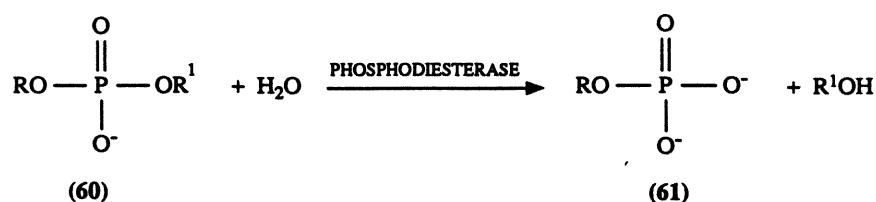
Scheme 1.15:



1.6.3. Phosphodiesterases: The phosphodiesterases are a very important group of enzymes since they catalyse reactions at the phosphorus groups of essential substrates such as DNA and RNA. These include various enzymes such as DNase, RNase, RNA and DNA polymerases. The majority of these phosphodiester transfer reactions proceed with inversion of configuration, which seems to imply a single in-line displacement mechanism. A general reaction for these enzymes is shown in **Scheme 1.16**, in which only one of the

R groups is cleaved from the phosphate diester (60) to yield the monophosphate ester (61).

Scheme 1.16:



1.6.4. Phosphatases: The phosphatases can be considered as a special class of phosphotransferases which catalyse reactions involving water as the nucleophile. Monosubstituted phosphate esters are hydrolysed to give inorganic phosphate and the corresponding alcohol. The simple hydrolytic enzymes that will be discussed are the acid (E.C. 3.1.3.2) and alkaline phosphatases (E.C. 3.1.3.1), both of which are nonspecific phosphomonoester hydrolases.

1.7. Alkaline Phosphatases:

The alkaline phosphatases are distinguished from their acid counterparts primarily by the pH dependence of their catalytic activity which is in the alkaline range rather than neutral or acid pH. A significant amount of information about the alkaline phosphatases and general phosphate ester hydrolysis can be found in several reviews.⁴⁴

1.7.1. Distribution and Function:

These phosphatases are widely distributed in fish, mammals, bacteria and fungi, though they are absent from higher plants. Within mammals, different levels of activity are found in various tissues. Tissue types in order of activity are: intestinal mucosa = placenta > kidney = bone > liver = lung.⁴⁵ Clearly alkaline phosphatases in mammals are

essential tissue specific enzymes. The phosphatases are found mainly on the outer membranes and absorptive surfaces of tissues such as the brush border of the intestine, implying that they play an important role in phosphate transport.⁴⁶ It has been observed that hardening cartilage is high in phosphatase activity suggesting that the catalysed release of orthophosphate in bone is responsible for its ossification.⁴⁷ *E. coli* alkaline phosphatase is a periplasmic enzyme whose production parallels an increase in orthophosphate consumption. It has been shown that high concentrations of orthophosphate inhibit the phosphatases.⁴⁷ Much of the early work in this field was done using the enzyme extracted from *E. coli*, which is readily isolated in large quantities. Consequently, a lot of what is understood about phosphatase activity is based on the *E. coli* enzyme.⁴⁸

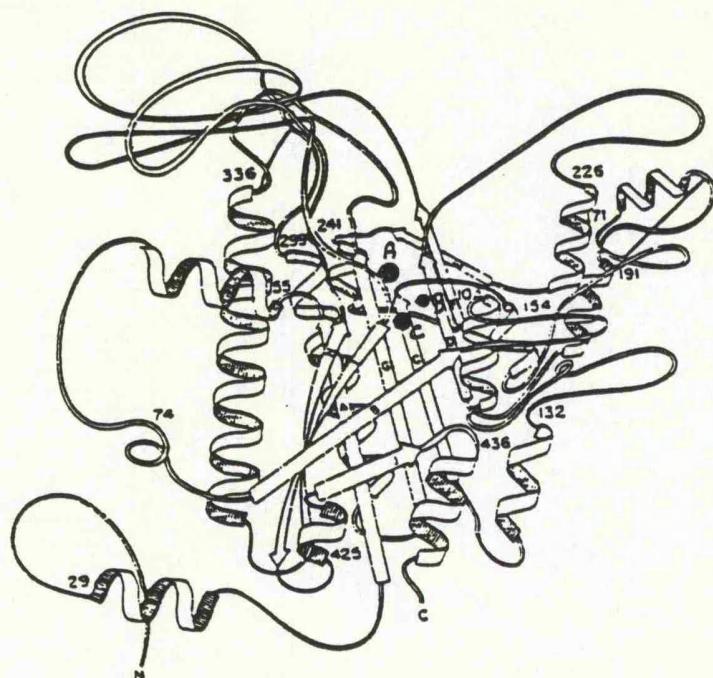
1.7.2. Structure:

The single chromosomal gene for alkaline phosphatase *pho A* is part of at least 18 genes on the *pho* regulon which have been located and mapped.⁴⁹ Alkaline phosphatase is synthesised as an inactive monomer on the plasma membrane bound ribosomes. It is made with a signal peptide which once cleaved enables dimerisation of the monomers to form the active enzyme. Each active site of the monomer contains three distinct metal binding sites identified as A, B and C and the phosphorylatable serine.⁵⁰ There is also a further metal site some distance from the active site. The monomers contain 449 amino acids in a single unglycosylated polypeptide chain.⁵¹ The relative percentages of amino acids within the monomer are 21% acidic, 14% basic, 14% hydrophilic and 51% nonpolar residues. The catalytic site is the serine 102 residue which is flanked by an aspartate and an alanine residue and is similar to the active centres of the serine proteases. Within the active site there are some ten histidine residues, four of which are involved in the coordination of the zinc ions. The X-ray structure of the alkaline phosphate enzyme

isolated from *E. coli* has been solved and shows the crystals to be of the P3₁2₁ space group, in which the subunits are related by two fold rotational symmetry.⁵²

The diagram shown below is taken from an article by Sowadski describing the three dimensional structure of alkaline phosphatase.⁵³ It shows the ten strand β-sheet labelled A through J and on either side of these are α-helices. Close to the active site are two more α-helices which are much more tightly twisted to favour dipole-dipole interactions. The three metal ions of the active site are identified as A, B and C. The phosphate is thought to locate itself between A and B, serine 102 and arginine 166, all of which are important for electrostatic stabilization of the substrate. The serine 102 is also a direct ligand to A and B, while threonine 155 is a ligand to C.

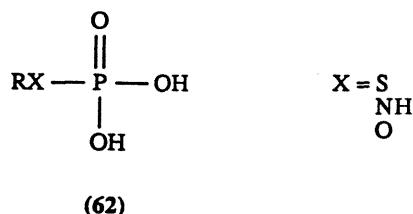
Besides a core of β-sheet, the secondary structure of the monomer consists of mainly 12 α-helices. Between the mammalian and *E. coli* enzymes, the amino acid sequences show a high degree of conservation around the active site.^{44b} This implies that the basic elements for the reaction have been conserved from bacteria to mammals. The *E. coli* dimer has a molecular weight of 94kD,⁵⁴ and that of the bovine intestinal mucosa has been determined as 120kD.⁵⁵



1.7.3. Substrate Specificity:

Besides catalysing the hydrolysis of monophosphate esters, alkaline phosphatase can also catalyse transphosphorylation reactions in which different alcohols can be substituted for water and act as the phosphoryl acceptor (e.g. propane-1,3-diol, glycerol). It has been observed that a variety of monoesters can be hydrolysed including the S-phosphorothioates⁵⁶ (X=S), N-phosphoramidates⁵⁷ (X=NH) and phosphates (X=O), which are shown below in **Scheme 1.17**.

Scheme 1.17:

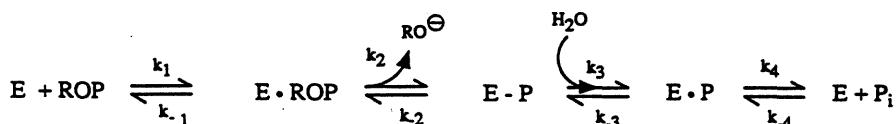


A comprehensive study by Heppel⁵⁸ and coworkers has shown that *E. coli* phosphatase can hydrolyse a wide variety of phosphate monoesters of the general structure (62), with R groups including glycinol, ribose, ADP and histidinol. Many different phosphate and S-phosphorothioate esters are hydrolysed at similar rates suggesting that they share a common intermediate. In contrast to the tolerance for the leaving groups with respect to itself, strict specificity for the phosphoryl group is shown. Di- and tri-esters are not hydrolysed, nor are mixed esters where the hydroxyl group is replaced with an alternative functionality. The phosphonates are not substrates since the phosphatases do not cleave P-C bonds. However, materials of this type including phenylphosphonate, are known to be competitive inhibitors by mimicking the natural substrate. Many amino acids are known to be inhibitors of the phosphatases and vary in the type of inhibition from competitive to mixed.

1.7.4. Kinetics and Mechanism of Action:

As mentioned earlier, the enzyme, alkaline phosphatase is able to hydrolyse P-S bonds just as rapidly as P-O bonds, which suggests an associative^{43,59}(nucleophilic S_N2) mechanism involving a common intermediate. This phosphoenzyme intermediate was first observed by Ergstrom and Agren,⁶⁰ and subsequently identified as a phosphoserine involving the serine 102 residue. **Figure 1.5** shows the sequential in-line mechanism of the hydrolysis of the phosphate monoester to the alcohol and inorganic phosphate. The E·ROP is the Michaelis complex with the dianion of the phosphate monoester. E-P is the phosphoryl-enzyme complex and finally the E·P Michaelis complex of the phosphate dianion product.

Figure 1.5:

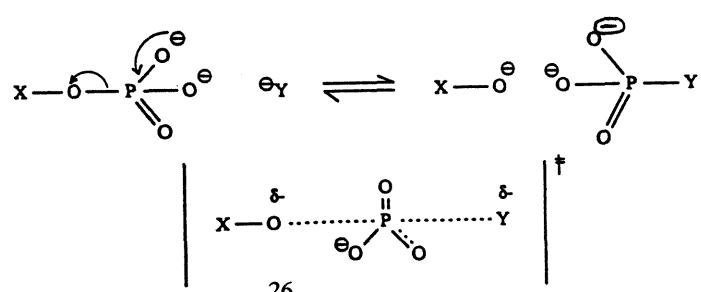


Under alkaline conditions the phosphoenzyme cannot normally be observed. Only at acidic pH is it possible to observe the phosphoserine intermediate by ³¹P NMR spectroscopy, since it accumulates as the dephosphorylation of E-P is rate limiting. As the enzymatic reaction proceeds there appears to be a burst of alcohol product^{54b} which is not only consistent with a phosphoenzyme intermediate, but also implies that the phosphate release is the rate limiting step of the reaction.^{44b} The stereochemical course of the reaction is known to proceed with overall retention of configuration at phosphorus, implying that both nucleophilic displacement steps must go with inversion of configuration, i.e. with "in-line" approach of the nucleophile.⁶¹ The enzyme must bind the

substrate in the Michaelis complex ($E \cdot ROP$) so that the phosphoryl group is in the correct orientation to allow nucleophilic attack by the serine residue. The phosphoserine is then hydrolysed in a subsequent nucleophilic displacement by water to give the second Michaelis complex ($E \cdot P$), which then dissociates as the rate limiting step. Site specific mutagenesis of the alkaline phosphatase has been performed in which cysteine was substituted for the serine 102 residue.⁶² The stereochemical course of the reaction was still retention of configuration about the phosphorus centre. This new mutant enzyme showed a decrease in the rate of phosphorylation by 10 fold, the turnover rate however, appears to remain the same, which implies that the rate limiting step is now that of phosphorylation and not the dissociation of the phosphoenzyme intermediate. Further site-specific mutagenesis experiments have been performed to explore the roles of the important side chains in the active site. The arginine 166 has been replaced with lysine, glutamine^{63a} and alanine,^{63b} to see if the guanidinium group plays an important role in catalysis. Also, the conversion of the lysine 328 residue to histidine and alanine have been investigated since they are known to be important in the binding of water molecules to the zinc ion centres (A and B).⁶⁴

The current view concerning the hydrolysis of phosphate monoesters is that it is a *dissociative-like* reaction in which a *metaphosphate-like* intermediate is involved as shown in **Figure 1.6**.⁶⁵ The bonds of the phosphate between X and Y are thought to be elongated. This reaction mechanism has been further supported by the work of Williams *et.al.*⁶⁶ while investigating the charge and functionality of the leaving alcohol group.⁶⁶

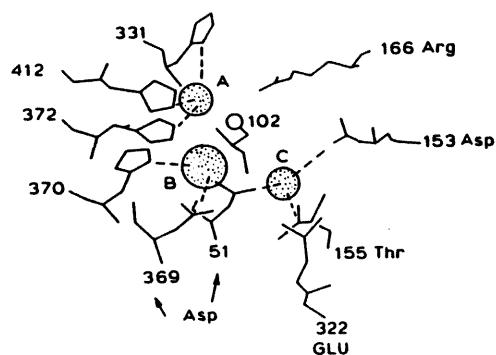
Figure 1.6:



1.7.5. Role of Zinc and Magnesium Ions:

Like most enzyme catalysts the alkaline phosphatases require metal ions for catalytic activity.⁶⁷ The alkaline phosphatases require two zinc (II) ions to occupy two definite binding sites within each monomer, identified as A and B, while a magnesium ion occupies the binding site C. These are shown in Figure 1.7 taken from a review by Coleman et.al.^{44b} These sites are identified by their decreasing order of binding affinity for the phosphoryl group during its transfer. They are thought to be involved in the general stabilization of the protein structure. It has been demonstrated that these phosphatases can be activated further by the addition of magnesium (II) ions.⁶⁸

Figure 1.7:



The metal ion in site A, initially coordinates the phosphate of the E·ROP complex prior to loss of the alkoxide ion, after which, following formation of the phosphoserine intermediate (E-P), the metal has a free coordination site. This allows a water molecule to coordinate to the A site to form a metal-bound hydroxide ion which can attack the phosphoserine intermediate, completing the catalytic cycle. The B site contributes to the phosphoryl binding and by assisting in the formation of the *serine-O*-anion. Since zinc is a spectrophotometrically silent metal most of these studies concerning

the binding sites have involved replacing the zinc ions with ^{113}Cd or ^{111}Cd , both of which have spins of one half and can be monitored by NMR spectroscopy.⁶⁹ Extensive investigations concerning these metal ion substitutions have been carried out but are too detailed to be discussed here. Several good reviews regarding these substitutions are available.^{44,53,68}

1.8. Acid Phosphatases:

The acid phosphatases hydrolyse monophosphate esters with a maximum rate observed near pH 5.0. Like their alkaline counterparts they are devoid of any di- or triesterase activity. The pH optimum and their inability to hydrolyse sulphur esters of phosphorothioates distinguishes them from the alkaline phosphatases. A great deal of work has been done with the prostatic, liver and spleen acid phosphatases to identify their activities using various substrates.⁷⁰ They are very versatile and nonspecific enzymes which can effect the transfer of the phosphoryl group to numerous alcohol acceptors,⁷¹ in place of the aqueous solvent. Acid phosphatases have also been isolated from other sources including bone, *E. coli*, fungi and plant seedlings. Hydrolysis of monophosphate esters by the acid phosphatases is known to proceed through the formation of a phosphohistidine covalent intermediate. This intermediate is analogous to the phosphoserine of the alkaline phosphatases. The enzymes are symmetrical dimers and have molecular weights in the range of 90-170kD.⁷² As with the alkaline phosphatases, competitive inhibition of the acid enzymes is observed with oxalate and vanadate. It has been shown by Reiner⁷³ and coworkers, that under various concentrations fluoride inhibition could be competitive or mixed. β -Hydroxycarboxylic acids are also known to be potent inhibitors of prostatic acid phosphatase.⁷⁴ If carboxylic acids are used without a β -hydroxy group they are found to have no effect on the enzyme and this is also true if the hydroxyl group is replaced with a thiol functionality.

1.8.1. Violet Acid Phosphatases:

This is a metalloenzyme, isolated from plants and various mammalian tissues, containing one manganese(III) ion per dimer. The intense violet colour is due to the manganese ion binding to a tyrosine residue within the active site. Both the tyrosine and Mn(III) are involved in the phosphate and substrate binding. The potato enzyme which has been isolated and crystallised has a molecular weight of 110kD as a dimer.⁷⁵ It has been shown to hydrolyse a broad spectrum of phosphate monoesters, although their activity varies from relative rates of 100 for *para*-nitrophenyl phosphate to 33 for glucose-6-phosphate.⁷⁵ Clearly, for the acid phosphatase, the rate limiting step is not the hydrolysis of the E·P, since the overall rate is influenced by the nature of the leaving group which is illustrated by the comparative reaction rates of *para*-nitrophenyl phosphate and glucose-6-phosphate.

1.8.2. Purple Acid Phosphatases:

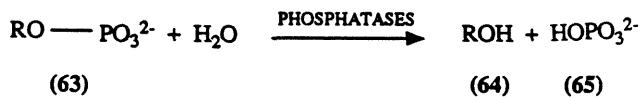
These are a relatively new class of phosphatases recently isolated from mammalian placenta and spleen.⁷⁶ They catalyse the hydrolysis of aryl phosphoric esters, phosphoric anhydrides and phosphoproteins containing phosphoserine residues.⁷⁷ The reaction catalysed by these phosphatases proceed with overall inversion at the phosphorus centre.⁷⁸ Therefore, in contrast to the other acid and alkaline phosphatases, which react *via* a phosphoenzyme intermediate, purple acid phosphatases catalyse the direct transfer of a phosphoryl group to water. They have an intense purple colour and two oxidation states, Fe(III) Fe(III) and Fe(III) Fe(II). The iron(III) is bound to two tyrosine residues within the active site, with at least one histidine residue acting as a ligand to each of the iron centres. In its oxidized form (purple), the enzyme shows no activity, but when reduced shows considerable phosphatase activity. The addition of inorganic phosphate causes inactivation of the enzyme converting it back to its oxidised form. Controversy still

surrounds the exact mechanism of these phosphatase reactions. They are believed to proceed either by metal catalysed release of metaphosphate,⁷⁹ or by direct attack of a metal-coordinated hydroxide at phosphorus.⁸⁰ Attack of an enzyme nucleophile to produce a phosphoenzyme which is subsequently hydrolysed is ruled out on the basis of the stereochemistry.⁸¹ Clearly, the phosphatase activity is coupled to the oxidation state of the iron centres and implies a complex function which is yet to be fully investigated and understood.

1.9. Proposed Plan of Study:

The aim of this work is to explore the use of selected alkaline phosphatases as routes to chiral building blocks. Alkaline phosphatases are a group of enzymes of broad specificity which catalyse the hydrolysis of a wide range of monophosphate esters (**63**) to the corresponding alcohols (**64**) and inorganic phosphate (**65**), as shown in **Scheme 1.18**.^{82,83}

Scheme 1.18:

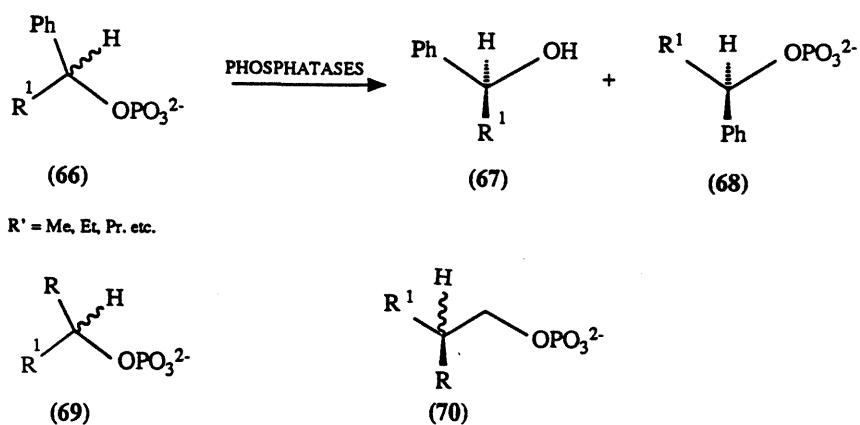


1.9.1 Enantioselectivity Using the Phosphatases:

As discussed earlier, a great deal of work has been done using the enantioselectivity of enzymes such as lipases to synthesise chiral products.¹²⁻¹⁸ It is intended that the phosphatases be investigated to determine their potential in terms of chiral discrimination leading to useful chiral structures. Recent work by Williams *et.al.*⁸⁴ has shown that the alkaline phosphatases show a degree of selectivity and reactivity towards various phosphate monoester derivatives. The structure and functionality of the

ester groups of these phosphates play an important role in determining the reactivity of the phosphate monoesters. It is thought that during the hydrolysis reaction the leaving substituent interacts with some sort of lipophilic binding site within the active site. The observations by Williams suggest that the differences in binding energies of the various primary alkyl and aryl phosphate substrates can be as much as 2.5kcal/mol (10.45kJ/mol). Clearly, this difference in the relative binding energies can effect the rates of reactions up to 65 fold. It is hoped that similar differences in the reaction rates of the (R)- and (S)-phosphate enantiomers for a series of racemic monophosphate substrates would be seen. These differences can be exploited by the alkaline phosphatases to effect high enantioselective reactions. For example, the kinetic resolution of the simple mixtures of racemic monophosphate esters (66, 69, 70) to produce chiral alcohols, as in Scheme 1.19, will be explored.

Scheme 1.19:



The scope of this approach will be explored by varying the nature of the ester function, eg. the size of the alkyl group (R^1) at the chiral centre; substituting alkyl groups

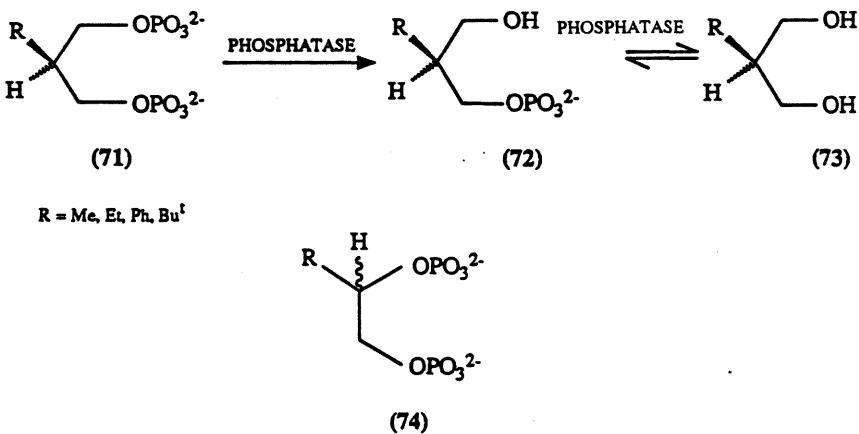
for the phenyl group and also by making the chiral centre more remote from the catalytic phosphorus centre as with (70) above. The effects of changing the various reaction conditions, such as solvent, pH and concentration on the chiral discrimination will also be explored.

1.9.2. Prochiral Selectivity Using the Phosphatases:

Described earlier was the considerable success that has been achieved through the use of esterases and lipases in the resolution of *meso*-1,2-and 1,3-disubstituted reactants.¹⁹

²¹ Similar strategies can be envisaged (Scheme 1.20) using the phosphatases, since they show a broad specificity for many substrates.²²

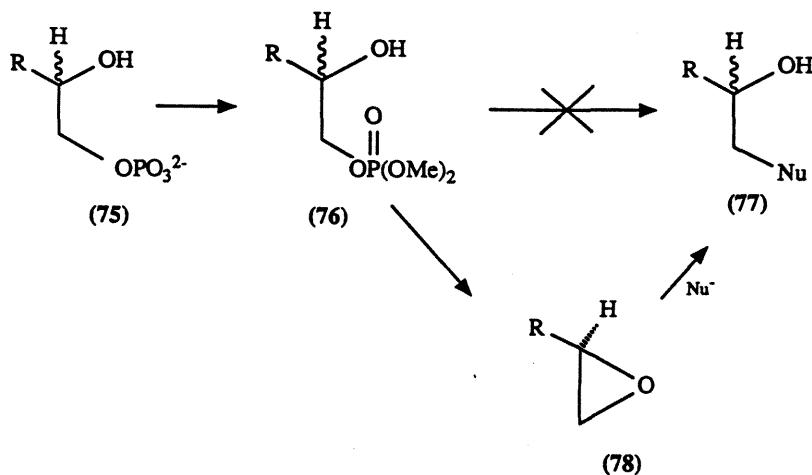
Scheme 1.20:



The phosphatases could be used to discriminate between the two enantiotopic phosphate monoester groups of a series of bisphosphates. The bisphosphate (71) is prochiral and the phosphatases might be expected to effect an enantioselective hydrolysis reaction to produce the chiral monophosphate ester (72), an important chiral synthon. The enzyme's stereoselectivity could be explored by varying the size and length of the alkyl group (R), as well as substituting it for other functional groups such as amines, esters and

halogens. The advantage of this type of transformation over the reactions with the racemic monophosphates, is that all of the symmetrical 1,3-bisphosphate can be converted into the desired chiral monophosphate product. It is also possible to investigate the selectivity of the phosphatases towards the racemic 1,2-bisphosphates (**74**) shown in **Scheme 1.20**. The use of the phosphatases for the synthesis of chiral building blocks offers the potential for converting the resultant phosphate functionality into a leaving group that can be chemically exploited. **Scheme 1.21** gives an example of such a chemical manipulation. Esterification of the monophosphate ester (**75**) will convert it to the corresponding triester (**76**). Direct nucleophilic displacement of the dimethylphosphate is unlikely because of the potential attack directly on the phosphorus. However, a two step process *via* the epoxide (**78**) might provide a route to a wide variety of substituted derivatives as well as the epoxides themselves.

Scheme 1.21:



Obvious advantages of using the phosphatases is that they are robust, easy to handle and readily available. In fact, Sigma has 45 alkaline phosphatase preparations from

some 20 different sources and a large inventory of acid phosphatases. Solubility is not a problem with these enzyme reactions since the phosphate substrates are readily soluble in aqueous media. In addition, the alcohol products from the hydrolysis reactions can easily be isolated by an organic extraction of the reaction mixture. The unreacted substrate remains in the aqueous solution. There is no need for expensive cofactors or the need to provide complicated ATP regeneration systems. The focus of this investigation will be to study the *regio-* and *stereo*-specificity of the phosphatases on a variety of substrates with the potential of developing routes to chiral synthons.

Chapter 2

Synthesis of Racemic Alkyl Monophosphates and 2-Alkylpropyl-1,3-Bisphosphate Derivatives.

2.1 Introduction:

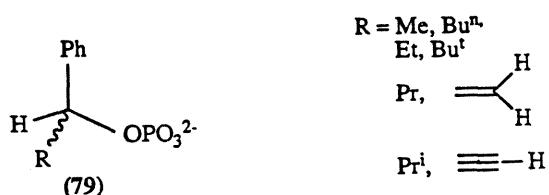
Included within this chapter are the syntheses of racemic alcohols, 1,3-propanediols and other essential intermediates required for the preparation of the monophosphate ester substrates used in the enzyme studies. The first section covers the preparation of racemic alcohols by various methods, which are subsequently used in the synthesis of the corresponding monophosphate esters. Also discussed are the syntheses of several enantiomerically pure alcohols used to evaluate the kinetic parameters of individual enantiopure chiral monophosphate esters with various alkaline phosphatases. The second part of the chapter deals with the selection and evaluation of different phosphorylating and phosphitylating reagents available used the synthesis of the phosphate monoesters. Also included are the preparations of various 1,3-propanediols *via* reduction of the corresponding diethyl-2-alkylmalonate derivatives, which are used to prepare the 2-alkylpropyl-1,3-bisphosphate substrates. Finally, an examination of the various derivatisation reactions used to determine the enantiomeric excesses of the alcohol products from the enzyme reactions is presented.

2.2 Synthesis of racemic alcohols:

The first part of this work was to study the enantioselectivity of the phosphatases with a series of racemic monophosphates of the general structure shown in Figure 2.1. The phenyl substituent was retained since previous work has shown that compounds containing aromatic groups are particularly good substrates for the alkaline phosphatases. The nature of the alkyl group (R), was varied to establish the effect on enantioselectivity. With the exception of the methyl derivative (R,S)-1-phenylethanol (**80**) which was commercially available, the remaining racemic alcohols were synthesised and converted

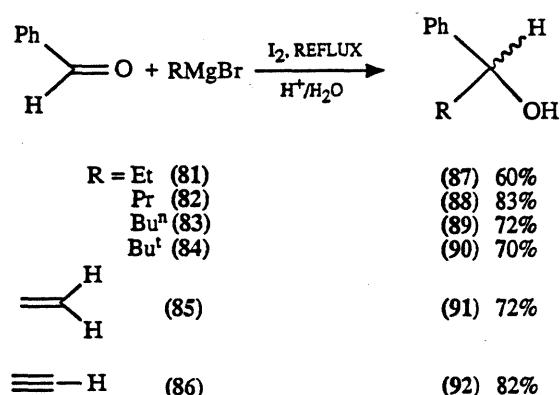
into their phosphate monoesters.

Figure 2.1:



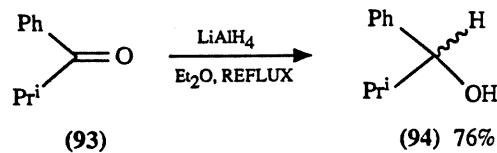
The ethyl,⁸⁵ propyl,⁸⁶ butyl,⁸⁷ *tert*-butyl,⁸⁸ vinyl⁸⁹ and acetylenic⁹⁰ derivatives (87-92) were obtained from the Grignard reaction of benzaldehyde with the corresponding alkyl, alkenyl and alkynyl magnesium bromide reagents (81-86), as shown in Scheme 2.1.

Scheme 2.1:



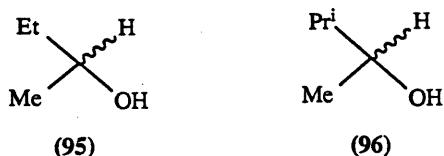
The *iso*-propyl substituted benzyl alcohol (94)⁹¹ was produced by the lithium aluminium hydride reduction of the ketone, 2-methyl-1-phenyl-1-propanone (93), shown in Scheme 2.2.

Scheme 2.2:



The other racemic alcohols used to prepare monophosphate esters were 2-butanol (95) and 3-methyl-2-butanol (96) shown in Figure 2.2, both of which are commercially available.

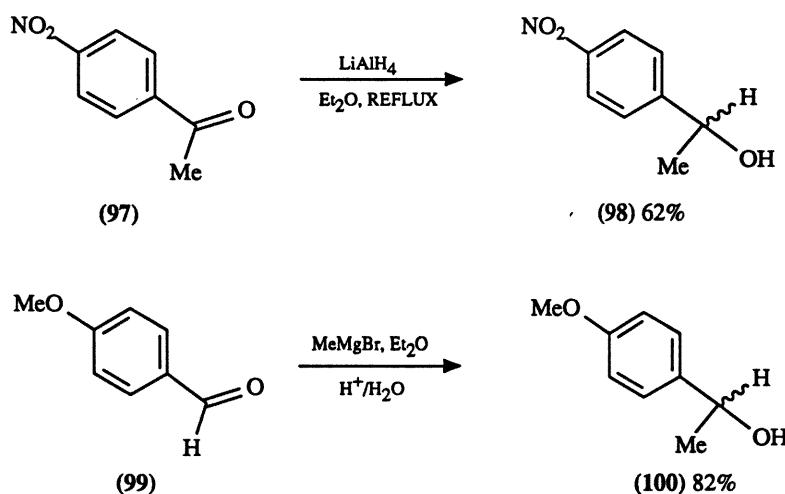
Figure 2.2:



The second stage of this investigation was to probe the effects that *para*-substituents on the phenyl ring of the alcohols (98, 100) might have on the enantioselectivity of the phosphatases. The basic template for these studies was 1-phenylethyl-1-phosphate substrate (179), substituting the ring with both electron withdrawing (NO₂) and electron donating (OMe) groups to explore the influence of electronic effects. The 1-(*para*-nitrophenyl)ethanol (98) was prepared by the reduction of the *para*-nitrophenylmethyl ketone (97) as shown in Scheme 2.3. Preparation of the 1-(*para*-methoxyphenyl)ethanol (100) was achieved by the Grignard reaction of anisaldehyde

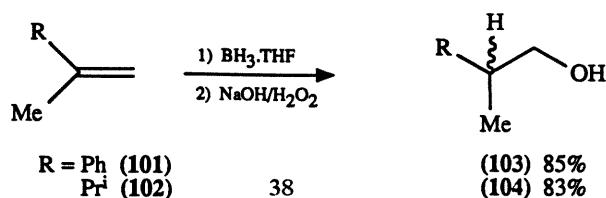
(99) with methyl magnesium bromide also shown in **Scheme 2.3**.

Scheme 2.3:



The final study carried out was to explore the enantioselectivity shown by the phosphatases with substrates where the chiral centre was more remote from the phosphoryl group. The synthesis of the corresponding racemic alcohols with the phosphate moiety and the chiral centre separated by a methylene group is shown in **Scheme 2.4**. Hydroboration reactions⁹² of the alkenes α -methylstyrene (**101**) and 2,3-dimethyl-1-butene (**102**) produced the respective alcohols 2-phenyl-1-propanol (**103**) and 2,3-dimethyl-1-butanol (**104**).

Scheme 2.4:



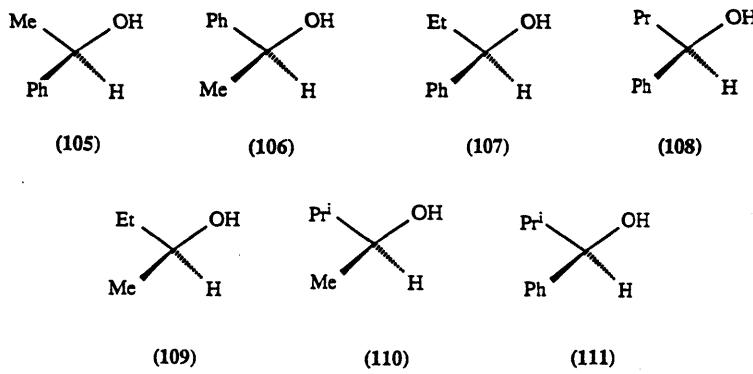
From this brief discussion, it is possible to see how all of the racemic alcohols were prepared for use as precursors in the synthesis of the racemic monophosphate substrates.

2.3 Synthesis of chiral alcohols:

Evaluating the selectivity of the phosphatase enzymes towards the various phosphate substrates, required the availability of single enantiomers of the alcohols as reference materials. The enantiopure phosphate esters were used to determine individual kinetic parameters, allowing the enantioselectivities to be optimised. Also, single enantiomers of the alcohols were used in subsequent derivatising reactions with (R)- α -methoxy-(α -trifluoromethyl)phenyl acetic acid chloride⁹³ (**209**) (page 63) and 2-chloro-3,4-dimethyl-5-phenyl-1,3,2-oxazaphospholidine-2-sulphide⁹⁴ (**217**) (page 71) to assign the absolute configuration of the predominant products from the enzymatic hydrolyses by ¹⁹F NMR and ³¹P NMR spectroscopy respectively.

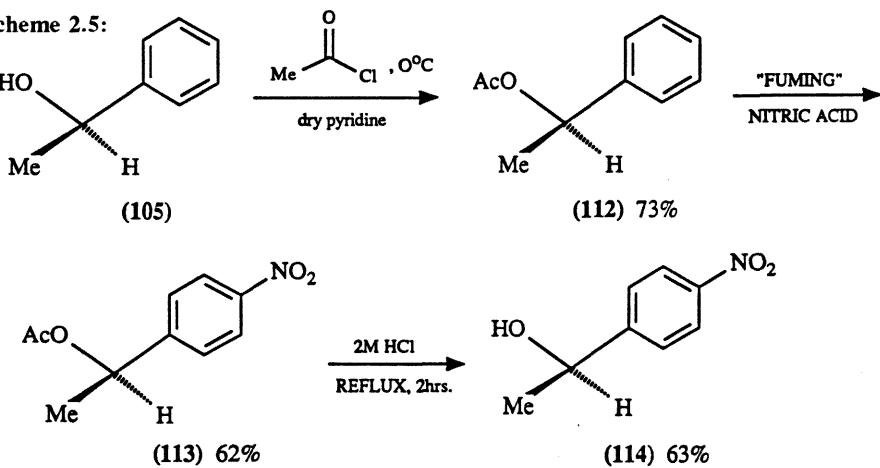
The chiral alcohols, (1R)-phenylethanol (**105**), (1S)-1-phenylethanol (**106**), (R)-1-phenyl-propanol (**107**), (R)-1-phenyl-1-butanol (**108**), (S)-2-butanol (**109**), (2S)-3-methyl-2-butanol (**110**) and (R)-2-methyl-1-phenyl-1-propanol (**111**), shown in **Figure 2.3**, are all commercially available.

Figure 2.3:



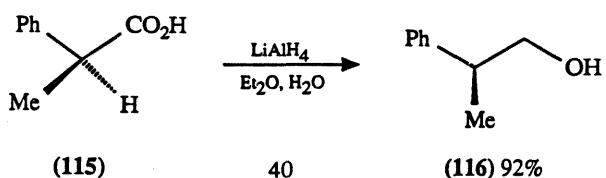
The (1R)-(para-nitrophenyl)ethanol (**114**) was synthesised from (1R)-phenylethanol (**105**) as shown in **Scheme 2.5**. The chiral alcohol was first protected with acetyl chloride to yield the acetate derivative (**112**) in 73% yield. This protected material was then treated with fuming nitric acid at 0°C to produce the *para*-nitro substituted phenylethyl derivative (**113**). A small amount of racemisation of the chiral benzylic centre under the acid conditions was observed yielding the (S)-enantiomer of (**113**). The acetate protecting group was subsequently removed by refluxing (**113**) in 2M hydrochloric acid to give the desired alcohol (**114**) in 63% yield.

Scheme 2.5:



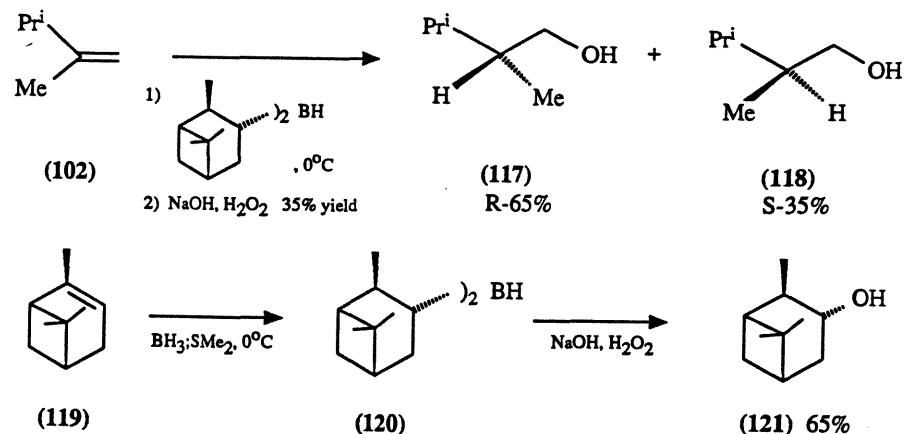
The (S)-2-phenyl-1-propanol (**116**) was obtained in a 72% yield from the lithium aluminium hydride reduction of the (S)-2-phenyl-1-propionic acid (**115**) shown below.

Scheme 2.6:



The synthesis of (*R*)-2,3-dimethyl-1-butanol (**117**) in 30% ee over the (*S*)-enantiomer (**118**), was achieved by reacting 2,3-dimethyl-but-1-ene (**102**) with the chiral hydroborating agent α -diisopinocampheylborane (**120**)⁹⁵ as shown in **Scheme 2.7**. The α -diisopinocampheylborane was synthesised from α -pinene (**119**) and borane dimethyl sulphide complex also shown in **Scheme 2.7**. The borane was analysed for optical purity by converting it to isopinocampheol (**121**).⁹⁵ Subsequent optical rotation measurements of this material indicated that it had an ee of 87%, and produced the 30% ee of (*R*)-2,3-dimethyl-1-butanol (**117**).

Scheme 2.7:



2.4 Phosphorylation of racemic alcohols:

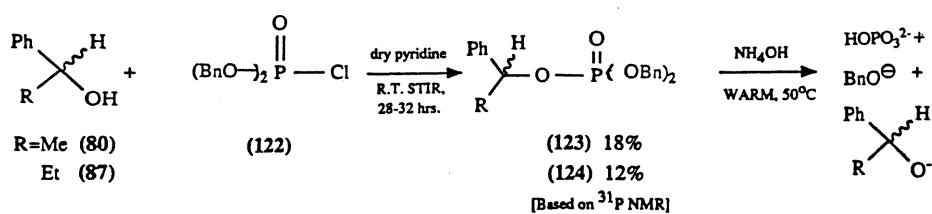
The next stage in the preparation of the phosphate substrates was the conversion of the racemic alcohols to their corresponding monophosphate esters. Two approaches to this synthetic transformation were undertaken; the first was to use stable readily available activated phosphates as phosphorylating agents. The other, more efficient phosphorylating procedure was to employ phosphite based reagents used in oligonucleotide synthesis,

which are known to be far more reactive. These would require an oxidation step to yield the desired monophosphate products.

2.4.1 Use of phosphorylating reagents:

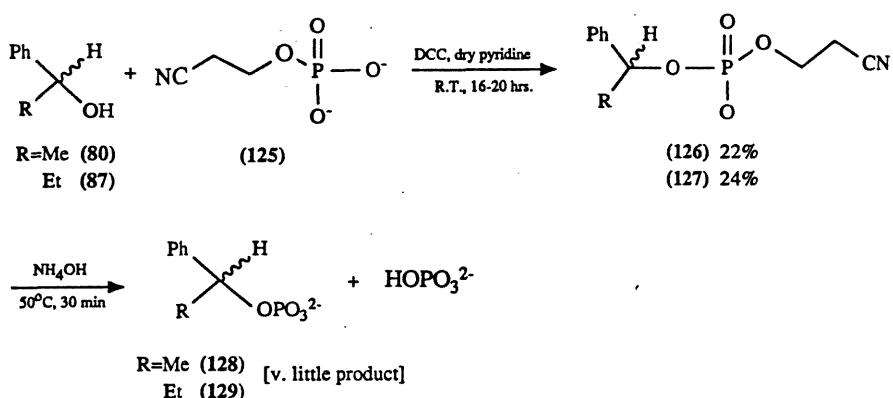
The use of multifunctional phosphorus reagents, such as phosphorus oxychloride and polyphosphoric acid were avoided during the synthesis of simple monophosphate esters. They tend to give a complex mixture of products, including pyro- and polyphosphorylated materials. The initial attempts to synthesise the phosphate esters centred around the use of dibenzyl phosphorochloridate⁹⁶ (122) in the presence of pyridine, as shown in **Scheme 2.8**. This method of phosphorylation was found to be ineffective. (122) Is not a very powerful phosphorylating agent and appeared to be very slow in reacting with the secondary alcohols. In general, it took between 28-31 hours to observe any significant amount of product, with numerous by-products being identified by ³¹P NMR spectroscopy. The low yields of 10-20% were thought to be due to the instability of the reagent (122). The prolonged reaction times in the presence of pyridine are reported to cause debenzylation.⁹⁷ Removal of the benzyl groups from the intermediate phosphotriester is usually achieved using palladium catalysed hydrogenolysis. Though the carbon linker of the alcohol group to the phosphorus centre is benzylic, it is a secondary carbon centre. The other benzyl groups attached to the phosphorus are primary centres. It was anticipated that this subtle difference would be enough to prevent cleavage of the secondary alkyl phosphorus bond. However, this reaction proved to be unsuccessful in most cases; the hydrogenolysis resulted in the production of mainly inorganic phosphate, and none of the desired phosphate esters.

Scheme 2.8:



The use of dibenzylphosphorochloridate (122),⁹⁶ to produce the phosphate monoesters proved to be inappropriate, therefore, 2-cyanoethyl phosphate (125)⁹⁸ was used as the phosphorylating agent. It was felt that it would be a superior reagent, since it is monofunctional and the 2-cyanoethyl group can be readily and selectively removed using mild alkaline conditions. Another advantage is its stability prior to reacting it with the dicyclohexylcarbodiimide (DCC), used in the coupling reaction. Shown in **Scheme 2.9** is the reaction between the racemic alcohols and the 2-cyanoethyl phosphate (125) in the presence of DCC, which yielded the phosphate diester intermediates (126, 127). Again the reactions were monitored by ^{31}P NMR spectroscopy. The yields were again very poor and the reactions times slow, taking some 16-20 hours. The cleavage of the cyanoethyl group was carried out by heating the diester derivatives with ammonium hydroxide for 30 minutes. However, the low yields obtained in the first step meant that there was little of the phosphate monoester products obtained. The 2-cyanoethyl phosphate (125), did not prove to be as successful a phosphorylating agent as had been anticipated.

Scheme 2.9:

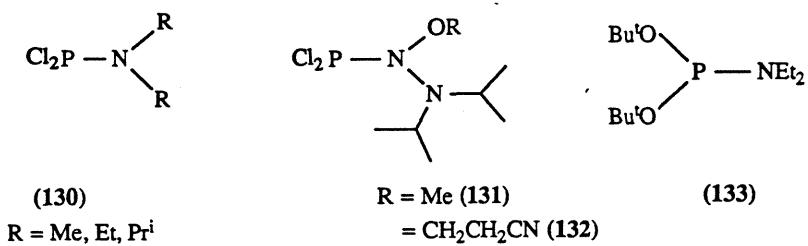


This brief description of the reactions carried out using the P^{V} phosphorylating reagents demonstrates the numerous obstacles and problems they present. These methods clearly did not give yields suitable for general access to the desired monophosphate esters from their respective alcohols. The problems seem to stem from the low reactivity of the secondary alcohols with P^{V} reagents, although no attempt was made to explore reactions of the corresponding alkoxides, which would have been more reactive. Many of these methods have been developed for the synthesis of various inositol derivatives. An important example of this type is tetrabenzylpyrophosphate (TBPP),⁹⁹ which has been used to phosphorylate multiple alcohol centres simultaneously. The sodium alkoxides are first formed using sodium hydride in THF, before the mixture is treated with TBPP and imidazole to yield the desired tri- and tetra-phosphate products.

The considerable success of P^{III} in oligonucleotide synthesis led to investigations of P^{III} phosphite based reagents for the preparation of the phosphate monoester needed in this study. Shown in Figure 2.4, are some of the numerous dichlorophosphoramidites

(130),¹⁰⁰ chlorophosphoramidites (131, 132)¹⁰¹ and the phosphoramidite (133),¹⁰² reported in the literature. These have previously been used to prepare *myo*-inositol phosphates and phosphorylate serine and tyrosine residues during solid phase peptide synthesis.¹⁰³

Figure 2.4:



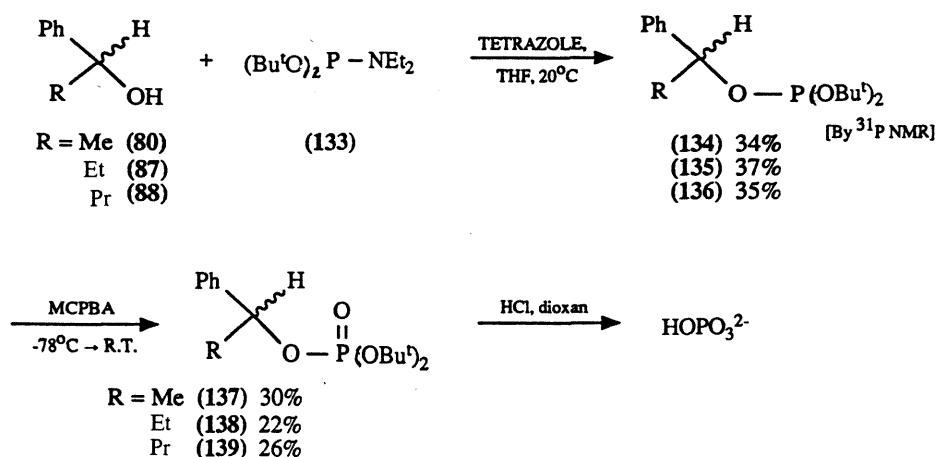
The *N,N*-dialkylchlorophosphoramidites (**130**)¹⁰⁰ were not used as phosphorylating reagents. They are too reactive and lead to the formation of disubstituted phosphoramide intermediates. Further transformations produce phosphate diester products and not the desired monoester materials. A representative sample of the chlorophosphoramidites (**131**, **132**) and the di-*tert*-butyl-*N,N*-diethylphosphoramide (**133**)¹⁰² are discussed in the following section.

2.4.2 Use of phosphitilating reagents:

Di-*tert*-butyl diethylphosphoramidite (**133**),¹⁰² was the first phosphitilating agent to be investigated as shown in Scheme 2.10. To some extent it proved successful in preparing the phosphite triesters (**134-136**), and the phosphate triesters (**137-139**). However the numerous attempts to treat the crude triesters with various concentrations of hydrogen chloride and dioxan failed to produce the monophosphate products. Cleavage of the benzylic C-O bonds also occurred to yield the undesired inorganic phosphate. The difficulties encountered with these experiments prevented any further investigation of this

method to produce the monophosphate substrates.

Scheme 2.10:

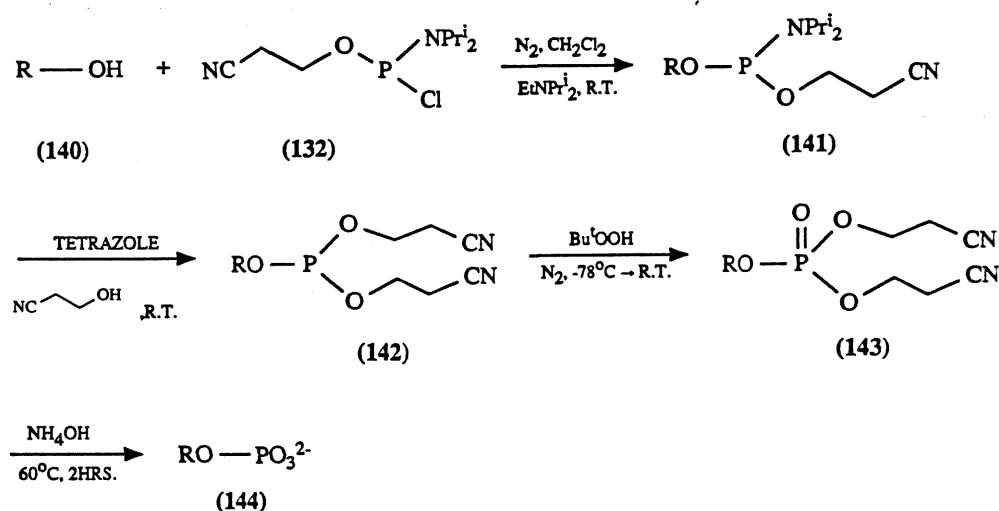


2-cyanoethyl-*N,N*-diisopropylphosphoramidochloridite (132) was found to be the most efficient and effective phosphorylating agent. (132) Could be readily synthesised in gram quantities, and has been widely used in the automated syntheses of oligonucleotides and in the *myo*-inositol field because of the easy of handling and the comparative stability of the P^{III} intermediates.

The advantages of using 2-cyanoethyl-*N,N*-diisopropylphosphoramidochloridite (132) can be appreciated by considering the reaction sequence shown in **Scheme 2.11**. In the first step, the alcohol readily reacts with the phosphite (132) to produce the phosphoramidite (141). The phosphoramidite is converted to the phosphite triester (142) by replacing the *N,N*-diisopropylamino functionality with a second cyanoethyl group catalysed by the weak acid tetrazole. Oxidation of the phosphite triester (142) using *tert*-butylhydroperoxide¹⁰⁴ affords the phosphate triester (143). The phosphate triester (143) is

stable and easily purified by flash chromatography. These first three steps can all be performed sequentially in a single flask. In the final step the 2-cyanoethyl groups can be selectively cleaved by a β -elimination in base giving the desired phosphate ester product (144) in good yields, and acrylonitrile as a by-product.

Scheme 2.11:

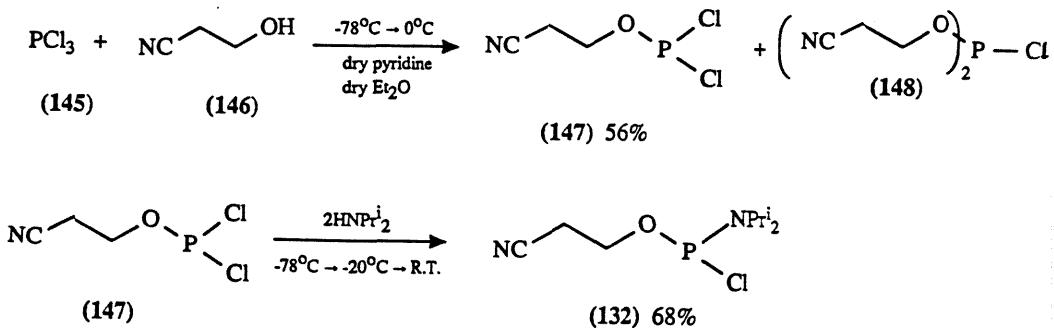


2.4.3. Synthesis of 2-cyanoethyl-N,N-diisopropylphosphoramidochloridite (132):

This reagent has been successfully used in the synthesis of *myo*-inositol derivatives,¹⁰¹ and was used to prepare the monophosphate ester substrates. The chlorophosphoramidite (132), was readily prepared in gram quantities using a variation of the method developed by Sinha *et.al.*¹⁰⁵ The chlorophosphoramidite (132) was synthesised according to the reaction sequence shown in Scheme 2.12. 2-Cyanoethanol (146) was treated with phosphorus trichloride (145) to give the 2-cyanoethylphosphorodichloridite product (147),^{100a} and separated from the bis-(2-cyanoethyl)phosphorochloridite (148) by-

product by fractional distillation. The dichloride (**147**) was then treated with *N,N*-diisopropylamine and left for 12 hours. After removing the amine hydrochloride salt, the desired phosphorylating agent (**132**) was isolated as a clear liquid by distillation under reduced pressure. The chlorophosphoramide (**132**) was then used in subsequent reactions as an effective phosphorylating reagent.

Scheme 2.12:

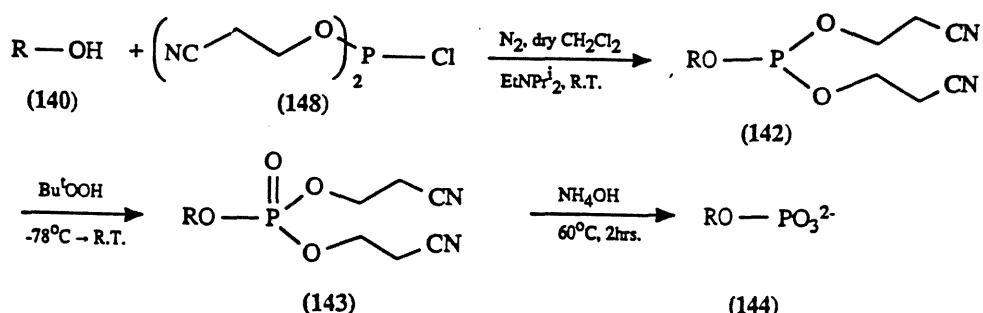


2.4.4. Synthesis and reactions of bis-(2-cyanoethyl)phosphorochloridite (**148**):

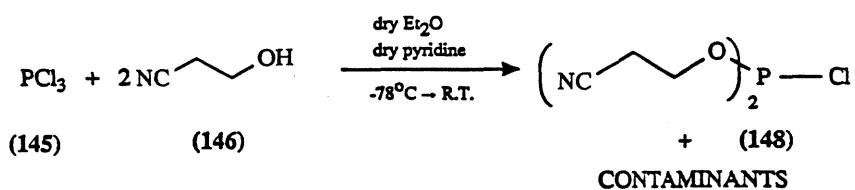
As described in **Section 2.4.2**, (**132**) has many advantages as a phosphorylating agent. However, during the first step of its preparation, besides producing the desired intermediate (**147**), a significant amount of the by-product (**148**) was obtained. This was characterised by a peak in the ³¹P NMR spectrum at δ_P(CH₂Cl₂) = 166.02 ppm (lit.,¹⁰⁶ δ_P = 165.87 ppm) and confirmed as the chloridite (**148**). It has been shown by Van Boom¹⁰⁶ and Evans¹⁰⁷ that this crude material can also be used as an effective phosphorylating agent. As shown in **Scheme 2.13a**, it should be possible to incorporate this material into a synthetic strategy to produce the desired monophosphate substrates. The reagent (**148**) offers advantages over (**132**) in that it can be prepared in one step from phosphorus trichloride and two equivalents of 2-cyanoethanol (**146**). In the synthesis of the phosphate

monoesters the use of (148) is preferable since it removes the need to introduce a second cyanoethyl group (see **Scheme 1.11**). **Scheme 2.13a** shows the overall synthesis of the phosphate monoester using the bis-(2-cyanoethyl)phosphorochloridite (148).

Scheme 2.13a:



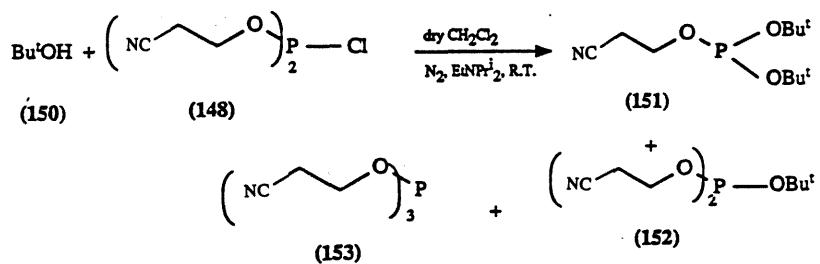
Scheme 2.13b:



Dry phosphorus trichloride (145) is reacted with two equivalents of freshly distilled 2-cyanoethanol (146) at $-78^\circ C$ to give (148) as shown in **Scheme 2.13b**. Purification of the product proved to be very difficult. Distillations at reduced pressures and increased temperatures gave poor results. One problem with bis-(2-cyanoethyl)phosphorochloridite (148) is that on distillation it disproportionates to 2-cyanoethylphosphorodichloridite (147) and tri-(2-cyanoethyl)phosphite (153).^{100a} As mentioned earlier, previous work had been carried out with this material as a crude mixture without any purification.^{106,107} It has been suggested that it is contaminated with only 10% 2-cyanoethylphosphorodichloridite

(147).¹⁰⁷ However, there was no indication of any tri-(2-cyanoethyl)phosphite (153) contaminant being present, which would be expected if (147) were detected. The crude material was used in trial experiments with *tert*-butanol (150) to evaluate its potential as a phosphitylating agent, as shown in **Scheme 2.14**. The reaction of the crude material with *tert*-butanol (150) in order to synthesise the phosphite triester (152) intermediate produced a complicated ^{31}P NMR spectrum. Several major peaks were identified at 136.26 ppm, 135.73 ppm and 133.51 ppm, these were thought to be the respective products (151), (152) and (153), since the phosphite triesters are known to have ^{31}P NMR chemical shifts in this region. A few smaller peaks were also observed indicating that the reaction mixture contained a considerable number of products. The mixture was then oxidised using *tert*-butylhydroperoxide to yield two major peaks at $\delta_{\text{p}} = -6.85$ ppm and -10.68 ppm as well as three other minor peaks at -1.73 ppm, -3.68 ppm and -2.82 ppm. The peak at -2.82 ppm was thought to be the desired *tert*-butyl-(di-2-cyanoethyl)phosphate product, since previous 2-cyanoethyl-phosphate derivatives are known to appear in this region of the ^{31}P NMR spectrum.

Scheme 2.14:



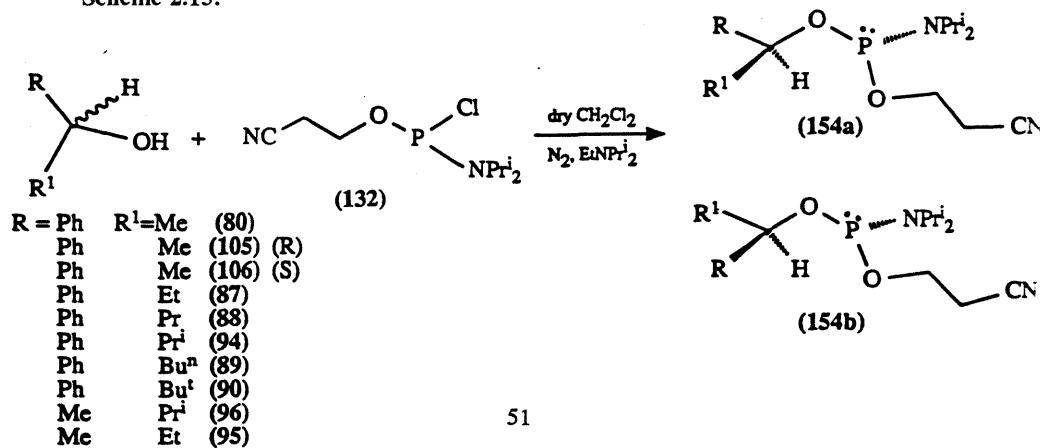
It was apparent that the crude (148) was proving a difficult reagent to handle. The numerous by-products and the inability to purify the mixture were the main problems. Its use as a phosphorylating agent was abandoned in favour of (132), since the main

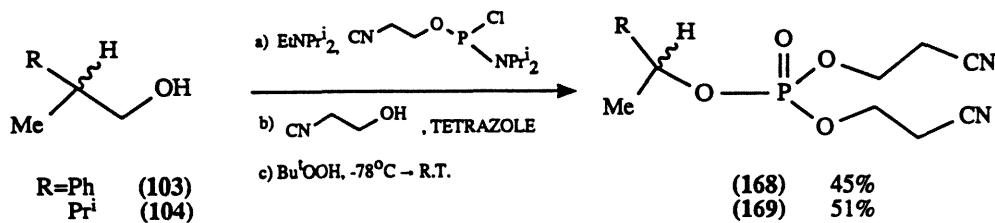
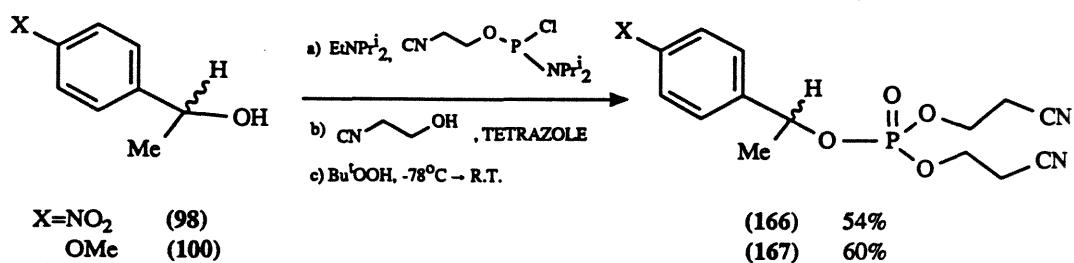
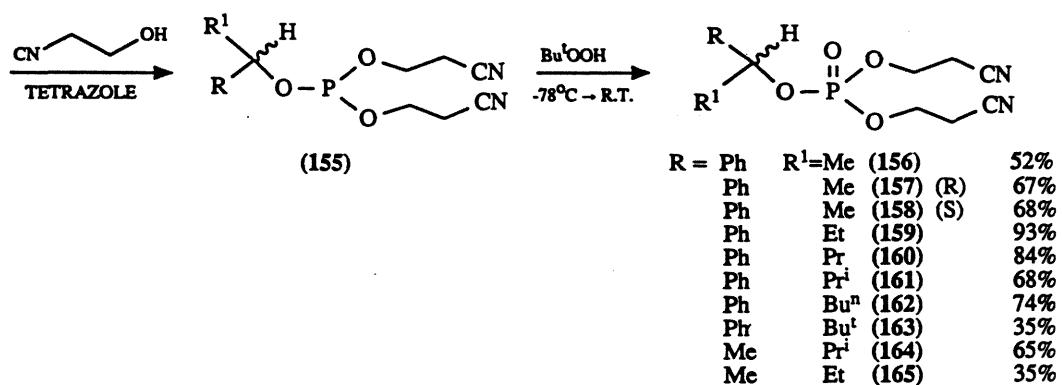
objective of these synthetic strategies was to prepare the monophosphate substrates in high yields.

2.5 Synthesis of (R,S)-alkyl-(di-2-cyanoethyl)phosphates:

The racemic alcohols were converted to the respective phosphate triesters using the phosphitylating agent (132) as shown in Scheme 2.15.^{101b} The alcohol was treated with one equivalent of the chlorophosphoroamidite (132) in the presence of *N,N*-diisopropylethylamine to yield the four phosphoramidite diastereoisomers which were identified by peaks at around $\delta_p(\text{CH}_2\text{Cl}_2) = 146.00\text{-}148.00\text{ppm}$. Two of these diastereoisomers (154a, 154b) are shown below. The areas of the two ^{31}P NMR peaks were measured to ensure that the mixtures remained racemic with equivalent amounts of both diastereoisomers. The phosphoramidites were subsequently treated with tetrazole and 2-cyanoethanol to yield the racemic phosphite triester (155), with a chemical shift around $\delta_p(\text{CH}_2\text{Cl}_2) = 136.00\text{-}138.00\text{ppm}$. Oxidation of the phosphate triester with *tert*-butylhydroperoxide¹⁰⁴ yielded the phosphate triester products (156-169). The phosphate triesters were isolated by flash chromatography after an aqueous workup and CH_2Cl_2 extraction. Optical rotations of these phosphate triester products were taken to confirm that they were still racemic mixtures.

Scheme 2.15:

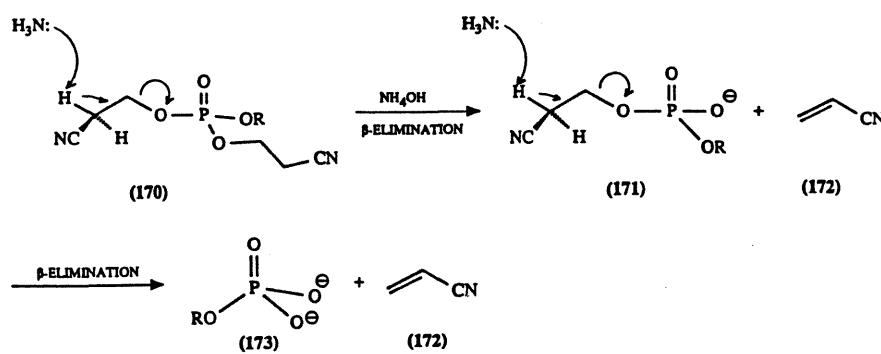




2.6 Synthesis of (R,S)-monophosphate esters (179-192):

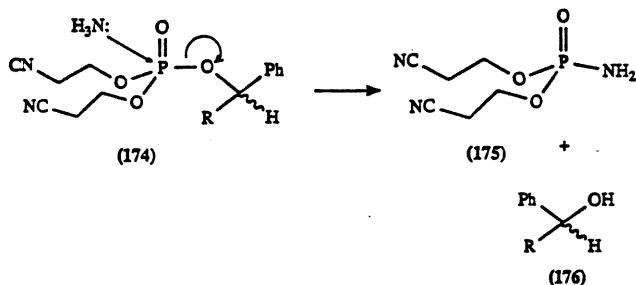
Conversion of the phosphate triesters to the desired monophosphate ester substrates was achieved by treatment with a solution of ammonium hydroxide at 60°C for 2 hours. A general mechanism for this reaction is shown in **Scheme 2.16**. The 2-cyanoethyl groups are removed by two consecutive β -elimination reactions. The ammonium removes the acidic β -hydrogen leading to the formation of the phosphate diester (171) and loss of acrylonitrile (172). Then a second reverse Michael-addition of the second 2-cyanoethyl group is seen to produce the desired phosphate monoester (173). Loss of the 2-cyanoethyl groups is dependent of the pKa values of the β -hydrogen. The hydrogen of the first 2-cyanoethyl group has a low pKa and is readily lost, probably when the phosphate triester is added to the basic solution. However, the β -hydrogens of the second 2-cyanoethyl group are much less acidic, and involve transferring electrons towards the newly created electron rich centre at phosphorus which is highly unfavourable. Therefore, the solution has to be heated to 60°C to allow loss of the second 2-cyanoethyl group from the phosphate diester (171).

Scheme 2.16:

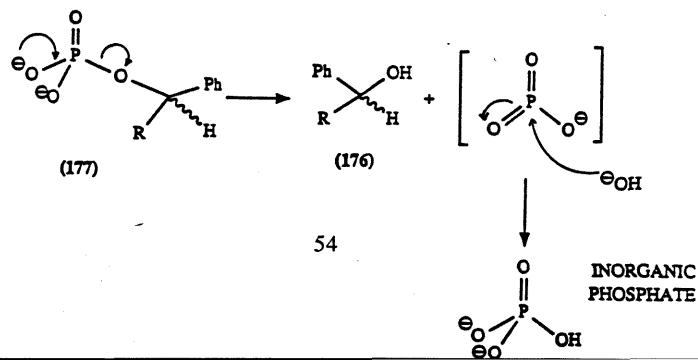


These monophosphate esters (**179-192**) were further purified and isolated by ion exchange chromatography on DEAE-sephadex using aqueous triethylammonium bicarbonate as the eluent. All attempts to improve this reaction and accelerate the loss of the cyanoethyl groups failed, for example increasing the temperature resulted in the cleavage of the desired alkyl group producing free inorganic phosphate material. There are two possible mechanisms by which cleavage of the benzylic group may occur at elevated temperatures. The first is shown in **Scheme 2.17a**, in which direct attack of the phosphorus by the ammonia leads to loss of the benzylic group and formation of a phosphoramide intermediate (**175**). This subsequently decomposes with loss of the 2-cyanoethyl groups to yield inorganic phosphate. The second, and more likely pathway shown in **Scheme 2.17b** at elevated temperatures, is simple dissociation of the benzylic group from the phosphate monoester (**177**) which readily goes on to produce free inorganic phosphate.

Scheme 2.17a:

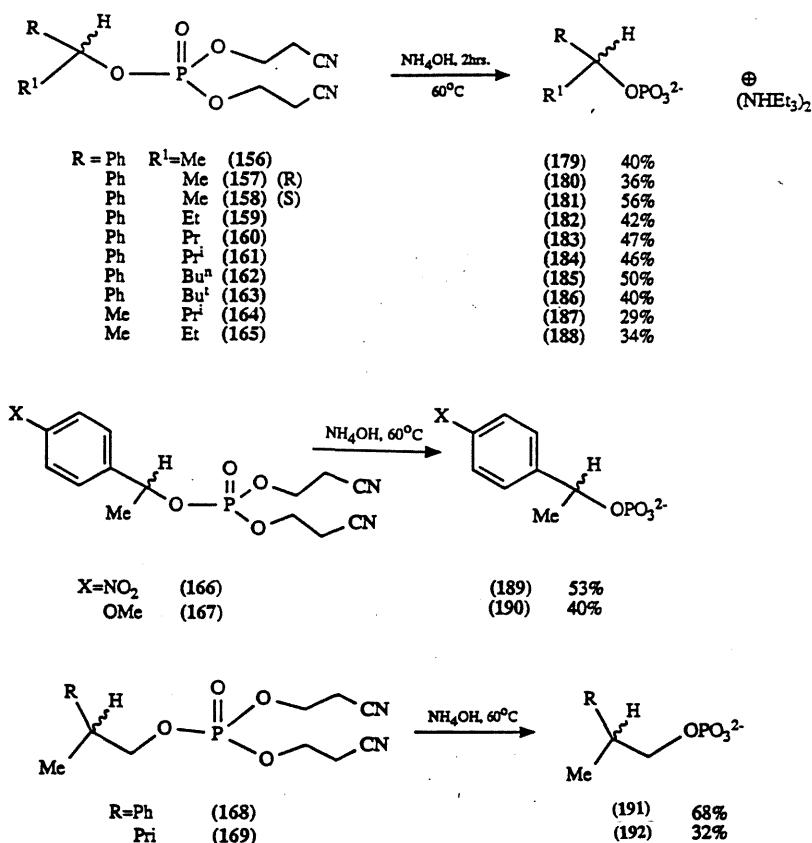


Scheme 2.17b:



Optical rotation measurements of the monophosphate ester products were taken to attempt to confirm that they were racemates. The monophosphates thus obtained were now available to study the enantioselectivities of the alkaline phosphatases.

Scheme 2.18:



2.7. Synthesis of allyl and propargyl phosphate derivatives:

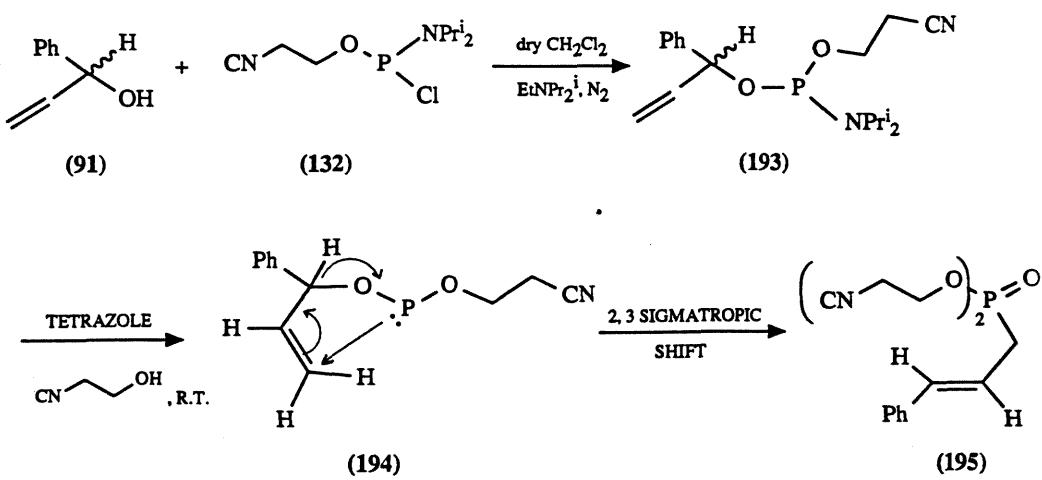
Attempts were made to synthesise the racemic monophosphate esters of 1-phenyl-2-propene-1-phosphate and 1-phenyl-2-propyne-1-phosphate from the phosphorylation of the corresponding racemic alcohols, 1-phenyl-2-propen-1-ol (**91**) and 1-phenyl-2-propyn-1-

ol (92) (page 36). However, in trying to make these phosphate substrates, several obstacles were encountered that made their synthesis impossible.

2.7.1. Decomposition of 1-phenyl-2-propene-1-(di-2-cyanoethyl)phosphite (194):

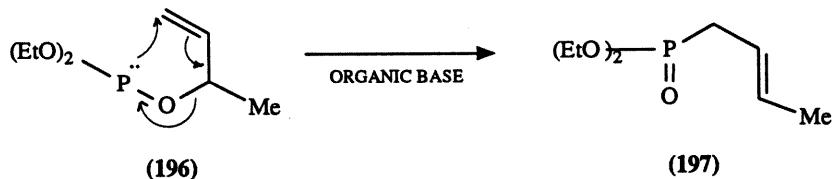
The phosphoramidite (193) was produced from the reaction of the alcohol (91) with the chlorophosphoramidite (132) as shown in **Scheme 2.19**. The addition of tetrazole and 2-cyanoethanol to this mixture resulted in the formation of the phosphite triester derivative (194). If this solution was left stirring for 45 minutes the peak at $\delta_p = 138.27\text{ppm}$ in the ^{31}P NMR spectrum corresponding to (194), was reduced in intensity and gave rise to several other peaks between 30-45ppm suggesting the formation of a phosphonate intermediate. These observations can be explained by the occurrence of a 2,3-sigmatropic rearrangement,¹⁰⁸ as shown in **Scheme 2.19**.

Scheme 2.19:



The intramolecular rearrangement by which the phosphonate derivative (**195**), is produced is known to be a thermal reaction.¹⁰⁸ Shown in **Scheme 2.20** is work by Lemper and coworkers,¹⁰⁹ in which diethyl- α -methylallylphosphite (**196**) undergoes a related rearrangement reaction in which the allyl group inverts during the formation of the diethyl crotylphosphonate (**197**). It has also been shown that the reaction can be catalysed by traces of organic base. The presence of *N,N*-diisopropylethylamine in the phosphitylating reaction is thought to be the catalyst for this rearrangement in which (**194**) produces (**195**).

Scheme 2.20:

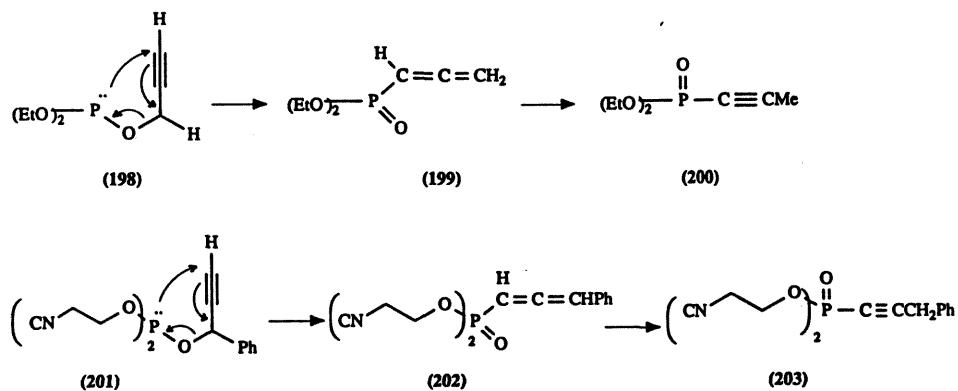


2.7.2. Decomposition of 1-phenyl-2-propyne-1-(di-2-cyanoethyl)phosphite (201):

During the synthesis of the propyne-1-phosphate derivative, problems were encountered with the decomposition of the phosphite triester intermediate (**201**). This was analogous to the decomposition observed with the allyl derivative (**194**) discussed in the previous section. The ^{31}P NMR spectrum became complicated with the appearance of extra peaks at around $\delta_{\text{p}} = 20\text{-}30\text{ ppm}$ which accompanied a decrease in intensity of the phosphite triester peak. Work by Pudovik and coworkers^{110a,b} has shown that propargyl derivatives of this type can undergo two step exothermic rearrangements as shown in **Scheme 2.21**. The diethyl-2-propynyl phosphite (**198**), was converted to the diethyl-1-propynyl phosphonate (**200**) by an intramolecular reaction involving the allene intermediate (**199**). Observations of this type have also been made by Letsinger^{110c} in

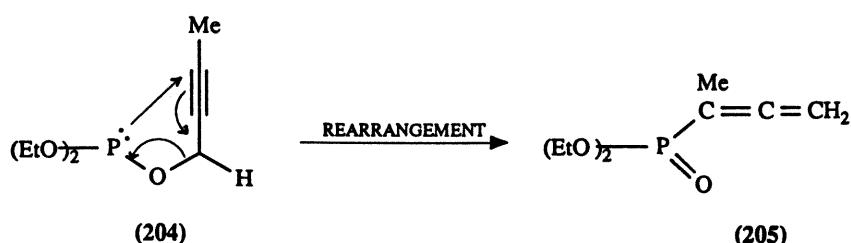
preparing allenic phosphonates from the rearrangement of acetylenic phosphites. A similar intramolecular reaction can be envisaged for the 1-phenyl-2-propyne-1-(di-2-cyanoethyl)phosphite (201) via the allene (202) to produce the phosphonate (203) as shown in Scheme 2.21.

Scheme 2.21:



It has been suggested^{110,111} that the rearrangement of the allene intermediate to the phosphonate product goes *via* a prototropic isomerisation which is catalysed by an organic base. This is supported further by the observations of Pudovik¹¹¹ which are shown in Scheme 2.22. This shows that the transformation of diethyl-2-butynyl phosphite (204) to the phosphonate is stopped at the allenic stage. The absence of protons at C-1 prevents the prototropic isomerisation from occurring, resulting in the formation of diethyl-methylallenyl phosphonate (205).^{110a}

Scheme 2.22:



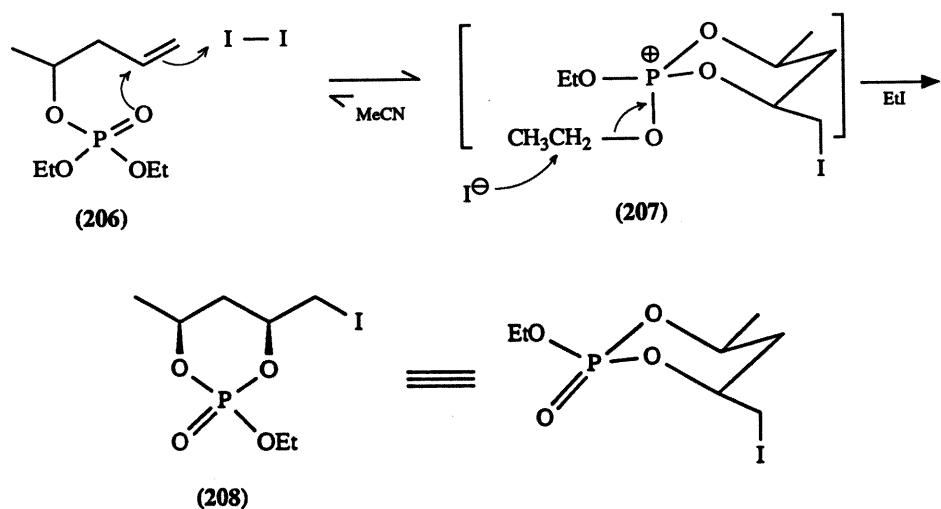
2.7.3. Problems associated with the oxidation of 1-phenyl-2-propene-1-(di-2-cyanoethyl) phosphite (194):

As indicated in Section 2.7.1. there were problems associated with the rearrangement of the allyl phosphite derivative (194) to the phosphonate (195). Attempts to avoid this problem involved quickly performing the oxidation step without allowing the reaction mixture to stand. On attempting this further difficulties were encountered. The phosphite peak at $\delta_p = 138.39\text{ppm}$ was seen to diminish and give rise to a major peak at $\delta_p = 7.2\text{ppm}$ and a minor peak at $\delta_p = 8.1\text{ppm}$. The phosphate triester peak usually appear in the range of $\delta_p(\text{CH}_2\text{Cl}_2) = -2.5$ to -3.1ppm ; whereas, $\delta_p(\text{NH}_4\text{OH}) = 2.8$ to 3.8ppm is in the range in which hydrolysis products appear. Further problems surrounded this oxidation, since *tert*-butylhydroperoxide is known to react with olefins to form epoxides,¹⁴ which could have been a competing reaction for the vinyl phosphite derivative (194). There were clearly too many problems associated with this oxidative step and proceeding intramolecular rearrangement for it to be pursued any further.

It is known that the oxidation of phosphites to phosphates can be achieved using iodine-water.¹¹² This oxidative method proceeds with retention of configuration *via* a two step process involving a phosphonium salt intermediate.¹¹³ One of the difficulties of using

this method with the vinylic phosphite (194) is the potential for an iodolactonisation reaction involving an Arbuzov rearrangement. Difficulties of this type have been encountered by Bartlett¹¹⁴ in the treatment of diethyl-4-pentene-2-phosphate (206) with iodine. This is shown in **Scheme 2.23** in which the undesired cyclic phosphate (208) was produced from an Arbuzov rearrangement of the cyclic phosphonium salt (207). A similar rearrangement would be expected with the phosphite (194) and so it was decided not to attempt this type of oxidation.

Scheme 2.23:

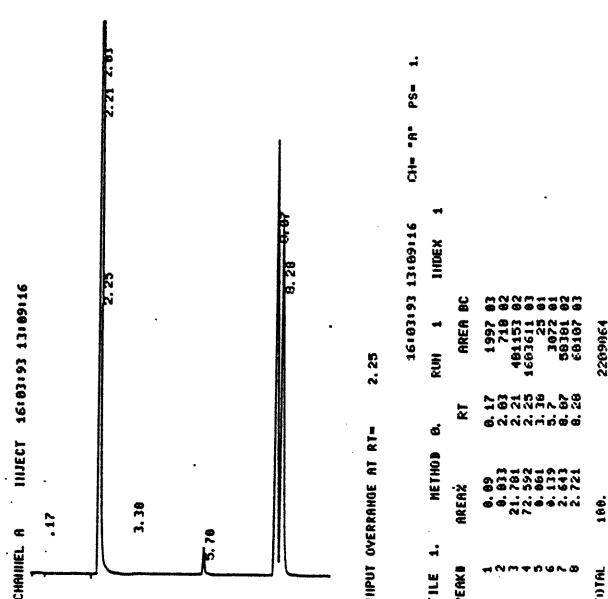
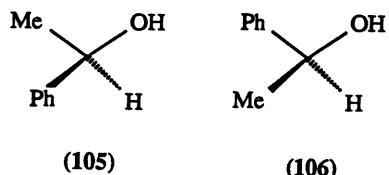


These brief discussions illustrate the problems that were encountered during the synthesis of the propene and propyne phosphate derivatives. The problems encountered with intramolecular rearrangements and alternative oxidation reactions prevented any further investigations of these monophosphate targets. Although these attempted syntheses have raised some interesting mechanistic questions, these were not pursued further.

2.8. Determination of enantiomeric excesses of the phosphatase-catalysed reactions:

The enantiomeric excesses (ee) of the alcohol products obtained from the enzyme reactions were measured in order to determine the selectivity of the phosphatases with the various monophosphate substrates. The ratios of the (1R)-(105) and (1S)-(106)-phenylethanol products of these enzyme reactions were determined by gas chromatography (GC). Using a chiral cyclodex B column it was possible to obtain two separate baseline resolved peaks of the respective enantiomers of the alcohol as shown in **Figure 2.5**, using 2-cyanoethanol as an internal standard in determining the enantiomeric excesses.

Figure 2.5:



However, the chiral column proved unsuccessful in separating the remaining alcohol products from the reactions of the phosphatases with the monophosphate substrates. In most cases, the two peaks were poorly separated and no baseline resolution was achieved. Consequently, the ees of these alcohol products were determined by converting them to diastereoisomers which could be identified by NMR spectroscopy.¹¹⁵ This was achieved by reacting the alcohols with various optically active reagents to afford the diastereoisomers. In order to determine the ees accurately, using the chiral derivatising reagents in conjunction with NMR spectroscopy, several criteria had to be met. Firstly, the reagents had to be available in an enantiomerically pure form. The substrate and the reagent had to react to produce the required adducts in quantitative yield, and should not have to be subjected to purification techniques. It is important that there should be no racemisation of the chiral centres and the differences in the chemical shifts of the diastereomeric products should be large enough to allow accurate measurements to be obtained.

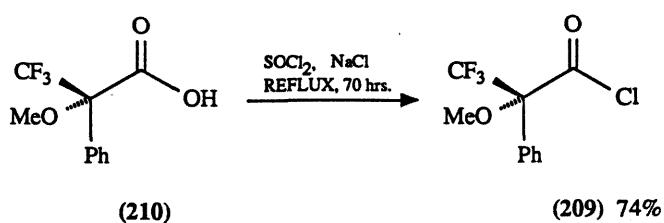
Enantiomeric excesses can be determined using NMR spectroscopy; employing ¹H, ¹³C, ¹⁹F and ³¹P nuclei. These methods usually involve the use of chiral lanthanide shift reagents,¹¹⁶ or by derivatisation with achiral reagents,¹¹⁷ prior to analysis. Methods based on ¹⁹F and ³¹P NMR spectroscopy are most commonly used since they have excellent diastereomeric shift differences in their decoupled NMR spectra. Evaluation of the ee's by ¹H using chiral shift reagents such as tris[3-(*tert*-butylhydroxymethylene)campharato] europium were not attempted since it has been reported by Whitesides et.al.,¹¹⁸ that the CHOH resonances of the enantiomers of 1-phenylethanol do not produce distinguishable shift differences. Consequently, as many of the other alcohols studied are derivatives of 1-phenylethanol (**80**) no work with lanthanide chiral shift reagents was undertaken.

2.8.1. Derivatisation of racemic alcohols with (R)-(-)- α -methoxy-(α -trifluoromethyl)phenyl acetic acid chloride (209) MTPA-Cl:

MTPA⁹³ (Mosher's acid) (**210**) is a widely used chiral derivatising agent which converts alcohols to diastereomeric esters.¹¹⁹ The separate signals for the diastereoisomers can be identified in the ¹⁹F NMR spectra. In such derivatisations, ¹⁹F NMR spectroscopy has advantages over other nuclei in that it has a large chemical shift range usually leading to large differences between the diastereoisomers and the ¹⁹F NMR spectra remain uncongested. The shifts are measured relative to an internal standard of trifluoroacetic acid.

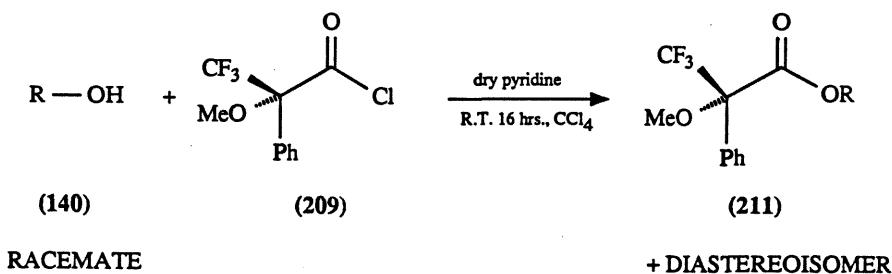
Initial attempts to react MTPA (**210**) with the racemic 1-phenylethanol (**80**) using dicyclohexylcarbodiimide and dimethylaminopyridine resulted in kinetic resolution of the reaction. The (*S*)-enantiomer was seen to react much faster with the acid than the (*R*)-isomer. Analysis of the peak areas of the diastereomeric products showed a ratio of *S* (1.75 ; 64%) : *R* (1.00 ; 36%). This clearly shows the inequivalence in the reactivity of the alcohol enantiomers with (**210**) and the limitations of this derivatisation method. A review of the work by Mosher shows that with numerous racemic alcohols, the reagent of choice is MTPA-Cl (**209**).¹²⁰ This material was readily prepared from the reaction of MTPA (**210**) with thionyl chloride as shown in **Scheme 2.24**.

Scheme 2.24:



Scheme 2.25 shows the general reaction of the racemic alcohols with MTPA-Cl (209) in pyridine.¹²⁰ The results of the reactions of all the racemic alcohols used are presented in **Table 2.1**. These results show that there is no kinetic resolution observed and the peak areas of the diastereoisomers identified in the ¹⁹F NMR spectrum are equivalent within the experimental error of NMR integration techniques. The MTPA-Cl (209) having been thoroughly evaluated, was used in derivatising the alcohol products of the *phosphatase-catalysed* reactions. This enabled the enantiomeric excesses (ee) to be determined from the observed de's of the esterified alcohols. The standard deviations of the percentages of the respective diastereomeric peaks was calculated to be $\pm 0.71\%$.

Scheme 2.25:



**TABLE 2.1 Ester Products from the Reactions of the Racemic Alcohols with
(R)-Methoxy-(α -trifluoromethyl)phenyl acetic acid chloride**

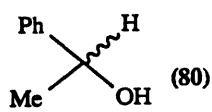
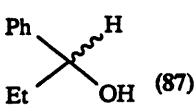
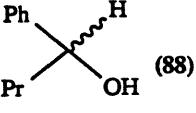
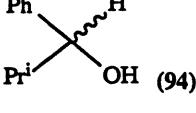
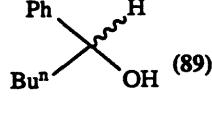
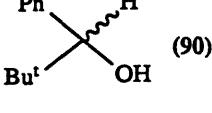
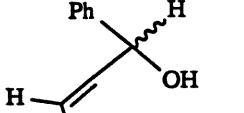
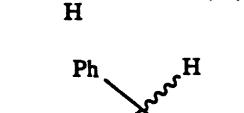
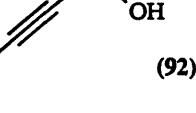
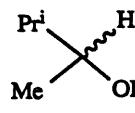
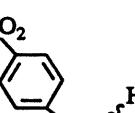
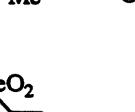
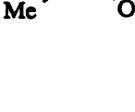
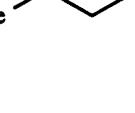
<u>Alcohol</u>	<u>δ_H Shift (ppm)</u>	<u>Peak Areas</u> <u>cm²</u>	<u>% Peak</u>
 (80)	(R-S) -71.91 (R-R) -72.11	18.96 20.00	48.66 51.33
 (87)	(R-S) -71.76 (R-R) -72.03	35.62 35.30	49.99 50.01
 (88)	(R-S) -71.77 (R-R) -72.02	40.54 39.99	50.25 49.75
 (94)	(R-S) -71.54 (R-R) -71.85	26.51 25.24	51.22 48.78
 (89)	-71.75 -71.97	40.80 40.75	50.04 49.96
 (90)	-71.21 -71.63	12.59 14.40	51.27 48.72
 (91)	-71.87 -72.04	15.06 16.98	49.17 50.83
 (92)	-72.19 -72.25	61.10 63.12	49.20 50.80
 (95)	-71.95	46.10	100%

TABLE 2.1 continued

<u>Alcohol</u>	<u>δ_H Shift (ppm)</u>	<u>Peak Areas cm²</u>	<u>% Peak</u>
 (96)	(R-R) -71.84 (R-S) -71.87	32.42 32.02	50.25 49.75
 (98)	(R-R) -71.80 (R-S) -71.66	13.71 12.98	50.40 49.60
 (100)	(R-R) -72.11 (R-S) -71.88	11.82 10.87	51.00 49.00
 (103)	(R-R) -72.05 (R-S) -72.10	18.12 19.74	48.07 51.93
 (104)	(R-R) -72.07 (R-S) -72.18	19.50 20.00	49.50 50.50

2.8.2. Derivatisation of the pure enantiomers with (R)-(-)- α -methoxy-(α -trifluoromethyl) phenyl acetic acid (210) MTPA:

For convenience and simplicity all the pure enantiomeric alcohols obtained were reacted with Mosher's acid (210) in the presence of dicyclohexylcarbodiimide (DCC) and 4-dimethylamino pyridine (DMAP) as shown in **Scheme 2.26**. After working up the reaction mixture the resulting diastereoisomers were analysed by ^{19}F NMR spectroscopy. The results of these reactions together with the derivatisations of the alcohol products obtained from the enzyme reactions were used to identify the respective (R-R) and (R-S) peaks in the ^{19}F NMR spectrum. Besides determining the des of the *phosphatase-catalysed* reaction, it was also possible to assign the absolute configuration of the preferred substrate by spiking with the Mosher's acid derivative of the respective pure enantiomeric alcohol. The results of these chiral alcohol derivatisations are shown in **Table 2.2**, in which the chiral centre is indicated. The peaks for the (R)-phenylethyl-(105) and (R)-1-(*para*-nitrophenyl)ethyl-(114) ester derivatives are both shown to be shifted in the same direction, i.e. downfield from the (S)- derivatives. Since there are similarities between these two alcohols and the 1-(*para*-methoxyphenyl)ethanol, the assumption was made that the (R)-1-(*para*-methoxyphenyl)ethyl derivative would also be shifted in the same direction downfield. So the assignment of the (R)- and (S)-1-(*para*-methoxyphenyl)ethyl ester derivatives is based on this extrapolation and not on the independent synthesis and derivatisation of either the (R)- or (S)-enantiomers of 1-(*para*-methoxyphenyl)ethanol.

Scheme 2.26:

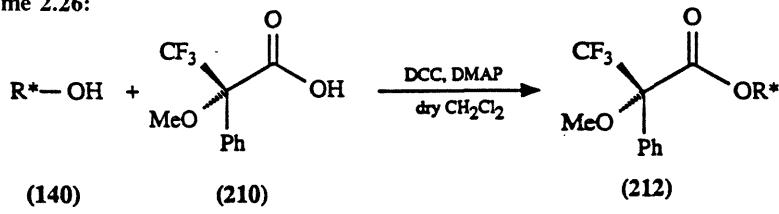


TABLE 2.2 Ester Products from the Reactions of the Chiral Alcohols with Methoxy-(α -trifluoromethyl) phenyl acetic acid.

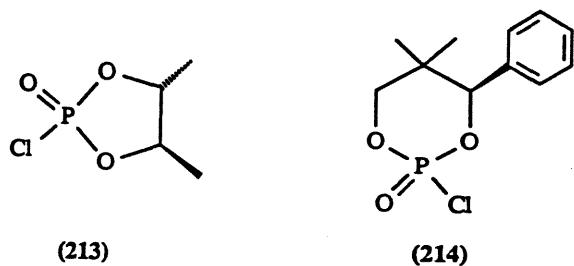
<u>Alcohol</u>	<u>Ester</u>	<u>δ_H Shift (ppm)</u>	<u>%Diastereoisomer</u>
 (105)		-72.15	(R-R) 100%
 (107)		-72.03	(R-R) 100%
 (108)		-72.02	(R-R) 100%
 (111)		-71.87	(R-R) 100%
 (110)		-71.98	(R-R) 100%
 (109)		-71.87	(R-S) 100%

<chem>CC(C)C(O)C</chem> (116)	<chem>CC(C)C(O)C(=O)OC(C)C(C(F)(F)F)(C(F)(F)F)C</chem>	-72.07	(R-R) 100%
<chem>Oc1ccc([N+](=O)[O-])cc1</chem> Me (114)	<chem>CC(C)C(O)C(=O)OC(C)C(C(F)(F)F)(C(F)(F)F)C</chem>	-71.80	(R-R) 100%
<chem>CC(C)C(O)C</chem> ⁱ Pr (117)	<chem>CC(C)C(O)C(=O)OC(C)C(C(F)(F)F)(C(F)(F)F)C</chem>	-71.94	(R-R) 65%
<chem>CC(C)C(O)C</chem> ⁱ Pr (118)	<chem>CC(C)C(O)C(=O)OC(C)C(C(F)(F)F)(C(F)(F)F)C</chem>	-72.12	(R-S) 35%

2.8.3. Derivatisation of alcohols with (2R,4R,5S)-2-chloro-3,4-dimethyl-5-phenyl-1,3,2-oxazaphospholin-2-sulphide (217):

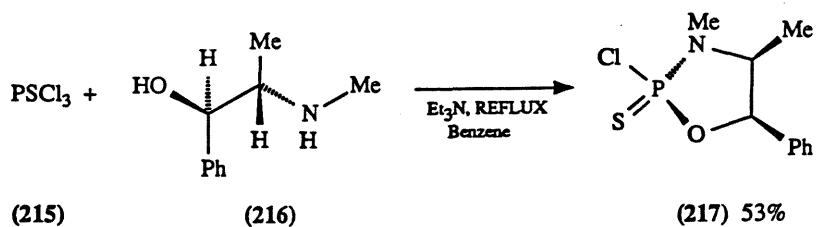
The results in **Table 2.1**, show that the diastereomeric ester derivatives of 2-butanol (**95**) could not be separated by ^{19}F NMR. Another method was therefore required to separate these enantiomers. A chiral phosphorus reagent was chosen since these can be readily analysed by ^{31}P NMR spectroscopy. These also give large diastereomeric shift differences and usually appear as singlets. Several chiral phosphorus derivatising agents are known in the literature including (4R,5R)-2-chloro-4,5-dimethyl-2-oxo-1,3,2-dioxaphospholane (**213**)¹²¹ and the more recently prepared Feringa reagent (**214**)¹²² which are shown in **Figure 2.6**.

Figure 2.6:



The chiral phosphorus reagent (**217**) was eventually chosen since it has been successfully used in determining enantiomeric purity of numerous alcohols and amines.⁹⁴ It was prepared according to the procedure of Inch et.al.¹²³ shown in **Scheme 2.27**. (1R,2S)-2-methylamino-1-phenyl-1-propanol (**216**) was reacted with thiophosphoryl trichloride (**215**) in the presence of triethylamine to yield both the *cis*- and *trans*- products, and the major *cis*-isomer was isolated by recrystallisation.

Scheme 2.27:



The racemic alcohols and enantiopure alcohols were treated with butyl lithium to form the anions which were reacted with (217) and refluxed for 15-20 hours as shown below in Scheme 2.28. The use of the non-nucleophilic base is important since Cullis¹²⁴ has shown that using (217) with nucleophilic bases such as pyridine causes an inversion of the configuration at phosphorus. An epimerisation of the *cis*-compound to the more favourable *trans*-compound is observed with a change in the ratio of *cis* : *trans* from 1:0 to 1:3. The *cis* and *trans* terms refer to the relationship between the chlorine atom and the methyl group at the C4 position. The results of these derivatisation reactions are shown in Table 2.3. (217) Was then used to analyse the ees of the alcohol products from the *phosphatase-catalysed* reactions. The standard deviations determined for the percentages of the oxy-phospholidine-2-sulphide diastereoisomers, determined from measuring the peak areas of the ^{31}P NMR spectra was calculated to be $\pm 1.15\%$.

Scheme 2.28:

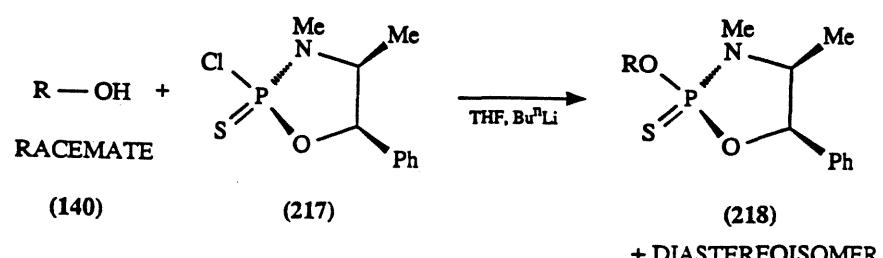
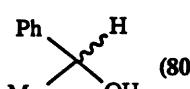
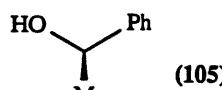
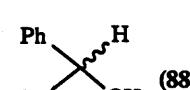
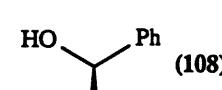
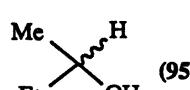
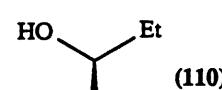


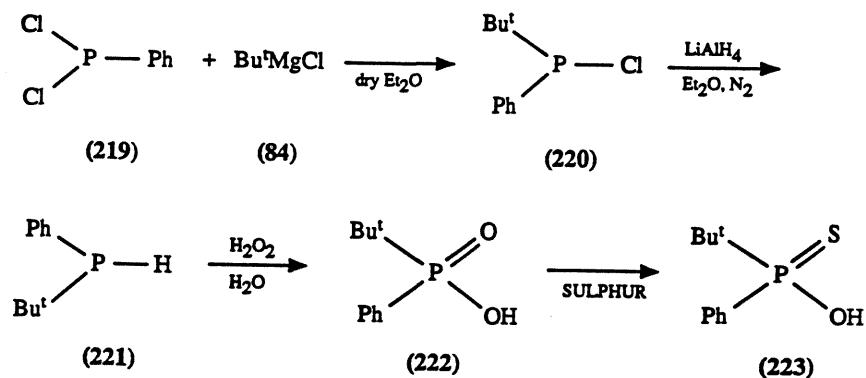
TABLE 2.3 Oxy-phospholidine-2-sulphide Products from the Reactions of the Alcohols with(2R,4R,5R)-2-Chloro-3,4-dimethyl-5-phenyl-1,3,2-oxazaphospholidine-2-sulphide

<u>Alcohol</u>	<u>δ_p Shift (ppm)</u>	<u>Diastereoisomer</u>
 (80)	(R-R) 81.91 (R-S) 81.71	51.32 48.68
 (105)	(R-R) 81.91	100.00
 (88)	(R-R) 81.89 (R-S) 81.67	50.12 49.87
 (108)	(R-R) 81.89	100.00
 (95)	(R-R) 82.38 (R-S) 82.18	52.01 47.98
 (110)	(R-R) 82.38	100.00

2.8.4. Use of chiral shift reagent (R)-phenyl-*tert*-butylphosphinothioic acid (223):

This material was kindly provided by Dr. M.J.P. Harger,¹²⁵ and had been prepared according to the method of Hoffmann and Schellenbeck,¹²⁶ as shown in **Scheme 2.29**. Dichlorophenylphosphine (219) was reacted with *tert*-butylmagnesium chloride (84) to produce chloro-(phenyl)-*tert*-butylphosphine (220). This was reduced with lithium aluminium hydride to yield phenyl-*tert*-butylphosphine (221) which on oxidation with hydrogen peroxide afforded phenyl-*tert*-butylphosphine oxide (222). Reacting this with sulphur yielded the desired racemic phenyl-*tert*-butylphosphinothioic acid. Recrystallisation of this material with (S)-1-phenylethylamine¹²⁵ provided the chiral (R)-enantiomer (223).

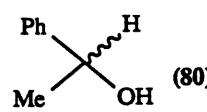
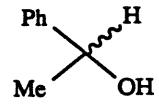
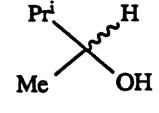
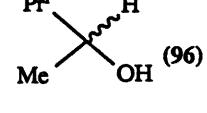
Scheme 2.29:



(223) Was added to the various alcohols and their ^1H NMR spectra were studied. The two enantiomers of 1-phenylethanol (80) were resolved by the formation of different diastereomeric complexes which were identified in the ^1H NMR spectrum. The doublet of the methyl group is seen to appear as two separate doublets representing the (R)- and (S)- enantiomers of the alcohol.

The phenyl-*tert*-butylphosphinothioic acid was also successfully used in resolving the enantiomers of 3-methyl-2-propanol (96). Unfortunately, the attempted resolution with other alcohols, including the propyl, ethyl and butyl proved to be unsuccessful. The ^1H NMR spectra of these mixtures were far too congested to locate a satisfactory set of resonances on which to determine the de's and hence the ee's. The results of the chiral shift reagent additions to the various alcohols are shown in Table 2.4. The standard deviation calculated for the percentages the diastereomeric peaks represent was determined to be $\pm 0.93\%$.

TABLE 2.4 Diastereomeric Complexes from the Addition of the Alcohols to (R)-Phenyl-*tert*-butylphosphinothioic acid.

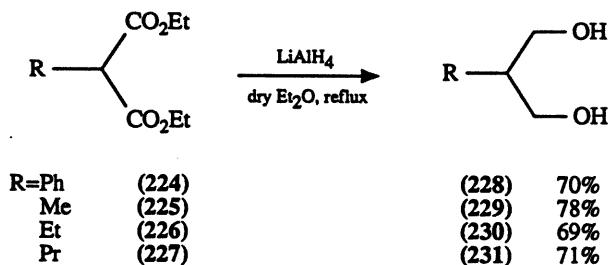
<u>Alcohol</u>	<u>% Enantiomer</u>	<u>δ_H Shift (ppm)</u>	<u>Peak Areas cm²</u>	<u>% Peak</u>
 (80)	50 (R) 50 (R)	1.39 1.43	11.62 11.61	50.03 49.97
	30 (R) 70 (S)	1.38 1.43	5.32 14.67	26.60 73.40
	50 (R) 50 (R)	0.852 0.895	28.57 30.43	48.43 51.57
	80 (R) 20 (S)	0.853 0.896	24.06 5.95	80.20 19.80

2.9. Synthesis of 2-alkyl-1,3-propanediols:

The synthesis of the series of racemic monophosphate esters has already been discussed earlier in this chapter. The purpose of this was to study the enantioselectivity of the phosphatase enzymes with the various substrates. The second part of this work involved investigating the potential of using these phosphatases to discriminate between the two enantiotopic phosphate monoester groups of a series of prochiral 1,3-bisphosphates. The initial step in the preparation of the 2-alkylpropyl-1,3-bisphosphates

involved the synthesis of the parent 2-alkyl-1,3-propanediols. These were converted to the 2-alkylpropyl-1,3-bisphosphate derivatives and subsequently used as prochiral substrates of the alkaline phosphatases. The 2-phenyl-(228),¹²⁷ 2-methyl-(229),¹²⁸ 2-ethyl-(230) and 2-propyl-(231)-1,3-propanediols¹²⁷ were prepared by the reduction of the respective diethyl 2-alkyl malonate derivatives (224-227) as shown in **Scheme 2.30**. The initial attempts to perform these reductions at room temperature proved to be unsuccessful, with a significant amount of the unreacted starting material being isolated. Refluxing the reaction mixtures proved to be effective leading to the 1,3-propanediol derivatives being isolated in high yields after purification by vacuum distillation.

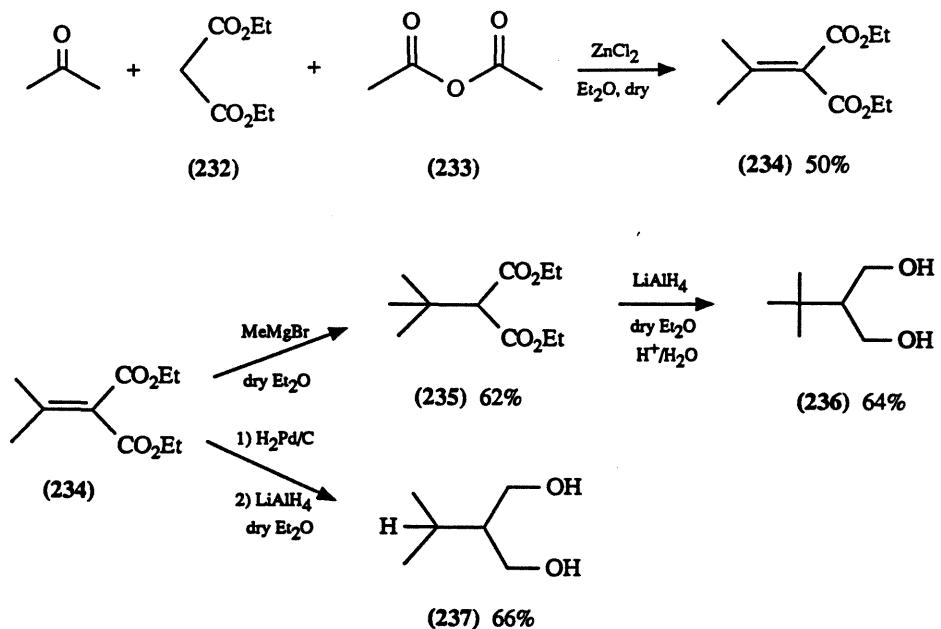
Scheme 2.30:



The two diols, 2-*iso*-propyl-(237) and 2-*tert*-butyl-(236)-1,3-propanediols were produced from the divergent synthesis, shown in **Scheme 2.31**, *via* the common intermediate diethyl *iso*-propylidenemalonate (234).¹²⁹ This material was produced from the reaction of acetone, acetic anhydride (233) and diethyl malonate (232) in the presence of zinc chloride. Addition of methylmagnesium iodide to this intermediate (234) gave the diethyl *tert*-butylmalonate (235),¹³⁰ which was subsequently reduced to the alcohol (236). Hydrogenation of the alkene (234) produced the diethyl *iso*-propylmalonate which was

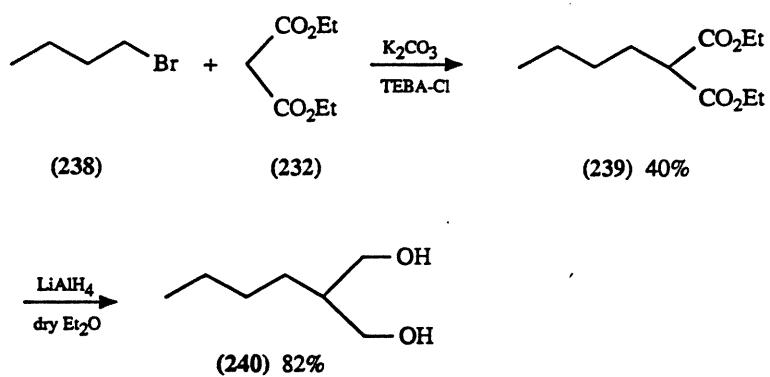
reduced without further purification to yield the *iso*-propyl derivative (**237**).

Scheme 2.31:



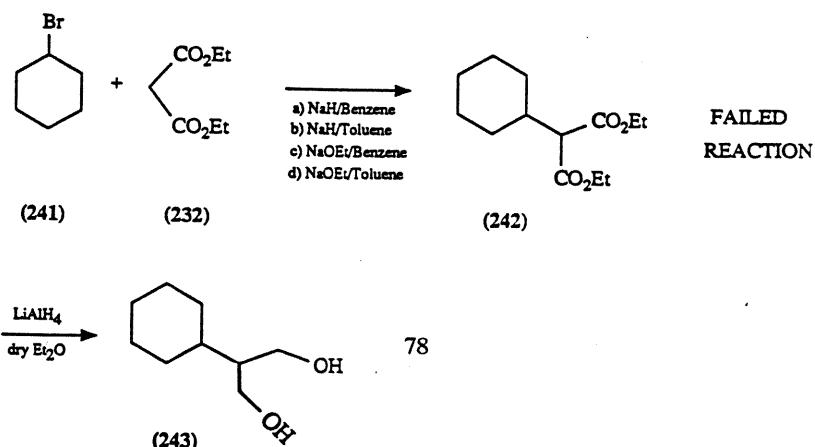
Scheme 2.32 shows how the 2-butyl-1,3-propanediol derivative (**240**) was prepared in two steps. The first step was the reaction of butyl bromide (**238**) and diethyl malonate (**232**) in the presence of potassium carbonate and triethylbenzylammonium chloride to yield the diethyl butylmalonate (**239**).¹³¹ This was converted to the 2-butyl-1,3-propanediol (**240**) in 82% yield by lithium aluminium hydride reduction.

Scheme 2.32:



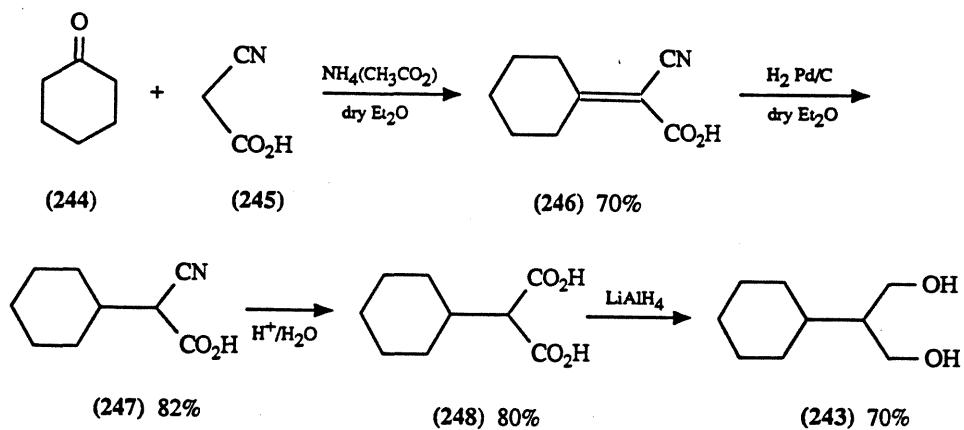
The initial attempts to prepare 2-cyclohexyl-1,3-propanediol (243) centred around the synthesis of diethyl cyclohexylmalonate (242). This would be prepared from the reaction of cyclohexyl bromide (241) with diethylmalonate (232) as shown in Scheme 2.33. It was envisaged that the malonate derivative (232) would be reduced to yield the desired 1,3-diol derivative (243). This synthesis proved very difficult, various bases such as sodium ethoxide and sodium hydride were used as well as making changes to the solvents (benzene, toluene). Other efforts involved the use of the phase transfer catalyst, triethylbenzylammonium chloride in aqueous sodium hydroxide. All of these had very limited success and were not pursued due to their low yields and poor reactivity.

Scheme 2.33:



From reviewing the literature, an alternative strategy for the synthesis of 2-cyclohexyl-1,3-propanediol (243) was obtained and is shown in **Scheme 2.34**. Cyclohexanone (244) and cyanoacetic acid (245) were reacted in the presence of ammonium acetate catalyst to yield cyclohexylidene cyanoacetic acid (246).¹³² Hydrogenation of the double bond using palladium on charcoal catalyst gave the cyclohexyl derivative (247) in 82% yield. Subsequent hydrolysis of the nitrile group with sulphuric acid produced the cyclohexyl malonic acid (248). This was readily reduced using lithium aluminium hydride and afforded the 2-cyclohexyl-1,3-propanediol derivative (243).

Scheme 2.34:

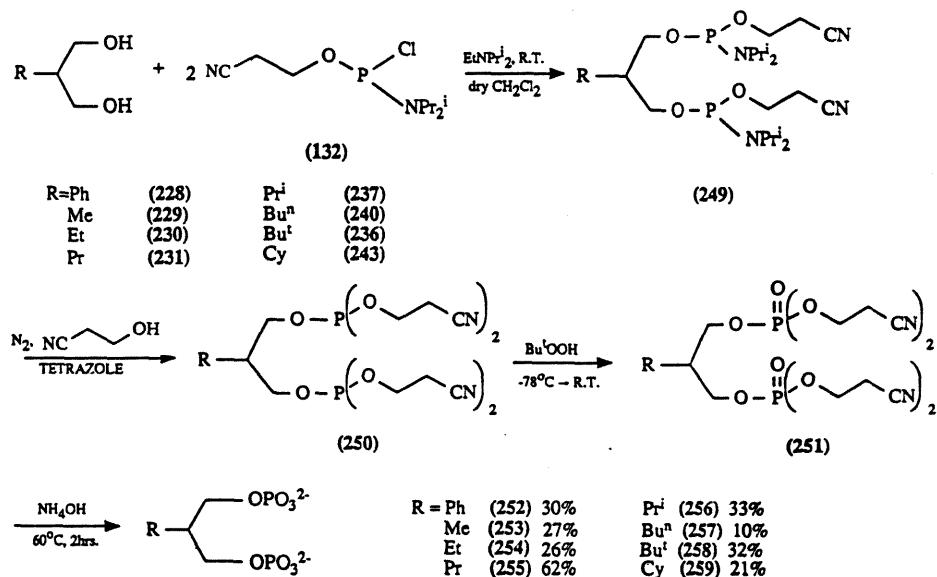


2.10. Synthesis of the 2-alkylpropyl-1,3-bisphosphates (252-259):

The next step in preparing the 2-alkylpropyl-1,3-bisphosphate derivatives was the phosphorylation of the 1,3-diol materials. This was achieved using the phosphitylating strategies previously employed in the synthesis of the racemic monophosphate esters. Conventional P^V phosphorylating agents were not used with these 1,3-diol materials since

this often leads to the formation of undesired cyclic 6-membered phosphates.¹³³ The synthetic sequence is shown in Scheme 2.35. The 1,3-propanediols were treated with two equivalents of the chlorophosphoramide¹⁰¹ (132), in the presence of the diisopropylethylamine to yield the bisphosphoramidite (249) as diastereoisomers with general shifts of around $\delta_p(\text{CH}_2\text{Cl}_2) = 146.00\text{-}148.00\text{ppm}$. Treatment of the diastereoisomers of (249) with tetrazole and 2-cyanoethanol produced the bisphosphite triester (250) with ³¹P NMR peaks around $\delta_p(\text{CH}_2\text{Cl}_2) = 138.00\text{-}135.00\text{ppm}$. Oxidation of this material with *tert*-butylhydroperoxide produced the bisphosphate triesters (251) with chemical shifts around $\delta_p(\text{CH}_2\text{Cl}_2) = -2.62\text{ppm}$. This was extracted from the reaction mixture using CH_2Cl_2 after an aqueous workup before being treated with ammonium hydroxide to afford the 2-alkylpropyl-1,3-bisphosphate products (252-259). These were purified further by ion exchange chromatography. The 1,3-bisphosphates were subsequently used in the investigations of the alkaline phosphatases.

Scheme 2.35:

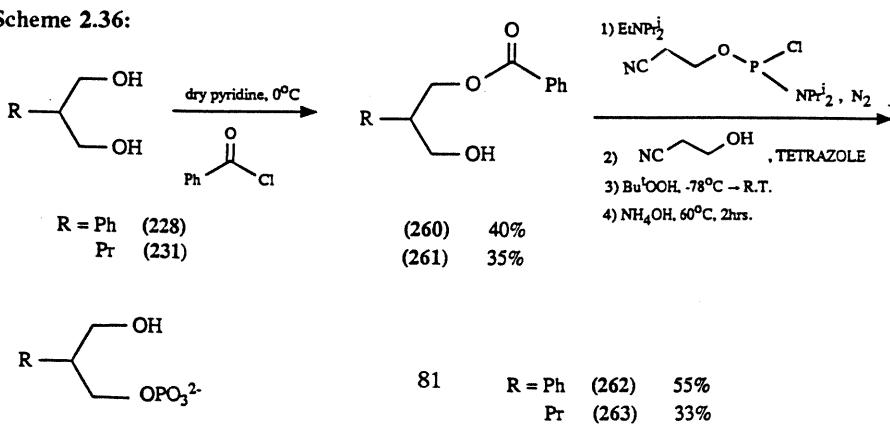


2.11. Synthesis of 3-hydroxyl-2-alkylpropyl-1-phosphates (262-263):

3-Hydroxyl-2-alkylpropyl-1-phosphates are the initial product of the hydrolysis of the 1,3-bisphosphates. In order to establish whether the *monophosphate* products would accumulate, the kinetics of the second hydrolysis step were independently measured. The synthesis of the racemic *monophosphates* (**262**) and (**263**) was achieved as described below.

Scheme 2.36 shows how 3-hydroxy-2-phenylpropyl-1-phosphate (**262**) and 3-hydroxy-2-propylpropyl-1-phosphate (**263**) were prepared from their respective 2-phenyl- (**228**) and 2-propyl- (**231**)-1,3-propanediols. The synthesis of these 1,3-diols has already been previously described. Monoprotection of the diols prior to phosphorylation was achieved by the reaction with one equivalent of benzoyl chloride in the presence of pyridine to afford the 3-benzoyl-2-phenyl-1-propanol (**260**) and 3-benzoyl-2-propyl-1-propanol (**261**) derivatives. These alcohol derivatives were converted to their corresponding monophosphate esters using the phosphorylating methods previously employed in the synthesis of the 2-alkylpropyl-1,3-bisphosphates. During the ammonolysis reaction step the benzoyl protecting groups were also cleaved circumventing the need for any further deprotection steps. The substrates synthesised, were now available for kinetic studies with alkaline phosphatases from various sources.

Scheme 2.36:



Chapter 3

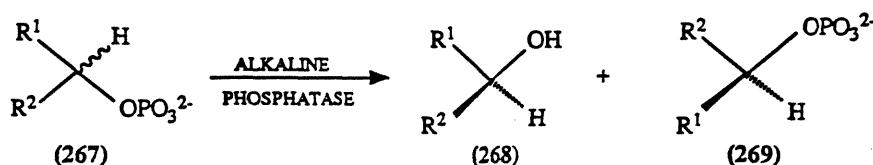
Studies of Alkaline Phosphatases with the Racemic Monophosphate

Ester Derivatives.

3.1. Introduction:

As discussed in detail in Chapter 1, the alkaline phosphatases are known to show a broad specificity towards various monophosphate ester derivatives,^{57a,58} catalysing the hydrolysis of the monophosphate ester substrates (264) to the corresponding alcohols (265) and inorganic phosphate (266) as shown in Scheme 3.1. The investigations described in this chapter concern alkaline phosphatases from *E.coli*, rabbit and bovine catalysing reactions with a variety of racemic monophosphate substrates (179, 182-192), whose syntheses have been described earlier in Chapter 2. It was hoped that the phosphatases would affect high enantioselectivity with the racemic phosphates (267) to produce chiral alcohol products (268) by kinetic resolution, with the remaining phosphate monoester enriched in the other enantiomer (269), as shown in Scheme 3.1.

Scheme 3.1:



3.2. Monitoring the enzyme reactions:

An efficient and accurate method was required to measure the reaction rates of the alkaline phosphatases with the various monophosphate ester derivatives shown in Scheme 3.1. Measuring this enzymatic hydrolysis has usually been achieved by measuring the

inorganic phosphate produced as a function of time.^{57a} These methods usually involve terminating the enzyme reaction at regular time intervals and then determining the amount of free phosphate present by colorimetric assays^{134,135} or coupled enzyme reactions.¹³⁶

3.2.1. Colorimetric assays:

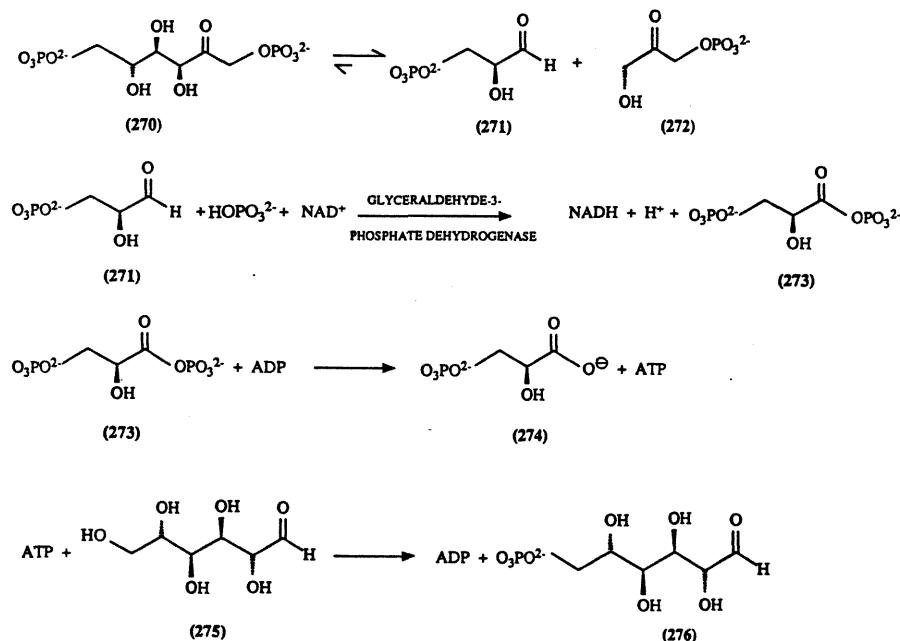
Most of the colorimetric assays require removal of the protein, usually by precipitation and centrifugation before the phosphate is quantified as a coloured complex of molybdophosphoric acid¹³⁴ or molybdoavanadophosphoric acid.¹³⁵ The molybdophosphoric acid derivatisations are variations of the Briggs^{134a} and Fiske-Subbarow^{134b} methods which have previously been used in connection with this work, to identify the phosphate monoester products from ion exchange columns. Further details of these protocols can be found in Chapter 7. The colorimetric assay is easier to perform, though there are some problems with the removal of the enzyme and its subsequent precipitation. Both of these problems are known to reduce the colour intensity observed, resulting in erroneous absorbance readings. Attempts to avoid these problems using the complexing reagent of molybdoavanadate¹³⁵ with sodium dodecyl sulphate as the denaturing agent have been used, but with poor results. It has been suggested by Trentham and Gutfreund¹³⁷ that kinetic data obtained by these methods are of doubtful significance due to the poor sensitivity of this method.

3.2.2. Coupled enzyme reactions:

Many assay systems have been developed to measure the production of inorganic phosphate. The advantage over the colorimetric assay is that they are less laborious and time consuming. Most of the coupling reactions allow specific and rapid determination of inorganic phosphate over a wide range of concentrations. A typical assay cocktail is shown in Scheme 3.2,¹³⁶ to which the inorganic phosphate product is added. The fructose-

1,6-diphosphate (270) is converted to dihydroxyacetone phosphate (272) and glyceraldehyde-3-phosphate (271). Reaction of (271) with the inorganic phosphate and NAD⁺ in the presence of glyceraldehyde-3-phosphate dehydrogenase yields the 1,3-diphosphoglycerate (273) and NADH. (273) Is coupled with ADP using phosphoglycerate kinase to yield ATP and 3-phosphoglycerate (274). The ATP is subsequently coupled to glucose (275) to yield ADP and glucose-6-phosphate (276). The calculations for inorganic phosphate are made directly from the NADH extinction coefficient, dispensing with the need for use of frequent standards.¹³⁶ However, this enzyme system is complex and the materials are expensive. Therefore, this method was not considered and ³¹P NMR spectroscopy was used instead.

Scheme 3.2:

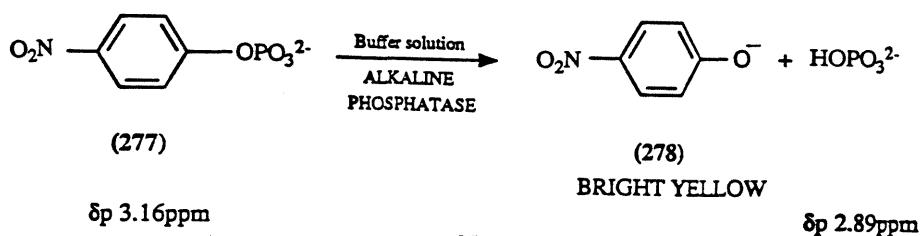


3.2.3. ^{31}P NMR spectroscopic techniques:

^{31}P NMR spectroscopy was used to monitor the alkaline phosphatase reactions of the various monophosphate ester substrates. It was efficient and accurate and did not suffer from the practical and tedious problems of the colorimetric assay and enzyme coupling methods. Using this technique to study biological systems is well documented.¹³⁸ It has been used in the past to determine the pK_a values of *myo*-inositol hexaphosphate, as well as the intracellular pH of various tissues.¹³⁹ Metabolic reactions within intact biological tissues have been followed by ^{31}P NMR spectroscopy, including the interaction of phosphates with haemoglobin.¹⁴⁰

Extensive ^{31}P NMR spectroscopic studies have been performed studying the equilibrium of the various intermediates involved in the hydrolysis reactions of the alkaline phosphatases.¹⁴¹ Coleman *et.al.*^{142a,b} has also applied this technique to measuring the rates of substrate turnover by the phosphatases.^{142a,b} It has been shown that the ^{31}P NMR chemical shifts of inorganic phosphate, *para*-nitrophenyl phosphate and other phosphate monoester derivatives are sufficiently different that the separate phosphorus signals can be followed on a time scale of minutes. The progress of the hydrolysis can be followed directly and continuously. This is illustrated by the reaction of *para*-nitrophenyl phosphate to *para*-nitrophenolate and inorganic phosphate as shown below in **Scheme 3.3**.

Scheme 3.3:

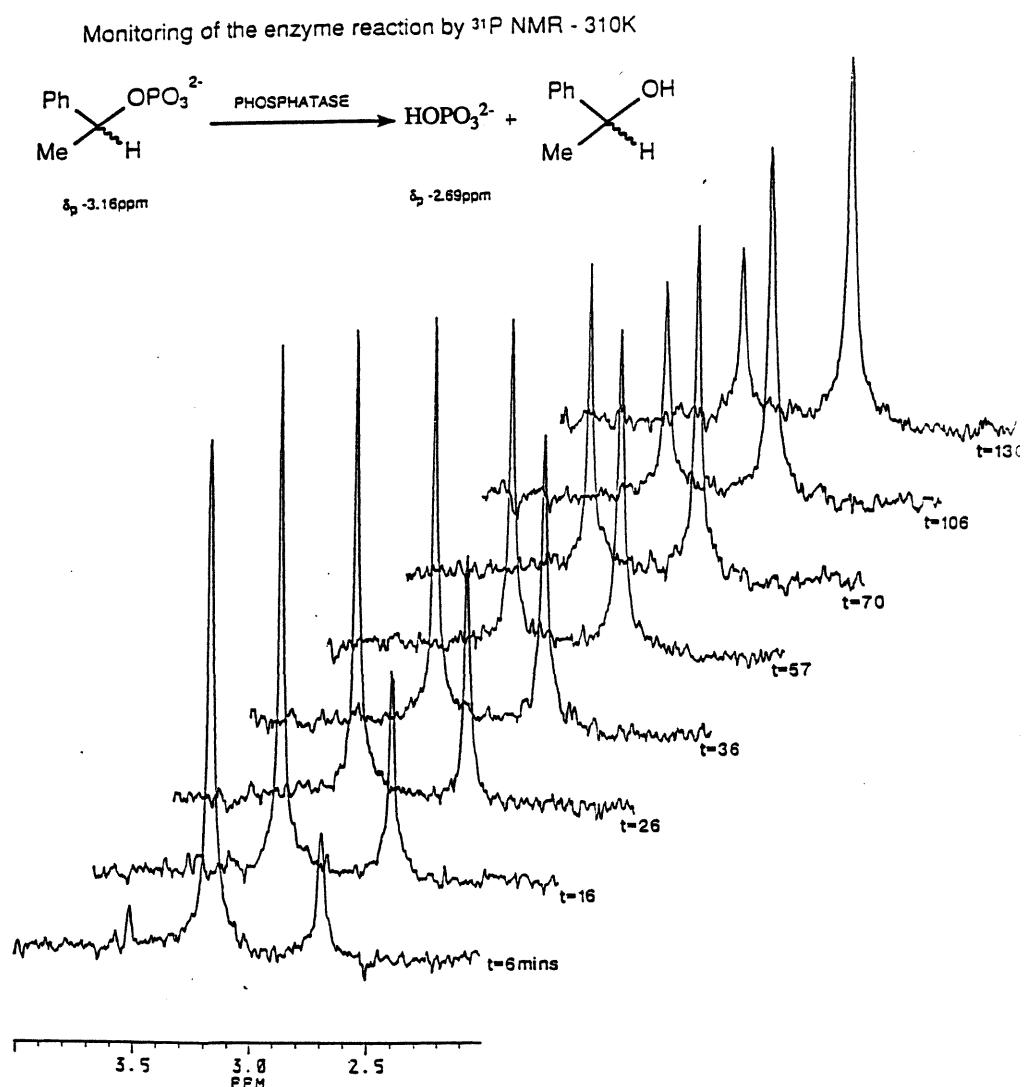


Work by Bock and Cohn, investigating the phosphate exchange catalysed by *E.coli* alkaline phosphatase, has also used ^{31}P NMR spectroscopy to detect isotopic labels^{142c} and to monitor phosphate-water exchange.^{142d} Unlike the other monitoring methods, it does not require samples to be removed at regular intervals, nor do further extractions and precipitations need to be carried out to determine the progress of the enzyme reaction.

Using ^{31}P NMR spectroscopy, the enzyme reaction is carried out in a NMR tube and the progress of the enzyme reaction monitored directly with substrate resonances typically at around $\delta_{\text{P}}(\text{buffer}) = 3.00\text{ppm}$ being replaced by the resonance at $\delta_{\text{P}}(\text{buffer}) = 2.69\text{ppm}$ corresponding to inorganic phosphate. This can be seen in **Figure 3.1**, in which the reaction progress of bovine alkaline phosphatase with the 1-phenylethyl-1-phosphate substrate (**179**) is monitored. Using an external standard of tetrahydroxyphosphonium ion ($\text{P}^+(\text{OH})_4$),^{143a} it is possible to measure the areas of the two peaks as the reaction proceeds, enabling the extent of reaction to be determined, as well as allowing kinetic data to be obtained. This circumvents the need to make extractions from the enzyme solution and derivatise the phosphate to deduce the rate of the enzyme reaction.

Using ^{31}P NMR spectroscopy to monitor the progress of the alkaline phosphatase reactions with the monophosphate ester substrates (**179**, **182-192**) is an accurate method. Measuring the peak areas of the inorganic phosphate product and monophosphate substrate to determine the extent of reaction completed has no significant error. The error in measuring the percentage completion using ^{31}P NMR spectroscopy is typically around $\pm 2.0\%$. This is based on the cumulative results for all of the experiments carried out to 50% completion. After the extended accumulation times of 200-300 scans, the peak areas measured will be a good representative sample of the actual amounts of substrate and product present in the reaction.

Figure 3.1:



The spin-lattice relaxation times (T_1) of the monophosphate esters are greater than those of inorganic phosphate ($T_1 = 0.4\text{sec}$).^{143b} Their spin-spin relaxation times (T_2) are also different. This is because the phosphorus centre of the monophosphate esters is coupled to the hydrogen atoms of the attached alkyl substituent ($^3J_{P-H}$). This coupling extends the spin-spin relaxation times of the phosphorus centre; though this coupling diminishes as the distance between the phosphorus centre and hydrogen atoms increases. The phosphorus atom of the inorganic phosphate is not coupled to any hydrogen atoms and consequently it has a shorter relaxation time. This difference in spin-spin and spin-lattice relaxation times is not a source of error since both are shorter than the 1 second pulse delay used in these studies. This extended pulse delay allows the molecules to fully relax before they are pulsed again at a 30° angle. The relatively long pulse delay and short angle of irradiation also means that the excited energy level will not be saturated when the sample is pulsed.

3.2.4. Enzyme reaction conditions:

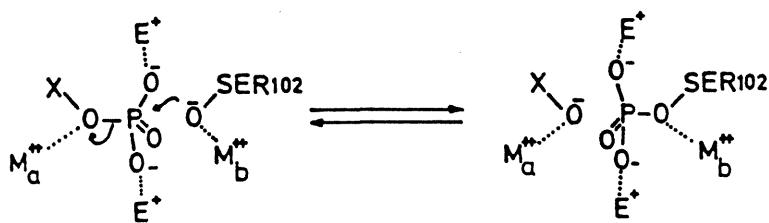
The buffer solution used in the enzyme reactions was as described by Knowles.^{61a} This solution was chosen since it did not contain any chloride ions, which are known to be a serious contaminant, since they are bound and eluted from the ion exchange columns. This is an important consideration when attempting to isolate the phosphate monoester product from the reactions of the 2-alkylpropyl-1,3-bisphosphates with the alkaline phosphatases. This bicarbonate buffer provides magnesium and zinc ions, both of which are known to play an important role in the stabilisation of the enzyme's tertiary structure.^{64b,144} The large quantities of carbonate and hydrogen carbonate provide a strong alkaline medium which is reduced to pH 8.0, by the addition of acetic acid, where alkaline phosphatase show a pH-rate maximum.^{48,56,58} At pH 8.0, k_{cat} is optimum and the K_m is low.

Knowles buffer solution does not contain any Tris-buffer, which is known to be a good acceptor of free phosphate and would affect the rate of reaction.^{48,56,57a,58} Most importantly, complications in the ³¹P NMR spectra are avoided. It has been shown by Coleman,^{142a,b} that using Tris-buffer leads to the formation of *ortho*-Tris phosphate as a by-product, as well as the formation of inorganic phosphate. This would obviously make measurement of the peak areas more difficult especially in determining the progress of the enzyme reactions.

3.3. Background:

It has been reported by Hall and Williams^{84a} that there is a difference in the reactivity of the primary alkyl phosphate and aryl phosphate esters with the *E.coli* phosphatase. Such observations are not unexpected since the pK_a of the leaving conjugate acids are very different. For example, the pKa of *para*-nitrophenolate (278), is around 7.0, much lower than that of a simple alkoxide group (e.g. Bn-O⁻), which has a pKa of around 14-16. Hall and Williams^{66,84a} have suggested that the enzyme reaction goes *via* an associative mechanism in which the phosphoryl group is transferred with retention of configuration. The explanation for this is one in which the formation and decomposition of the phosphoserine intermediate *each* occur with inversion of configuration involving "in-line" nucleophilic attack. This double inversion mechanism is shown below in Figure 3.2 in which the oxyanions are stabilised by the two zinc ion centres, M_a and M_b.

Figure 3.2:



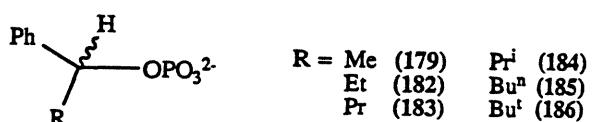
The hydrolysis of alkyl phosphates has been shown to be considerably slower than that of aryl phosphates. The rate limiting step for these two reactions differ; for the former, the cleavage of the monophosphate ester is rate limiting, while for the latter, the hydrolysis of the phosphoenzyme is rate limiting. For the alkyl phosphate a linear free energy relationship between the leaving group pKa and the rate has been demonstrated.^{66a} For alkyl phosphates with large lipophilic ester groups, the rate is slower than expected and this has been interpreted in terms of a "slow" off rate for the alcohol, consistent with the presence of a lipophilic binding site. It was hoped that this lipophilic binding site could be exploited to affect enantioselectivity in the hydrolysis of racemic phosphate monoesters.

Further evidence supporting the use of alkaline phosphatases to achieve enantiomerically pure alcohols from their reactions with the racemic monophosphate derivatives, is provided by Butler-Ranshoff *et.al.*^{61b} They have observed that the substrate leaving group (ROH) remains enzyme bound during the final phospho-transfer step to water. This slow "off" rate from the active site is dependent on the nature of the alcohol leaving group.^{61b} Clearly, if the substrate leaving group remains bound in the active site for an extended period of time, the orientation and interaction of the alkyl and aryl substituents of either enantiomer become important. Preferential binding of a particular enantiomeric phosphate substrate would be exploited by the alkaline phosphatases to afford high enantioselectivity. Although this study used the mutant alkaline phosphate (S102C) in which the serine 102 of the active site was replaced with a cysteine residue using site-directed mutagenesis,⁶² the authors state that the overall reaction mechanism of the mutant (S102C) enzyme is conserved.

3.3.1. Monophosphate ester substrates:

Williams *et.al.*^{84a} observed that derivatives with partial aromatic character, such as benzyl and phenylpropyl groups, occupy an intermediate position on the Brønsted plots ($\log k_{cat}/K_m$ versus pK_a) for the reactions of the *E.coli* alkaline phosphatase with a series of monophosphate esters. This suggests that the reactions of the benzyl derivatives lie between those of the alkyl and aryl phosphate esters, and for which the initial phosphorylation is the rate limiting step. As a consequence of these results and the existence of a lipophilic binding region, these investigations were initiated using racemic secondary benzyl phosphates. The simplest of these is the 1-phenylethyl-1-phosphate (179). The size of the alkyl group was increased to include the ethyl (182), propyl (183), *iso*-propyl (184), butyl (185) and *tert*-butyl (186) derivatives shown in **Figure 3.3**. This series of substrates was studied to identify any possible correlation between the size of the alkyl group (R) and the enantioselectivity seen with the various alkaline phosphatases. The larger alkyl groups have more hydrophobic character and would be expected to compete with the phenyl ring for the lipophilic binding site; consequently their reactivity and selectivity will be affected. The interaction of these monophosphate substrates with the active sites of the alkaline phosphatases will also be perturbed by the steric constraints of the different alkyl groups resulting in changes in their reactivity and selectivity.

Figure 3.3:



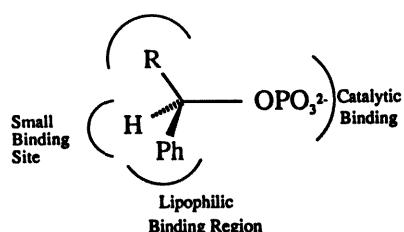
The reactions of the benzyl monophosphate esters (170, 182-186) with the various alkaline phosphatases were used to probe the binding constraints of the active site. Two possible models were explored to explain the binding of the monophosphate enantiomers within the active site. These are based on the two point attachment theory proposed by Vennesland and Westheimer.³ Two points of attachment between the substrate and the active site of the enzyme are all that is required for stereospecificity in enzyme catalysis to be observed. The active pocket of the phosphatase must therefore have at least two distinct binding regions that are fixed with respect to one another. The two fixed points of attachment of the monophosphate substrates within the active site of the phosphatases can be described by the two models. The models proposed are illustrated in Figure 3.4. In Model 1, the two fixed points of attachment of the benzyl phosphate derivative within the active site, are the phosphate group, which is bound to the catalytic site, and the hydrogen atom which is located in a small binding site. The (R)-phosphate enantiomer is orientated such that the phenyl ring is situated in the lipophilic binding region. Whereas with the (S)-phosphate enantiomer, the alkyl group (R), is located in the lipophilic binding region. The enantioselectivity of the phosphatases will depend on the preferential binding of either the phenyl or alkyl group within the lipophilic region. Clearly, this will depend upon the steric bulk and length of the alkyl group, and whether its interactions with an alternative binding site are more or less favourable than that of the phenyl group.

In Model 2, also shown in Figure 3.4, the two fixed points of binding of the substrate within the active site are the phosphate moiety, which is bound to the catalytic site, and the phenyl ring, which is bound to the lipophilic region. The (S)-phosphate enantiomer is orientated such that the alkyl group now occupies the small binding site, and the (R)-phosphate enantiomer has the alkyl group located in an alternative binding

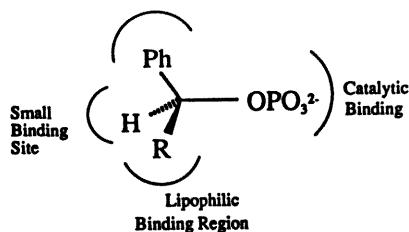
site. With this model, the enantioselectivity of the phosphatases will depend on the binding of either the alkyl group or hydrogen atom into the small binding site. The results will confirm how well this site can accommodate the different alkyl groups.

Figure 3.4:

MODEL 1

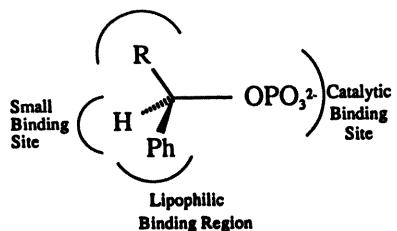


R-Enantiomer

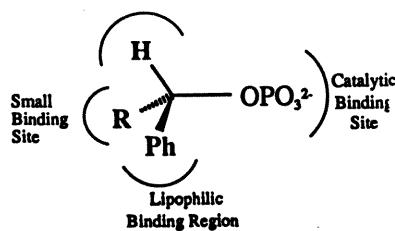


S-Enantiomer

MODEL 2



R-Enantiomer



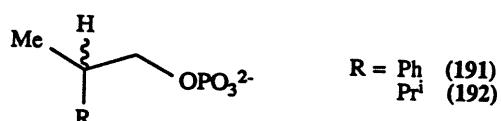
S-Enantiomer

As the size of the alkyl group (*R*), is increased, the ee's observed will depend upon which of the two models is used to explain the binding of the phosphate substrates within the active site. If Model 1 is envisaged as the binding orientation, the enantioselectivity should decrease; both the phenyl ring and large hydrophobic alkyl group will be competing to bind to the lipophilic region, resulting in reduced selectivity. If Model 2 is the binding conformation of the phosphate substrate within the active site, then the enantioselectivity would be expected to increase, since the large alkyl group is unlikely to occupy the small binding site. This brief discussion illustrates how the binding orientations of the two models oppose one another. These two models can therefore be used to cover all the possible binding orientations of the monophosphate esters within the active site. It is important to realise that these models are not mutually exclusive. The binding of the monophosphate esters within the active site is probably a combination of these models. They have been used to interpret the enantioselectivities of the alkaline phosphatases with the various monophosphate substrates. It is hoped that they would provide a better understanding of the binding interactions within the active site.

Further work involved studying the enantioselectivity of the phosphatases with a series of β -branched phosphate substrates (191) and (192). These derivatives possess a chiral centre, which is more remote from the phosphate functionality. The first derivative of this type was 2-phenylpropyl-1-phosphate (191). This is the logical progression from the secondary substrate (179) in which the phenyl and methyl group template is retained but a linker methylene group has been added. The other primary phosphate of this type was the 2,3-dimethylbutyl-1-phosphate (192) shown in Figure 3.5. A comparison of these two substrates, (191) and (192) was made. The balance between the important aromatic function and steric constraints were investigated through the observed selectivity. The

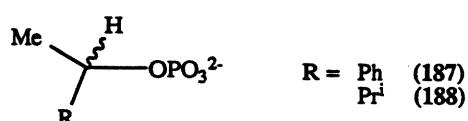
phenyl group of (191) was replaced with an *iso*-propyl group (192) to probe both steric and hydrophobic effects.

Figure 3.5:



The study was developed further to include the secondary alkyl phosphates 3-methylbutyl-2-phosphate (187) and butyl-2-phosphate (188) shown in **Figure 3.6**. Substrate (187) was chosen as a comparison to 2,3-dimethylbutyl-1-phosphate (192), in which the chiral centre is directly attached to the phosphate group, while retaining the *iso*-propyl and methyl groups yet having no aromatic character. Finally, the selectivity of phosphatases with the simple secondary phosphate butyl-2-phosphate (188) was investigated.

Figure 3.6:



3.3.2. Enantioselectivity:

Consider the racemic secondary phosphate substrate with its two enantiomeric forms (R) and (S). When hydrolysed in the presence of alkaline phosphatase, they can potentially produce the chiral alcohols (R) and (S). If the phosphatase is unable to

discriminate between the two enantiomeric substrates, the alcohol produced will be racemic. High enantioselectivity is achieved when the enzyme discriminates between the two enantiomeric phosphates. Enantioselectivity is generally expressed as an enantiomeric excess, ee.

e.g. For an enzyme reaction observed upon 50% completion:

$$\text{ee} = [(\text{R})-(\text{S})] / [(\text{R})+(\text{S})]^26 \quad (\text{R}) \text{ alcohol peak area } 17.5$$
$$\text{ee} = [17.5-7.5] / [17.5+7.5] \quad (\text{S}) \text{ alcohol peak area } 7.5$$
$$\text{ee} = 10 / 25 \quad \underline{\text{ee} = 0.4 = 40\%}$$

It has been shown by Sih^{26,145} that with biocatalytic reactions, the enantioselectivity can be expressed as an enantiomeric ratio (E value) which correlates the extent of conversion of racemic substrate (c), with the enantiomeric purity of product (ee(P)). The enantioselectivity value is defined as the ratio of the specificity constants V_{max}/K_m ²⁶ (page 139). An example of this is shown below.

$$\text{At } 50\% \text{ completion } c = 0.5 \quad \text{ee} = 40\% \quad \text{ee}(P) = 0.4$$

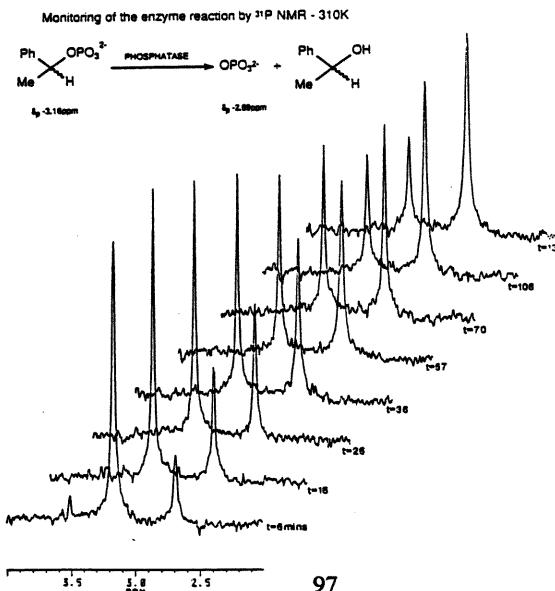
$$E = \ln[1 - c(1+ee(P))] / \ln[1 - c(1-ee(P))]^{26,26,145}$$
$$E = \ln[1 - 0.5(1+0.4)] / \ln[1 - 0.5(1-0.4)]$$
$$E = \ln[1-0.7] / \ln[1-0.3] = \ln(0.3) / \ln(0.7) \quad E = 3.37$$

Besides presenting the percentage ee in a different format, the E value enables the progress of the enzyme reaction to be monitored as it converts racemic substrate to chiral product. From their investigations of the lipases, Sih and coworkers have obtained a series of graphs expressing the various correlations of ee, c and E values.^{26,26,145} Using these plots it is possible to optimise a particular enzyme reaction to maximise the enantiomeric purity of the product. Enzymes with E values between 5-10 tend to show a synthetically useful selectivity. Theoretically, the product could be recycled several times to achieve even

higher optical purity.¹⁴⁵ However, this would be a problem with the phosphatases since it would necessitate repeating the phosphorylation sequence of reactions. When using enzymes in synthetic strategies, it is best to select enzymes with high E values of greater than 10.¹⁴⁵

3.4. Bovine alkaline phosphatase:

These experiments were carried out to explore the binding regions of the active site and their relationship to Models 1 and 2, as discussed previously. To the monophosphate substrates (100mM) was added the bovine alkaline phosphatase (25units) in the standard buffer.^{61b} These reaction solutions were incubated at 37°C and the reaction progress monitored by ³¹P NMR spectroscopy. Shown below is an illustration of how these reactions were monitored. The 1-phenylethyl-1-phosphate (179) which has a chemical shift at $\delta_p(\text{buffer}) = 3.16\text{ppm}$ is slowly converted to free inorganic phosphate, which is identified in the ³¹P NMR spectra by a peak at $\delta_p(\text{buffer}) = 2.69\text{ppm}$.



**TABLE 3.1 Enantiomeric Excesses Determined at 50% Completion of the Bovine
Alkaline Phosphatase Reactions with Racemic Monophosphate Substrates.**

<u>Substrates</u> <u>[100mM]</u>	<u>R : S Ratio</u>	<u>% e.e.</u>	<u>E value</u>	<u>Reaction Time</u>
 (179)	70 : 30	40	3.38	2 hr 10 min
 (182)	70 : 30	40	3.38	1 hr 50 min
 (183)	68 : 32	36	2.95	1 hr 30 min
 (184)	N/S	—	—	—
 (185)	68 : 32	36	2.95	1 hr 20 min
 (186)	N/S	—	—	—
 (191)	48 : 52	4	1.12	1 hr 30 min
 (192)	48 : 52	4	1.12	2 hr 30 min
 (187)	N/S	—	—	—
 (188)	N/S	—	—	—

N/S

Not a substrate

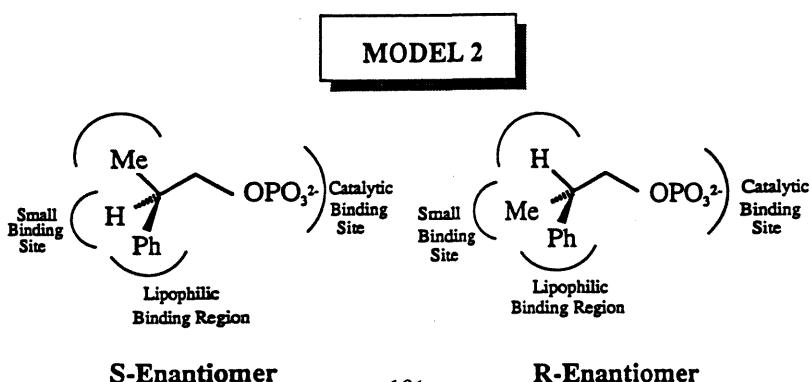
The results of these experiments, with the various monophosphate substrates are shown in **Table 3.1.** on page 98. The enantiomeric excesses were determined by reacting the alcohol products of the enzyme reactions with MTPA-Cl (209) as described in **Chapter 2.** The respective diastereoisomers produced were analysed by ^{19}F NMR spectroscopy and their relative amounts determined by measurement of the peak areas. The experimental error in determining the ee's by measuring the peak areas of the diastereomers is negligible. The ^{19}F NMR spectra were recorded on a Bruker FT NMR at 282MHz. From the reactions of the racemic alcohols with MTPA-Cl (209), the deviation calculated for the ees determined from these ratios is $\pm 0.94\%$. Since these diastereoisomers are the same, their relaxation times and saturation levels will be the same. The (R) and (S) alcohol assignments were made by comparing them with authentic samples of the racemic and chiral alcohol derivatives, also shown in **Chapter 2.** The upper section of the table, shows the results of the bovine phosphatase reactions with the 1-phenylalkyl-1-phosphate derivatives (179, 182, 183, 185). The enzyme shows a preferential turnover with the (R) phosphate enantiomer over the (S) derivative. This indicates that the (R)-enantiomer binding mode illustrated in **Model 1**, maybe the preferred orientation of the monophosphate substrate within the active site. In this (R) configuration, the phenyl group is bound to the lipophilic region and the alkyl group is located in an alternative binding region. This is also in agreement with **Model 2**, in which the hydrogen atom is situated in the small binding region. As the chain length of the alkyl group was increased, at 50% completion the selectivity was reduced from 40% ee for the methyl derivative (179), to 36% ee for the butyl substrate (185), although the magnitude of this change does not significantly exceed the experimental error. The selectivity of these substrates is methyl > ethyl > propyl > butyl. This would be consistent with the

more hydrophobic, larger alkyl groups competing with the phenyl ring for the lipophilic binding site, as proposed in **Model 1**. If considered in terms of **Model 2**, the longer reaction times maybe a reflection of the inability of the small binding pocket to accommodate the larger alkyl groups. Increasing the steric bulk of the alkyl groups to the 2-methyl-1-phenylpropyl-1-phosphate (**184**) and the 2,2-dimethyl-1-phenylpropyl-1-phosphate (**186**) has a profound effect on their reactivity, since neither of these derivatives are substrates of the bovine alkaline phosphatase. Various changes were made in the concentrations of these substrates and the units of bovine phosphatase used, but no reaction was observed.

The secondary alkyl monophosphates, 3-methylbutyl-2-phosphate (**187**) and butyl-2-phosphate (**188**), are not substrates of the bovine phosphatase. Numerous alterations to their reaction conditions were attempted but under none of these conditions was activity observed. Finally, the selectivity of the two primary monophosphates, 2-phenylpropyl-1-phosphate (**191**) and 2,3-dimethylbutyl-1-phosphate (**192**) was studied. The presence of the methylene linker between the phosphate group and asymmetric centre reverses and reduces the selectivity. With the primary phosphate substrates, the selectivity measured at 50% completion shows that the (S) phosphate enantiomer is preferentially turned over with an ee of 4%. The turnover of the 2-phenylpropyl-1-phosphate (**191**), fits **Model 2**, in which the phosphate moiety and phenyl group are the two fixed points of attachment for the substrate. This is illustrated in **Figure 3.7** in which the (S) enantiomer has the favoured binding mode. In this orientation, the methyl group is situated in an alternative binding site and the hydrogen atom is located in the small binding site. This conformation explains why the bovine phosphatase reacts preferentially with the (S)-enantiomer. However, the low ees do not significantly exceed the experimental error and this would

suggest that both orientations shown in **Figure 3.7**, are of comparable energies. This is difficult to interpret since the change in size from a hydrogen atom to a methyl group is dramatic. The only difference between the substrates 1-phenylethyl-1-phosphate (**179**) and the 2-phenylpropyl-1-phosphate (**191**) is the methylene functionality, however the difference in their reactivity is significant. The selectivity is reduced 10 fold, reversed and the reaction time of the primary monophosphate (**191**) is 1.5 times faster. This is not unexpected since either orientation of 2-phenylpropyl-1-phosphate (**191**) can be accommodated by the active site of the enzyme. Consequently the 2-phenylpropyl-1-phosphate (**191**) has a faster rate of reaction and reduced selectivity compared to the 1-phenylethyl-1-phosphate (**179**). Replacing the phenyl (**191**) group with an *iso*-propyl group (**192**) has no affect on the selectivity of the bovine phosphatase, as the selectivity of the two substrates is the same. The only difference is that the reaction time of the *iso*-propyl derivative (**192**) was extended by an additional hour. This can be attributed to the steric and hydrophobic differences between the *iso*-propyl and phenyl groups. Clearly, the more hydrophobic phenyl group is bound more readily to the lipophilic site, and this is reflected by its shorter reaction time.

Figure 3.7:



3.4.1. Rabbit alkaline phosphatase:

The rabbit alkaline phosphatase (6 units) was added to the various monophosphate esters (100mM) in the standard buffer solution.^{61b} The enzymes were incubated at 37°C and the enzyme's progress was monitored by ³¹P NMR spectroscopy.

The results of these experiment are shown in Table 3.2. The selectivity of the rabbit enzyme with the 1-phenylalkyl-1-phosphate derivatives is the same as that seen with the bovine enzyme, in that the (R)-enantiomer is the preferred substrate. Again, this indicates that the (R)-enantiomer illustrated in Models 1 and 2, are the favoured binding mode of the 1-phenylalkyl-1-phosphate substrates. In these orientations the phenyl group is bound to the lipophilic binding site and the R-group which has less hydrophobic character, is situated in an alternative binding site. The hydrogen atom and phosphoryl group occupy the small binding and catalytic binding sites respectively. The selectivity of the enzyme with the methyl (179), ethyl (182) and propyl (183) derivatives at 50% completion is consistent with ee values around 42% and high E values of 3.8. The ee decreases to 36% for the butyl derivative (185). The bulky derivatives 2,2-dimethyl-1-phenylpropyl-1-phosphate (186) and 2-methyl-1-phenylpropyl-1-phosphate (184) are not substrates of the rabbit phosphatase. Similarly, the secondary alkyl monophosphates, 3-methylpropyl-2-phosphate (187) and butyl-2-phosphate (188) are not turned over by the rabbit alkaline phosphatase.

Poor selectivity of the primary monophosphate esters, 2-phenylpropyl-1-phosphate (191) and 2,3-dimethylbutyl-1-phosphate (192) is observed with the rabbit phosphatase, with ee's of 4% and 6% respectively and low E values. The turnover of the 2-phenylpropyl-1-phosphate (191) and 2,3-dimethylbutyl-1-phosphate (192) are better represented by Model 2, in which the more hydrophobic groups, phenyl (191) and *iso*-

propyl (**192**) are located in the lipophilic binding region and the phosphate moiety is in the catalytic site, providing the two fixed points of attachment. This can be seen from the example in **Figure 3.7**, in which the (S)-enantiomer has the favoured binding. In this orientation the methyl groups are situated in the alternative binding site and the hydrogen atom is in the small binding site. However, the low ee's do not greatly exceed the experimental error suggesting that both binding orientations are favourable in which either the methyl group or hydrogen atom is located in the small binding site. Similar to the bovine phosphatase, the rabbit enzyme preferentially turns over the (S)-enantiomer of the primary monophosphates. The absence of an aromatic group in 2,3-dimethylbutyl-1-phosphate (**192**) does not affect its selectivity but the reaction time is extended by 45 minutes. Shown overleaf is a ^{31}P NMR spectrum showing the bovine alkaline phosphatase reaction with 1-phenylbutyl-1-phosphate (**183**), whose peak at $\delta_{\text{p}} = 3.09\text{ppm}$ is slowly converted to free inorganic phosphate, identified by a peak at $\delta_{\text{p}} = 2.69\text{ppm}$.

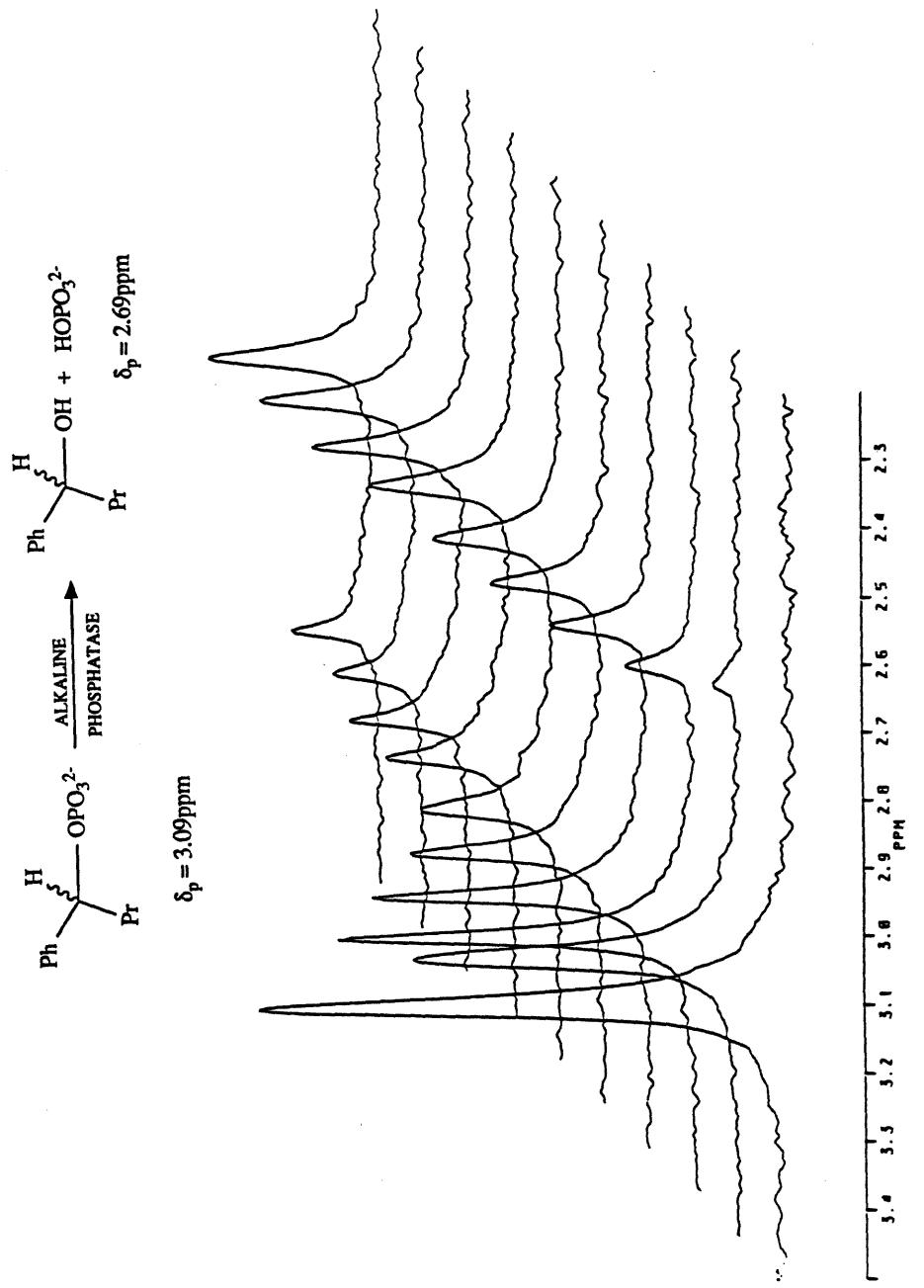
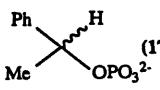
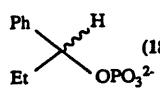
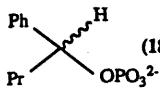
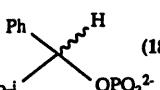
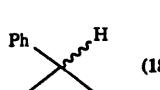
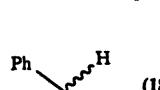
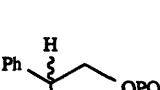
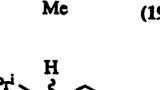
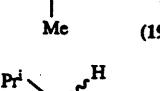
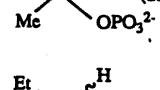


TABLE 3.2: Enantiomeric Excesses Determined at 50% Completion of Rabbit Alkaline Phosphatase Reactions with the Racemic Monophosphate Substrates.

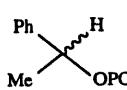
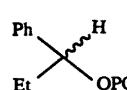
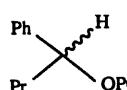
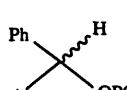
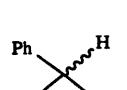
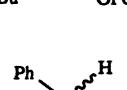
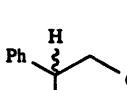
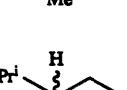
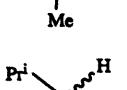
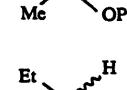
<u>Substrates</u> [100mM]	R : S Ratio	% e.e.	E value	Reaction Time
 (179)	71 : 29	42	3.61	2 hr 10 min
 (182)	72 : 28	44	3.88	1 hr
 (183)	71 : 29	42	3.61	45 min
 (184)	N/S	—	—	—
 (185)	68 : 32	36	2.95	40 min
 (186)	N/S	—	—	—
 (191)	48 : 52	4	1.12	55 min
 (192)	47 : 53	6	1.19	1 hr 40 min
 (187)	N/S	—	—	—
 (188)	N/S	—	—	—

N/S Not a substrate

3.4.2. *E.coli* alkaline phosphatase:

The *E.coli* enzyme (10 units) was added to the substrates (100mM) and the reactions monitored as described previously. The results of these experiments are shown in Table 3.3. The selectivity of the *E.coli* phosphatase is inferior to that seen with the rabbit and bovine alkaline phosphatases. This is reflected in low ee's of less than 25% and E values that fall within the range of 2.10-1.40. The reduced selectivity indicates that the alternative binding site of the *E.coli* phosphatase has a different lipophilic character than that of the bovine and rabbit phosphatases. The results of this are that the alkyl and phenyl moieties of the substrates are more readily accommodated with fewer constraints; consequently, the selectivity is reduced. The reaction times are much longer, usually requiring in excess of 5 hours to reach 50% completion. Like the mammalian phosphatase the bulky 1-phenylpropyl-1-phosphate derivatives, 2-methyl-(184) and 2,2-dimethyl-(186) are not substrates of the *E.coli* enzyme; nor are the secondary alkyl monophosphates, butyl-2-phosphate (188) and 3-methylpropyl-2-phosphate (187). Increased chain length of the alkyl groups again leads to reduced selectivity. At 50% completion the ee is halved from 24% for the methyl derivative (179) to 12% for the butyl substrate (185). In contrast to the mammalian enzymes, the reaction times are slightly increased as the length of the alkyl chain is increased. This may be due to a decrease in the size of the small binding site of the *E.coli* phosphatase compared to that of the mammalian phosphatase. The orientation of the phosphate substrate within the active site becomes essential as shown in Model 2, in Figure 3.4, in which the phenyl and phosphate groups are the fixed points of attachment of the substrate. Accommodating the larger alkyl chains within this smaller binding site is less favourable resulting in longer reaction times and improved selectivity of the primary monophosphates (191) and (192).

TABLE 3.3: Enantiomeric Excesses Determined at 50% Completion of *E.coli* Alkaline Phosphatase Reactions with the Racemic Monophosphate Substrates.

<u>Substrates [100mM]</u>	<u>R : S Ratio</u>	<u>% e.e.</u>	<u>E value</u>	<u>Reaction Time</u>
 (179)	62 : 38	24	2.02	5 hr
 (182)	58 : 42	16	1.59	5 hr 30 min
 (183)	56.5 : 43.5	13	1.46	5 hr 20 min
 (184)	N/S	—	—	—
 (185)	56 : 44	12	1.42	4 hr 20 min
 (186)	N/S	—	—	—
 (191)	39.5 : 60.5	21	1.85	6 hr 15 min
 (192)	41 : 59	18	1.69	9 hr 35 min
 (187)	N/S	—	—	—
 (188)	N/S	—	—	—

N/S Not a substrate

Surprisingly, the primary alkyl monophosphate substrates, 2-phenylpropyl-1-phosphate (191) and 2,3-dimethylbutyl-1-phosphate (192) show improved enantioselectivity with ee's of 21% and 18% respectively. Both of these derivatives show extended reaction times. This presumably reflects a difference in the lipophilic character of the binding sites of the mammalian and bacterial alkaline phosphatases, with a possible decrease in the small binding site of the *E.coli* phosphatase. The absence of an aromatic substituent in derivative (192) retards its reactivity, since the reaction time is 1.5 times longer than that of the phenyl derivative (191). This suggests that the more hydrophobic phenyl group is bound to the lipophilic region much better than the *iso*-propyl group.

3.4.3. Conclusions:

The secondary alkyl phosphates, 3-methylbutyl-2-phosphate (187) and butyl-2-phosphate (188) are not substrates for any of the alkaline phosphatases studied. As far as is known, these are the first secondary alkyl monophosphates to be studied with the alkaline phosphatases. The work of Williams and Hall involved investigating the *E.coli* alkaline phosphatase with primary alkyl phosphates. The results suggest that in order for secondary monophosphates to be turned over by the phosphatases, they must possess a large hydrophobic substituent which can interact with the lipophilic binding region. In the absence of a large hydrophobic group, the binding is presumably too weak for significant turnover. Consequently, the secondary alkyl monophosphates (187) and (188) remain unreacted. These observations are in agreement with those of Hall and Williams.^{66a,84a} They also noted that the reactivity of the alkyl phosphates is slower than that of the aryl substituted phosphates, and determined that this was not purely due to pK effects, but is the result of special binding between the substrate and enzyme.^{84a} The lipophilic binding is an essential prerequisite for substrate turnover.

is an essential prerequisite for substrate turnover.

2-Methyl-1-phenylpropyl-1-phosphate (**184**) and 2,2-dimethyl-1-phenylpropyl-1-phosphate (**186**) remain unreacted. Neither binding orientation of these substrates described by **Models 1** and **2** can be tolerated by the active site. This suggests that there are steric constraints within the binding site that can accommodate the long alkyl chain derivatives (**179**, **182**, **183** and **185**), but not branched alkyl groups such as *iso*-propyl (**184**) and *tert*-butyl (**186**). It has been reported by Sowadski and coworkers,⁵³ that there is little accessible surface area inside the binding pocket. If this holds true, then the branched chain derivatives will be too bulky and sterically hindering to allow the phosphate moiety into the active site; as a result they remain unreacted. However, the turnover of both enantiomers of the 1-phenylalkyl-1-phosphates (**179**, **182**, **183** and **185**) implies all the orientations described by **Models 1** and **2** are possible. These models have been proposed to describe the binding of the monophosphate enantiomers within the active sites of the various alkaline phosphatases. It is important to appreciate that neither model exclusively applies. The binding sites indicated are not as well defined as they appear in the models. These regions should be viewed with some flexibility, the monophosphate esters are also mobile and freely rotating within the active site. The binding orientation of one group to a particular binding region may disrupt or enhance other interactions of the monophosphate substrate to other binding regions.

The results of the reactions of the secondary 1-phenylalkyl-1-phosphates with the alkaline phosphatases indicate that the basic elements of reaction have been conserved from bacteria to mammalian sources, which is in agreement with the work of Coleman *et.al.*^{44b} With the *E.coli* phosphatase, the order of selectivity of these derivatives is methyl- (**179**) > ethyl- (**182**) > propyl- (**183**) > butyl- (**185**). Since the larger alkyl groups are more

hydrophobic, they are just as likely to bind to the lipophilic binding site as the phenyl moiety. Consequently, either orientation of these larger benzyl phosphate derivatives will be *more* readily accommodated within the active site. This is reflected by a decrease in selectivity as the size of the alkyl group of the 1-phenylalkyl-1-phosphates is increased. This implies that **Model 1** best describes the binding of the benzyl-1-phosphate enantiomers within the active site, although it is important to remember that both models make contributions in describing the overall binding orientations of the monophosphate esters within the active site.

The overall ee results suggest that there are minor differences in the active sites of the *E.coli* phosphatase and those of the mammalian enzymes. The *E.coli* phosphatase shows a greater selectivity with the 2-phenylpropyl-1-phosphate (**191**) and 2,3-dimethylbutyl-1-phosphate (**192**) when compared to the mammalian enzymes. In contrast, the mammalian enzymes show a far greater selectivity with the secondary 1-phenylalkyl-1-phosphates (**179**, **182**, **183** and **185**). This presumably reflects differences in the lipophilic character of the alternative binding site and a decrease in the size of the small binding site of the *E.coli* phosphatase. This is supported by the work of Kantrowitz *et.al.*⁶⁴ and Coleman *et.al.*^{44b} who have reported variations in the amino acid sequences of the active sites of the bacterial and mammalian phosphatases. Further evidence supporting this explanation are the different ways the alkaline phosphatases stabilise the zinc ion (site A) within the active pocket. The *E.coli* enzyme stabilises the ion centre through a hydrogen bonding pattern involving the lysine 328 residue.^{64a} However, the mammalian enzymes stabilise the zinc ion through direct binding to the histidine 153 residue.^{64b}

The poor selectivity of the mammalian phosphatases with the 2-phenylpropyl-1-phosphate (**191**) and 2,3-dimethylbutyl-1-phosphate (**192**), were not unexpected since the

chiral centre is more remote from the catalytic centre. However, not only was there a 10 fold decrease in ee for the reaction of (191), compared with the 1-phenylethyl-1-phosphate (179), but the selectivity was reversed. The (S)-enantiomer of the primary monophosphate (191) is the preferred substrate, though the low ees seen do not exceed the error suggesting that both orientations illustrated in Figure 3.7, are equivalent in energy.

Model 2 best describes the binding orientations of 2-phenylpropyl-1-phosphate (191) and 2,3-dimethylbutyl-1-phosphate (192) within the active site. The different binding orientations are the result of the methyl group and hydrogen atom competing to bind to the small binding site. Since they are both relatively small, either can be accommodated within this region and this is reflected by the poor selectivity observed. Although both **Model 1** and **2** contribute in describing the binding of the methylene linker substrates (191) and (192), within the active site, **Model 2** more accurately represents the binding reflected by the enantioselectivity observed. The models presented are an oversimplification of the actual binding. The extent to which either model contributes to the actual binding undoubtedly depends on the reacting monophosphate substrate.

The explanation for the ees and enantioselectivity values determined is based on the proposed binding **Models 1** and **2**. However, it is important to understand that the different monophosphate enantiomers have different effects on the catalytic event. Though one particular enantiomer may bind well and have a low K_m , it may have a poor effect on the catalytic event/turnover. This will clearly affect the enantiomeric excesses observed, however determining the effects the enantiomers have on the catalytic events is very difficult and often subjective.

An alternative approach to understanding the degree of hydrophobicity of the alternative binding site, is to consider it in terms of the work of Hansch and Coats.^{144c}

By determining the partitioning of a large series of compounds between octanol and water, Hansch and Coats,^{144c} have found that the given substituents produce a constant and additive contribution to the hydrophobicity of the parent compound. The observed partition coefficient is dependent on the competing tendencies of the hydrophobic regions of the molecule to prefer the organic phase, and the polar regions to be heavily solvated and prefer the aqueous phase. They have shown that the hydrophobicity constant can be correlated to the Gibbs energy of transfer of the substituent R group from octanol to water. An example of this is shown in Table 3.4.

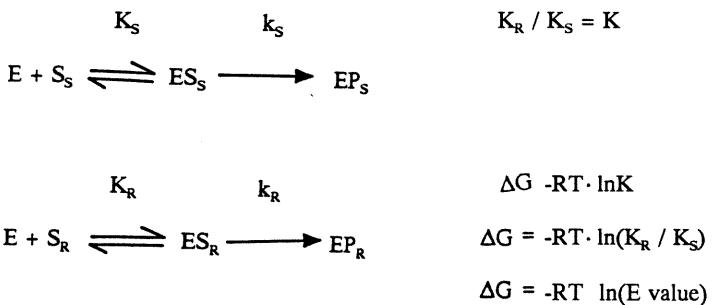
Table 3.4: Increased Gibbs Free Energy of Transfer from Octanol to Water

<u>Substituent</u>	<u>kJ · mol⁻¹</u>	<u>kcal · mol⁻¹</u>
methyl	2.85	0.68
ethyl	5.71	1.36
propyl	8.56	2.05
butyl	11.41	2.73

A. Fersht, *Enzyme Structure and Mechanism*, 1985, p.300.

It can be seen from Table 3.4 that the addition of each additional methylene adds a further 2.84kJ/mol (0.68kcal/mol) to the Gibbs energy. This indicates that the hydrophobic contributions from the substituents are significant as the compounds are transferred from octanol to water. This is frequently used as a model for the binding of substrates to an enzyme's active site, since this latter process involves distribution of a substrate from water to a more organic like phase that constitutes an enzyme active site. The enantioselectivity value (E) of the alcohol enantiomers produced at 10% reaction reflects the $V_{max}/K_m^{26,26}$ ratio for the individual enantiomers of the starting material. This is related to the change in Gibbs energy seen between the reactions of the (R) and (S) monophosphate substrates. Using the expression shown below, it is possible to determine

the apparent energy difference for the turnover of the enantiomers of the 1-phenylethyl-1-phosphates.



Bovine alkaline phosphatase: 10% completion

1-phenylethyl-1-phosphate (**179**) 1-phenylpentyl-1-phosphate (**185**)

E value = 3.38 E value = 2.95

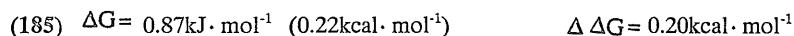
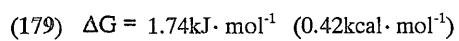
$$\begin{array}{ll}
 \Delta G = -RT \cdot \ln(3.38) & \Delta G = -RT \cdot \ln(2.95) \\
 \Delta G = 3.02 \text{ kJ} \cdot \text{mol}^{-1} (0.72 \text{ kcal} \cdot \text{mol}^{-1}) & \Delta G = 2.68 \text{ kJ} \cdot \text{mol}^{-1} (0.65 \text{ kcal} \cdot \text{mol}^{-1}) \\
 \Delta \Delta G = 0.72 - 0.65 = \underline{0.07 \text{ kcal} \cdot \text{mol}^{-1}}
 \end{array}$$

The calculated differences in Gibbs energy for the bovine enzyme reactions are only 0.07kcal/mol which is much lower than those seen in **Table 3.4**. When the alkyl substituent is changed from methyl to butyl, equivalent to the addition of three methylene groups, the Gibbs free energy remains essentially the same. This suggests that either the hydrophobic contributions from the alkyl substituents are small, perhaps because the alkyl binding site is on the surface and does not get sequestered away from water. Alternatively, the methyl and butyl substituted substrates are bound in different ways for steric reasons with some of the potential increased hydrophobic interactions being offset by a disruption

of other binding interactions. These results probably imply that the alkyl groups are not involved in any significant hydrophobic interaction.

Applying this equation to the reaction of the *E.coli* phosphatase with 1-phenylethyl-1-phosphate (179) and 1-phenylpropyl-1-phosphate (185) produces the following: At 10% completion (179) E value = 2.02

$$(185) \text{ E value} = 1.42$$



The differences in the Gibbs free energy for *E.coli* alkaline phosphatase are also small when the alkyl substituent is changed from methyl to butyl, being only 0.20kcal/mol. This is far smaller than the 0.68kcal/mol calculated of an additional methylene group bound to a substrate molecule. The implications of this are that the hydrophobic contributions from the alkyl groups are very small. This suggests that the alkyl substituents must remain in contact with the aqueous environment during catalysis.

These results suggest that the alkyl portion of the monophosphate ester substrates does not come into contact with the hydrophobic region. During catalysis, the alkyl group may reside in a region that is in contact with a hydrophilic environment, while the phosphate moiety fits into the active pocket. This would explain the small hydrophobicity changes seen with the alkyl substituents of the 1-phenylalkyl-1-phosphates. Thus the alkyl portion of the substrate could potentially lie partially exposed on the surface of the enzyme and does not enter the active site.

The apparent energy differences seen with this series of substrates are very small (0.07kcal/mol). This figure is of the same magnitude as a single hydrogen bond or a single methylene group in a hydrophobic interaction. Clearly, the broad specificity of

alkaline phosphatase is reflected in the lack of precise binding interactions to the variable ester substituents. This is the usual "trade-off" faced in the field of exploiting enzymes in organic synthesis.

The selectivity of the *E.coli* phosphatase with the various monophosphate substrates was disappointing with low enantiomeric excesses and long reaction times. The reactions of the 2-phenylpropyl-1-phosphate (191) and 2,3-dimethylbutyl-1-phosphate (192) with the mammalian phosphatases were poor, with enantiomeric excesses around 4% and E values of 1.12 at 50% completion. The outcome of these investigations were unsatisfactory. However, the results of the reactions of the mammalian phosphatases with the secondary 1-phenylalkyl-1-phosphate derivatives (179, 182, 183, 185) showed modest selectivity with E values in the range of 3.8-2.9 and enantiomeric excesses between 36-44%. These E values do not fall in the range of 5-10 suggested by Sih,^{26,145} and as discussed in Section 3.3.2, in order for enzymes to be useful in synthetic strategies, to achieve enantioselective transformations, they should have E values > 10.¹⁴⁵

In order to enhance the enantioselectivity of the phosphatases and achieve higher E values, further studies were conducted. The 1-phenylethyl-1-phosphate (179) showed the best enantioselectivity with the various alkaline phosphatases. Clearly, it is the obvious substrate with which to conduct further experiments with the phosphatases, as described in Chapter 4.

Chapter 4

*Studies of (R,S)-1-Phenylethyl-1-Phosphate with the Alkaline
Phosphatases.*

4.1. Introduction:

The results of Chapter 3 show that the alkaline phosphatases' affect their greatest enantioselectivity with the racemic 1-phenylethyl-1-phosphate (179) substrate. It is the simplest aryl substituted chiral phosphate derivative, containing a small methyl group to constitute the secondary phosphate centre. Both (R)- and (S)-1-phenylethanol are readily available as reference materials to compare with the products of the enzyme reactions. The availability of both (1R)-phenylethyl-1-phosphate (180) and (1S)-phenylethyl-1-phosphate (181) also allows for detailed analysis of the kinetic parameters for the individual enantiomers with the bovine and *E.coli* alkaline phosphatases. The monophosphate ester (179), was used to extend the study of enantioselectivity of the alkaline phosphatases from various sources, including those from mammals, bacteria, birds and fish. Once completed, the alkaline phosphatase with the best selectivity was investigated further and this is discussed in Chapter 5.

4.2. Initial kinetics:

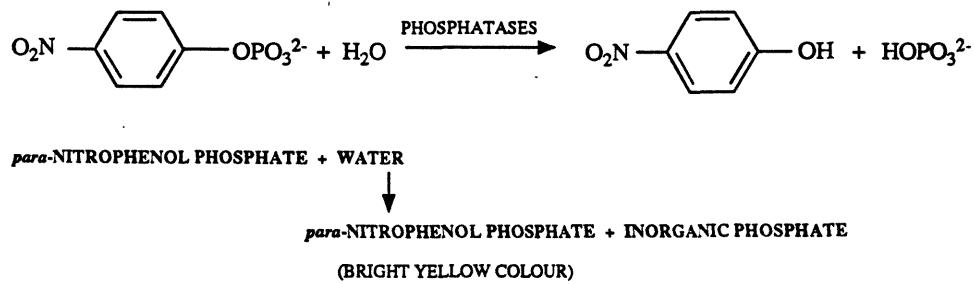
To evaluate further the enantioselective applications of the alkaline phosphatases, the kinetic constants of their reactions with the (R)-phosphate monoester (180) and (S)-phosphate monoester (181) derivatives were determined. Sih^{2c} has already shown how a knowledge of K_m and k_{cat} for individual enantiomers can be manipulated to optimise enantiodiscrimination. It has already been shown with the lipases¹³ and the esterases¹⁵ that the essential requirement for the separation of a racemic mixture, is significant differences in the rate constants for the transformations of the two enantiomers. The Michaelis constant, K_m , is invaluable in identifying the minimum concentration of substrate required to achieve an effective rate of reaction. V_{max} indicates the most rapid rate of product

formation that can be achieved for a given amount of enzyme. Clearly, the kinetic constants for the reactions of (R)-phenylethyl-1-phosphate (**180**) and (S)-phenylethyl-1-phosphate (**181**) substrates with the alkaline phosphatases, needed to be determined independently. Analysis of this kinetic data would provide the K_m , k_{cat} and V_{max} values which would be used to determine the optimum concentrations of enzyme and substrate.

4.2.1. Kinetic studies of the bovine and *E.coli* alkaline phosphatases:

Prior to carrying out the kinetic studies of the (R)-phosphate monoester (**180**) and (S)-phosphate monoester (**181**), the kinetic parameters of the alkaline phosphatases with the usual assay substrate, *para*-nitrophenyl phosphate was undertaken. This was necessary to ensure that the commercial enzyme samples were active and had rates comparable to those reported in the literature.^{84a} The reaction rates of the bovine and *E.coli* enzymes were measured with various concentrations and determined by monitoring the absorbance of the *para*-nitrophenolate product spectrophotometrically as shown in **Scheme 4.1**.

Scheme 4.1:



The results were then plotted. The first graphs on pages 121 and 122 show the Michaelis-Menten plots of the rate of reaction (V) against substrate concentration (S). The results were also converted into double reciprocal plots. The *E.coli* alkaline phosphatase

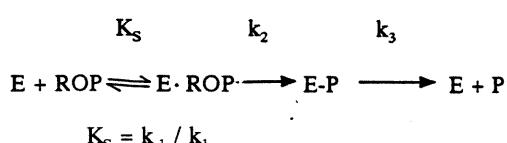
Lineweaver-Burk plot is shown on page 123. Using these two graphical representations, the K_m , V_{max} and k_{cat} values of the bovine and *E.coli* phosphatases were determined, and are shown in **Table 4.1**.

Table 4.1:

<u>Kinetic Parameters</u>	<u>Alkaline Phosphatase Enzymes</u>	
	<u>Bovine</u>	<u><i>E. coli</i></u>
K_m (μM)	13.6	5.6 (lit., ^{62,63a} 5.5)
V_{max} ($\mu mol \cdot min^{-1}$)	0.028	0.018
specific activity ($\mu mol \cdot min^{-1} \cdot mg^{-1}$)	133.0	17.3
k_{cat} (sec^{-1})	204.0 (lit., ¹⁴⁶ 200)	26.0 (lit., ^{62,63a,146} 24.8, 19)
k_{cat}/K_m ($\mu M^{-1} \cdot sec^{-1}$)	14.7	4.64

The values of kinetic parameters determined are in good agreement with those reported in the literature.

K_m values: K_m , the Michaelis constant, is the concentration of substrate at half the maximum rate of reaction. From the Lineweaver-Burk plot, $-1/K_m$ is the intercept on the horizontal axis. For enzymes that obey simple Michaelis-Menton kinetics, $K_m = K_s$, the true dissociation constant of the enzyme-substrate complex. Shown below is an example of how K_m is a measure of the amount of enzyme that is bound in any form whatsoever to the substrate.



K_m is a function of all the rate constants of a reaction, but since k_2 and k_3 are small the binding equation is approximated to $K_m = K_s$. This is the Michaelis-Menten approximation in which there is no special type of pre-binding only the equilibrium between the substrate (ROP) and the Michaelis complex (E ROP).

The K_m values measured for the reactions of the alkaline phosphatase with *para*-nitrophenyl phosphate are low, in the μM range. These K_m values are not a true measure of the binding of the alkaline phosphatases and monophosphate but rather the *apparent* binding of the *para*-nitrophenyl phosphate.

The K_m values determined for the reactions of the (R)- and (S)-1-phenylethyl-1-phosphate enantiomers with the bovine and *E.coli* alkaline phosphatase are approximately 10^4 times higher than the K_m values of the *para*-nitrophenyl phosphate. These K_m values are a good approximation of the true dissociation constants. The slow step is the formation of the phosphoserine intermediate (E-P). This means that the Michaelis complex (E·ROP) is the predominant enzyme-substrate species. K_s makes the largest contribution since both k_2 and k_3 are small; i.e. $K_m = K_s$. Therefore the K_m values closely represent the actual binding constant of the 1-phenylethyl-1-phosphate enantiomers to the alkaline phosphatases. The smaller this value, the tighter the binding of the substrate to the enzyme.

V_{max} values: V_{max} is the maximum velocity of the enzyme reaction when it is saturated with substrate. From the Lineweaver-Burk plot, $1/V_{max}$ is the intercept on the vertical axis. The results from Table 4.1 show that the bovine alkaline phosphatase turns over the natural substrate about 1.5 times faster than the *E.coli* alkaline phosphatase.

k_{cat} values: k_{cat} is often referred to as the turnover number of the enzyme.^{146,147} It represents the maximum number of substrate molecules converted to product per active

site per second. The larger this number, the greater the number of substrate molecules that are turned over per second by the enzyme. From **Table 4.1**, it can be seen that the bovine enzyme turns over more of the *para*-nitrophenyl phosphate substrate per second than the *E.coli* alkaline phosphatase.

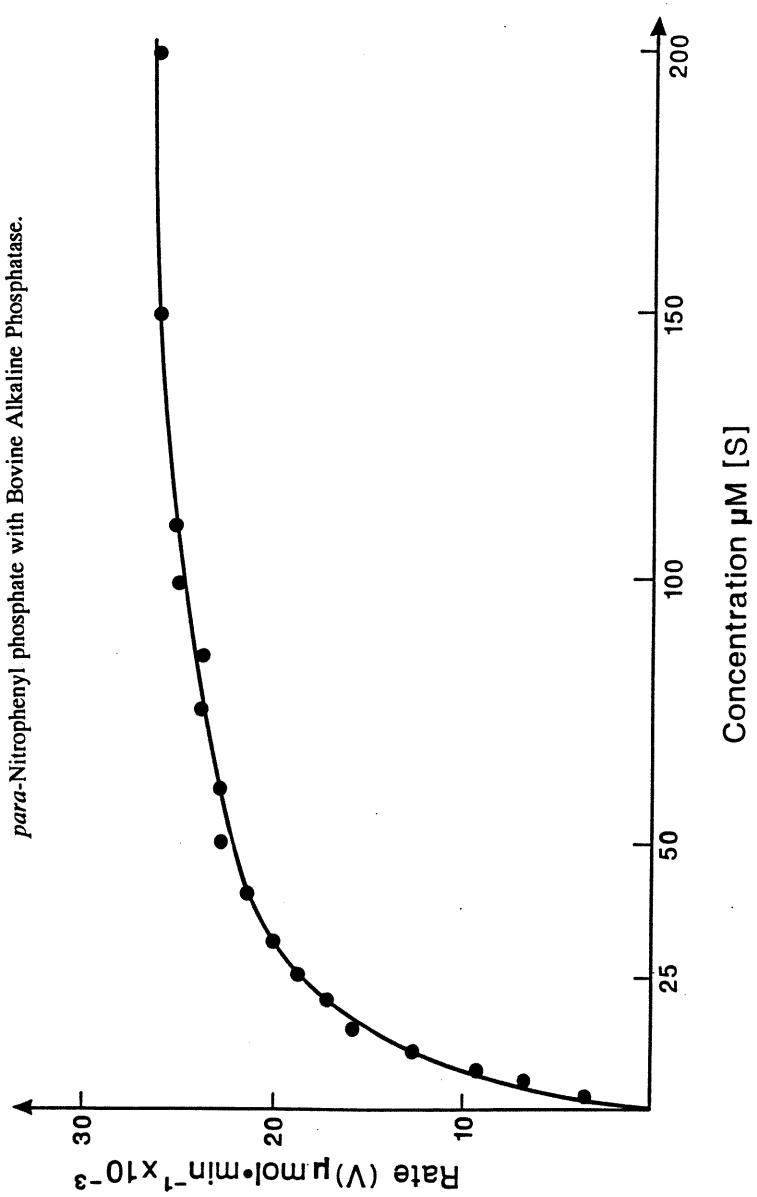
k_{cat} is calculated as follows: $V_{max} = 28\mu M \cdot min^{-1} = 28.0 \times 10^9 mol \cdot min^{-1}$ $K_m = 13.6\mu M$

$$k_{cat} \cdot [E_0] = V_{max}^{148} \quad [E_0] = \text{mass/RMM} = 2.105 \times 10^{-7} / 94,000 = 2.239 \times 10^{-12} \text{ mol}$$

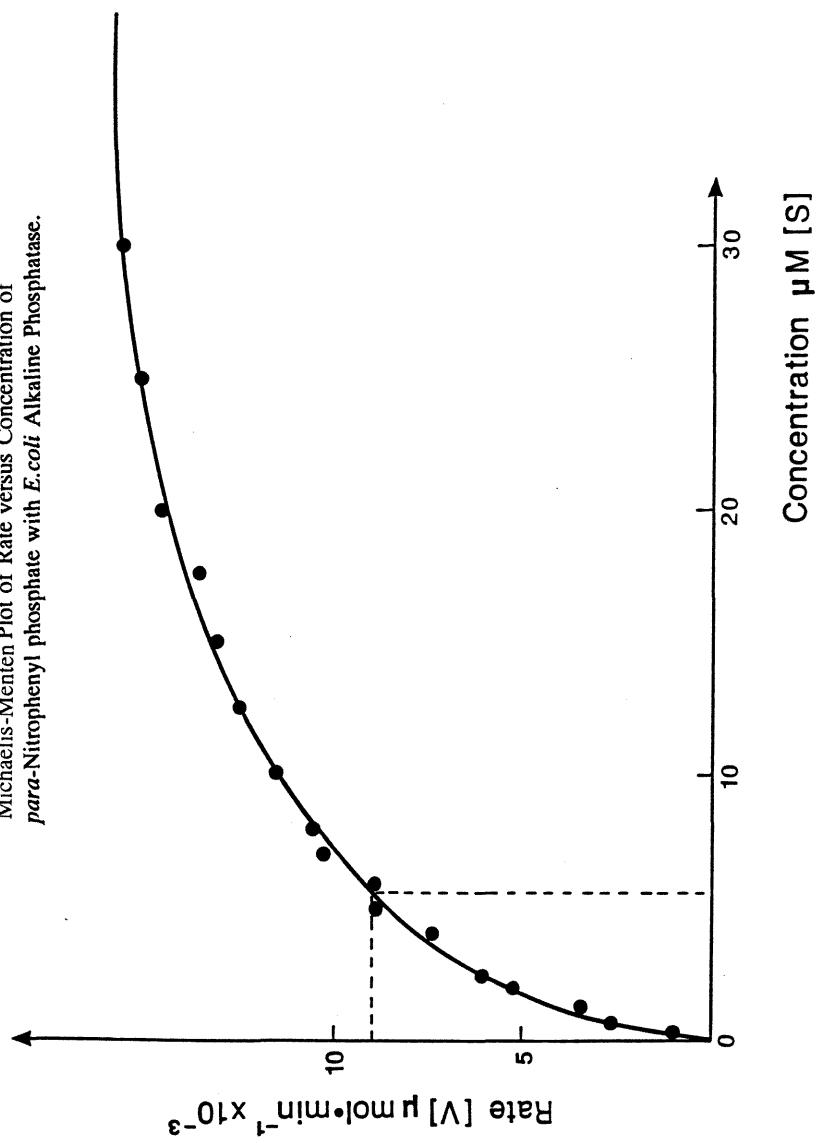
$$k_{cat} = V_{max}/[E_0] = 28 \times 10^9 / 2.288 \times 10^{-12} = 204 \text{ sec}^{-1} \quad k_{cat} = 204 \text{ sec}^{-1}$$

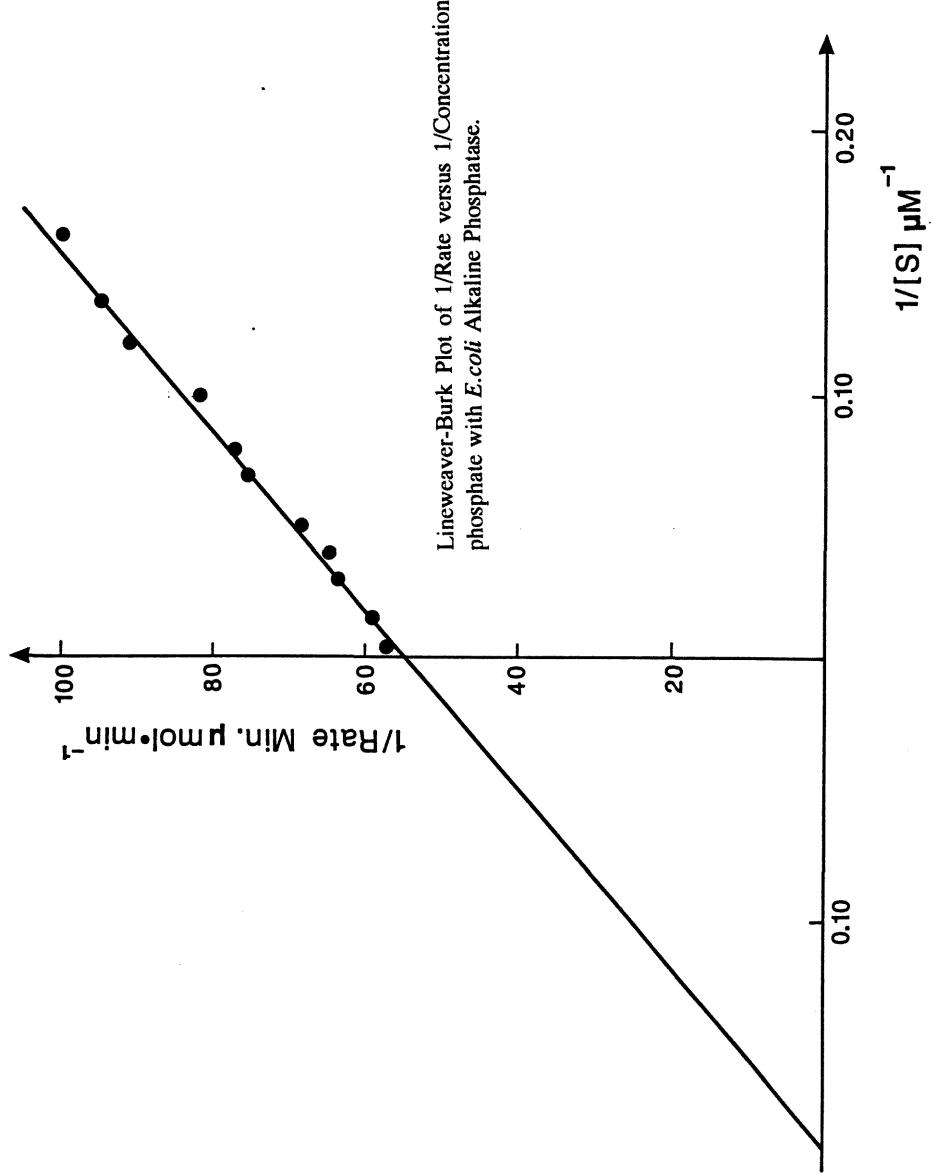
k_{cat}/K_m is a second order rate constant. It relates the reaction rate to the concentration of free enzyme, rather than total enzyme. It shows that at low substrate concentration the enzyme remains largely unbound. However, more importantly, it determines the specificity of the enzyme in the presence of competing substrates. This is important in assessing the activity of the bovine and *E.coli* alkaline phosphatases with the enantiomeric substrates, (R)-phenylethyl-1-phosphate (**180**) and (S)-phenylethyl-1-phosphate (**181**).

Michaelis-Menten Plot of Rate versus Concentration of
para-Nitrophenyl phosphate with Bovine Alkaline Phosphatase.



Michaelis-Menten Plot of Rate versus Concentration of
para-Nitrophenyl phosphate with *E.coli* Alkaline Phosphatase.





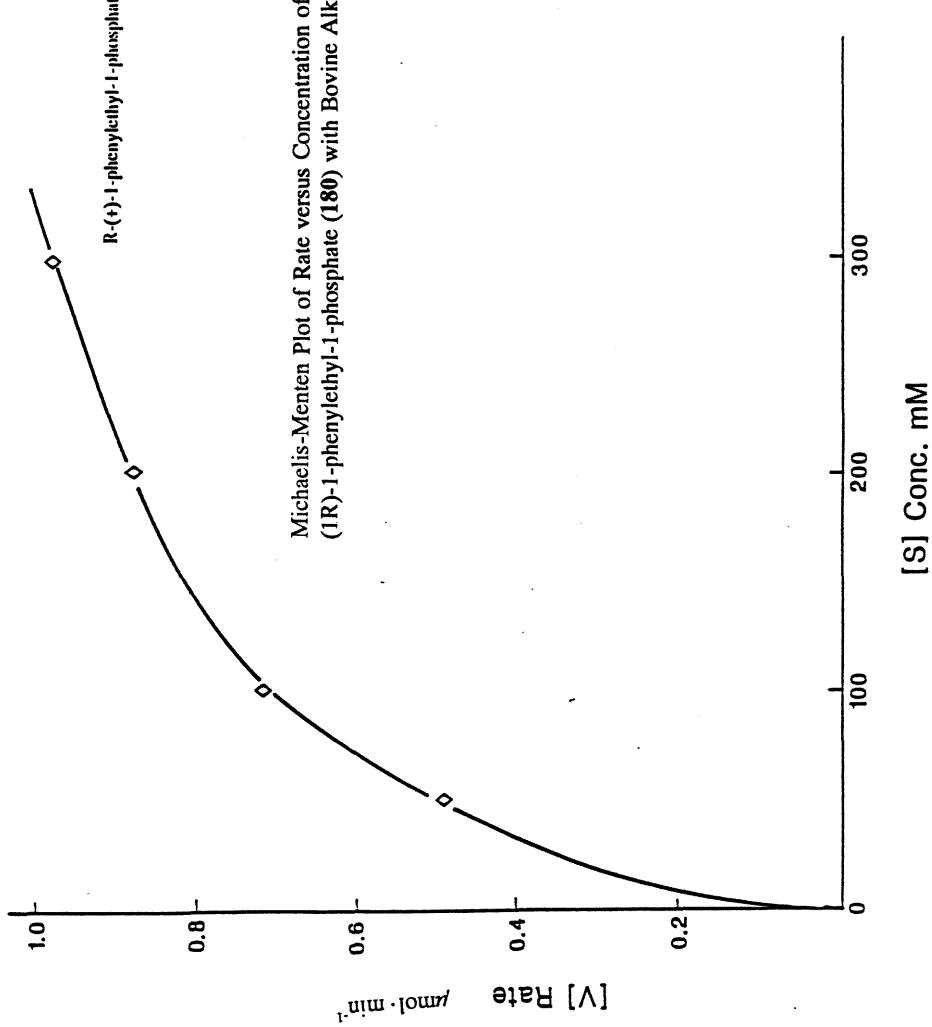
4.3. Kinetic studies of the bovine alkaline phosphatase with (R)-(180) and (S)-1-phenylethyl-1-phosphate (181):

These studies were carried out using ^{31}P NMR spectroscopy as described previously in Chapter 3. The reactions were monitored to 30% completion using concentrations of the individual enantiomeric monophosphates (R)-(180) and (S)-(181) from 5mM to 300mM. At regular time intervals, the progress of the reaction was monitored and the areas of the substrate and product peak were measured to determine the extent of reaction. From these results, plots of product concentration versus time were drawn and from the slopes of these lines, the reaction rates ($\mu\text{mol}/\text{min}$) were determined. From these results, Michaelis-Menten plots of rate ($\mu\text{mol}/\text{min}$) versus concentration (S) were produced as shown on pages 125 and 126. Lineweaver-Burk plots were produced from which the K_m , V_{max} and k_{cat} were determined for the individual phosphate enantiomers. The cumulative results of these reactions are shown below in Table 4.2.

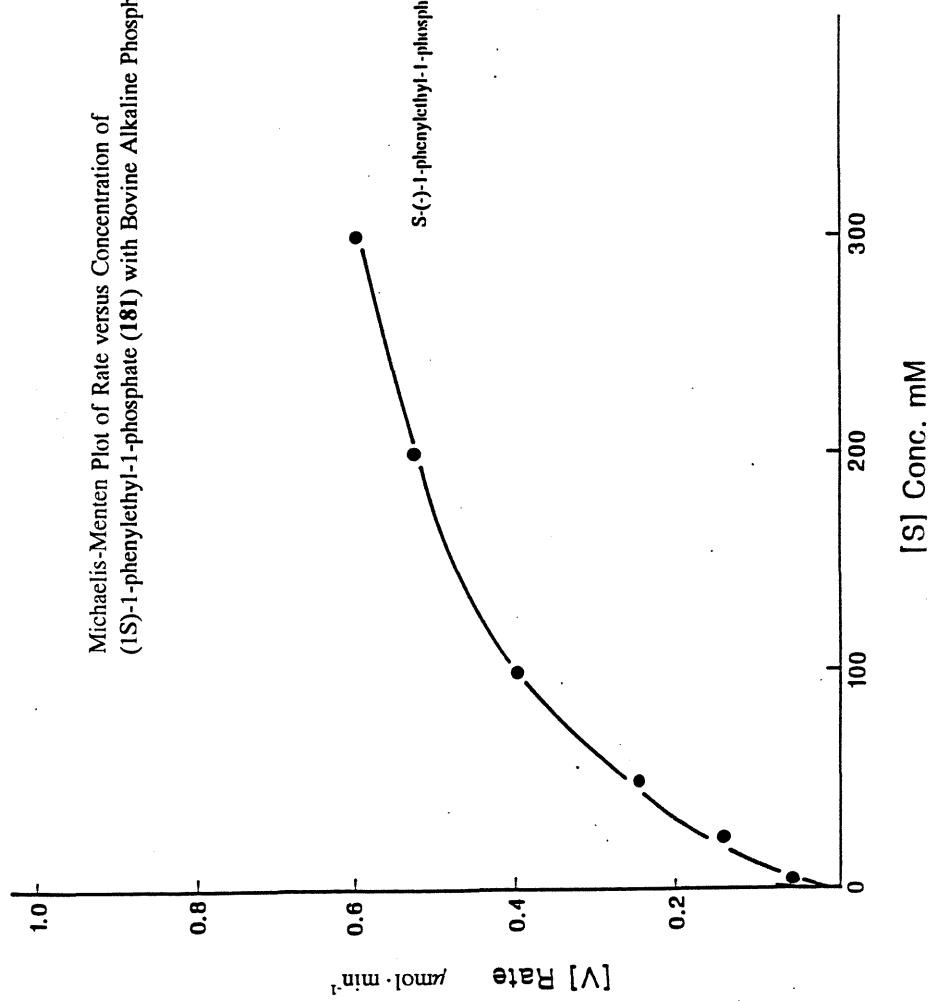
Table 4.2: Bovine Alkaline Phosphatase

1-phenylethyl-1-phosphate substrates

<u>Kinetic parameters</u>	(R)-(+)	(S)-(-)	pNPP
K_m (mM)	65.8	161.3	0.0136
V_{max} ($\mu\text{mol} \cdot \text{min}^{-1}$)	1.18	1.00	----
specific activity ($\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$)	112.0	95.0	133.0
k_{cat} (sec^{-1})	171	146	204
k_{cat}/K_m ($\mu\text{M}^{-1} \cdot \text{sec}^{-1}$)	2.59×10^{-3}	9.0×10^{-4}	14.7



Michaelis-Menten Plot of Rate versus Concentration of
(1S)-1-phenylethyl-1-phosphate (181) with Bovine Alkaline Phosphatase.



The k_{cat}/K_m values for the (R)-(180) and (S)-(181)-1-phenylethyl-1-phosphate substrates are much lower than that of the *para*-nitrophenyl phosphate. This clearly shows that the (R)-(180) and (S)-(181) enantiomers of 1-phenylethyl-1-phosphate are poorer substrates of the bovine alkaline phosphatase. Using the Gibbs free energy equation, the difference in the binding energy of the (R)-1-phenylethyl-1-phosphate (180) and *para*-nitrophenyl phosphate was calculated and is shown below.

$$G = -RT \cdot \ln (K_{pNPP} / K_R) \quad K_{pNPP} = (k_{cat} / K_m)_{pNPP}$$

$$G = -RT \cdot \ln (14.7 / 2.59 \times 10^{-3}) \quad K_R = (k_{cat} / K_m)_R$$

$$G = 22.28 \text{ kJ} \cdot \text{mol}^{-1} = 5.30 \text{ kcal} \cdot \text{mol}^{-1}$$

This difference in binding energy between the (R)-1-phenylethyl-1-phosphate (180) substrate and the *para*-nitrophenyl phosphate substrate is very large. The reason for the disparity in the reactivity of these substrates is the differences in the pKa values of their leaving groups. The *para*-nitrophenolate has a lower pKa, since it is resonance stabilised and consequently it is readily lost. Clearly, the electron withdrawing nitro substituent also decreases the phosphate pKa inductively, however, this is not important since the phosphate group is completely ionised at pH 8.0. The 1-phenylethyl-1-phosphate substrate (179) is a benzyl derivative and the leaving group a simple alkoxide. The pKa of this leaving group is much higher than that of the *para*-nitrophenolate and consequently it is less reactive.

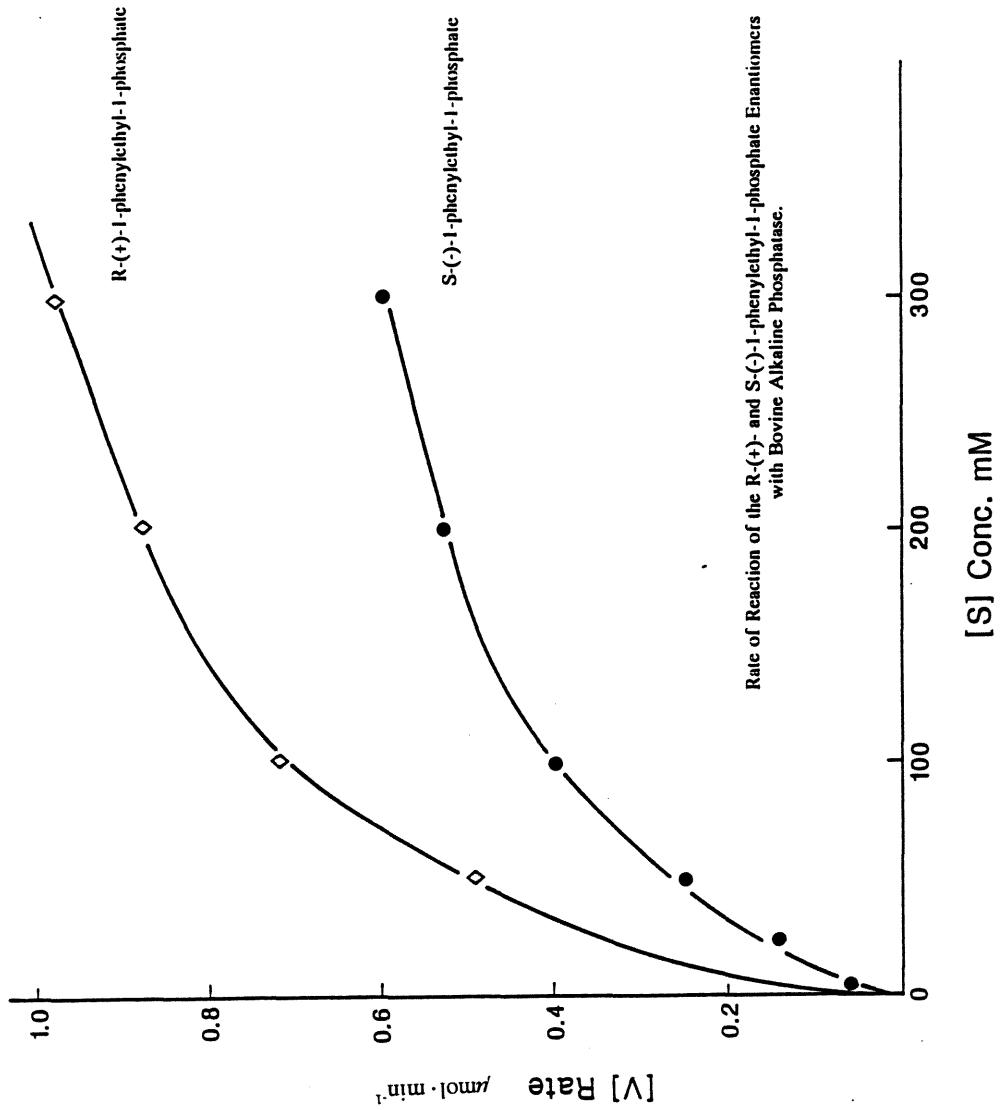
There are also differences in the steric bulk of these substrates. The *para*-nitrophenyl phosphate only has a nitrophenyl group attached to the reacting phosphate group. The 1-phenylethyl-1-phosphate is a secondary benzyl derivative, which besides having a phenyl group has an additional methyl group attached to the benzylic carbon centre. The *para*-nitrophenyl phosphate has only the phenyl and phosphate groups with

which to bind to the active site of the enzyme. The phosphate is bound to the catalytic site and the phenyl, being highly hydrophobic, is bound to the lipophilic binding site.^{84a} The 1-phenylethyl-1-phosphate substrate on the other hand, has three groups which need to be accommodated within the active site. Obviously, the phosphate group must be bound to the catalytic site for turnover. The orientation of the methyl and phenyl groups within the active pocket is important in terms of any enantioselectivity. This can be discussed in terms of **Model 1** for the binding orientation for the 1-phenylethyl-1-phosphate enantiomers. In this model the greatest discrimination is between the methyl and phenyl groups competing to bind to the lipophilic binding site. These steric requirements and orientation of the 1-phenylethyl-1-phosphate substrate within the active site will clearly slow down its turnover.

The k_{cat} / K_m value for the (R)-phosphate monoester (**180**) is 2.8 times larger than that of the (S)-phosphate monoester (**181**). This suggests that of the two enantiomers the (R)-1-phenylethyl-1-phosphate (**180**) is a better substrate and more tightly bound to the enzyme. The differences in the k_{cat} values calculated for the (R)-(180) and (S)-(181)-1-phenylethyl-1-phosphate enantiomers also reflects this. This is also supported by the V_{max} values. It then follows that the formation of the (R)-1-phenylethanol product must be faster than that of the (S)-1-phenylethanol product. Exploitation of the differences in kinetic parameters between the (R)-(180) and (S)-(181)-1-phenylethyl-1-phosphates can be used to optimise enantioselectivity of the bovine alkaline phosphatase. Combined Michaelis-Menten plots of the two phosphate enantiomers are shown on page 130. An examination of this graphical representation indicates that as the concentration of substrate is increased above 100mM, the differences in reaction rates of the (R)-phosphate monoester (**180**) and (S)-phosphate monoester (**181**) remain constant. The two rate profiles

of the (R)-(180) and (S)-(181) substrates with the alkaline phosphatases are similar which indicates that manipulating the substrate concentrations will have little effect on the observed ee, because the enantioselectivity (E) is independent of the substrate concentration.

From the kinetic constants obtained, a concentration of the racemic phenylethyl-1-phosphate (179), of 75mM was used. Higher concentrations of 1-phenylethyl-1-phosphate (179) were investigated with bovine alkaline phosphatase, but their reaction rates and enantioselectivities were the same as those determined at 75mM. At this concentration, the rate of turnover of the (R)-phosphate monoester (180) is 1.6 times faster than that of the (S)-phosphate monoester (181). At this concentration, the rate of turnover of the (R)-phosphate enantiomer (180) is half its maximum at $0.6\mu\text{mol}/\text{min}$, while the rate of the (S)-phosphate monoester (181), is only $0.32\mu\text{mol}/\text{min}$. At lower concentrations, the reaction rates of both enantiomers (180) and (181), are too close to one another to achieve any reasonable selectivity.



4.3.1. Kinetic studies of the *E.coli* alkaline phosphatase with (R)-(180) and (S)-1-phenylethyl-1-phosphate (181):

These experiments were carried out as described earlier in Section 4.3.1., using the (R)-(180) and (S)-phosphate (181) esters and 10 units of *E. coli* alkaline phosphatase. The result of these investigations are shown in the Michaelis-Menten plots on pages 134 and 135. The kinetic parameters are shown in Table 4.3.

Table 4.3: *E.coli* Alkaline Phosphatase

<u>1-phenylethyl-1-phosphate substrates</u>			
<u>Kinetic parameters</u>	<u>(R)-(+)</u>	<u>(S)-(-)</u>	<u>pNPP</u>
K_m (mM)	39	55	0.0056
V_{max} ($\mu\text{mol} \cdot \text{min}^{-1}$)	0.59	0.38	-----
specific activity ($\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$)	2.44	1.83	17.30
k_{cat} (sec^{-1})	4.20	2.72	26.00
k_{cat}/K_m ($\mu\text{M}^{-1} \cdot \text{sec}^{-1}$)	1.08×10^{-4}	4.9×10^{-5}	4.64

The table of results show that the k_{cat}/K_m values of both the (R)-phosphate monoester (180) and (S)-phosphate monoester (181), are smaller than that of the *para*-nitrophenyl phosphate, again showing that the alkyl phosphates (180) and (181) are poorer substrates of the *E.coli* phosphatase. The k_{cat} for the (R)-(180) and (S)-(181) phenylethyl-1-phosphates are about 8 times lower than that of the *para*-nitrophenyl phosphate at 26sec^{-1} . The difference in k_{cat}/K_m values for the (R)-phosphate monoester (180) and the *para*-nitrophenyl phosphate can be expressed as the difference in Gibbs free energy for the catalytic process involving the two substrates. This is shown overleaf.

$$\Delta G = -R T \cdot \ln (K_{pNPP} / K_R)$$

$$K_{pNPP} = (k_{cat} / K_m)_{pNPP}$$

$$\Delta G = -RT \cdot \ln (4.64 / 1.07 \times 10^4)$$

$$K_R = (k_{cat} / K_m)_R$$

$$\Delta G = 27.59 \text{ kJ} \cdot \text{mol}^{-1} = 6.56 \text{ kcal} \cdot \text{mol}^{-1}$$

The explanation for these large differences are the same as those given for the bovine phosphatases. The major contribution being the different pKa values of the leaving groups and steric effects of the methyl moiety of the (R)-1-phenylethyl-1-phosphate (180).

The k_{cat}/K_m value for the (R)-phosphate monoester (180), is double that of the (S)-phosphate monoester (181) which implies that the (R)-1-phenylethyl-1-phosphate (180) is a better substrate and probably more tightly bound to the active site of the enzyme than the (S)-phosphate monoester (181). Summarising these results, the (R)-1-phenylethyl-1-phosphate (180), is a better substrate for the *E.coli* alkaline phosphatase than the (S)-enantiomer (181). The kinetic constants measured are comparable to those reported by Williams *et.al.*^{84a,149} in which the kinetic parameters of the *E.coli* enzyme with various primary alkyl and aryl phosphates were determined. A comparison of the kinetic data from the enzyme reactions of the (R)-phosphate monoester (180) and (S)-phosphate monoester (181) with those obtained by Williams and coworkers,^{84a,149} are shown in Table 4.4.

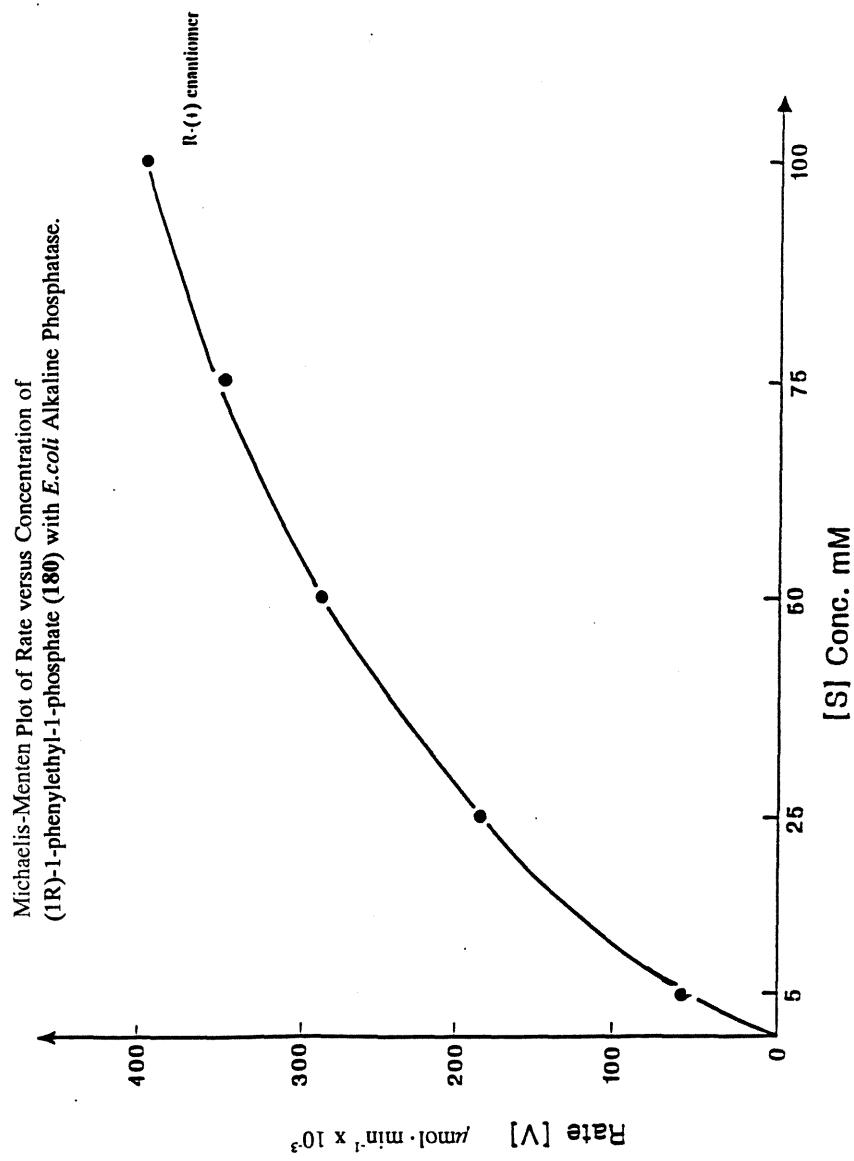
Table 4.4. Kinetic Parameters

<u>Substrates</u>	<u>$k_{cat}(\text{sec}^{-1})$</u>	<u>$K_m(\text{mM})$</u>
aryl substituted phosphate substrates ^{84a,149}	80.0	0.013
(R)-1-phenylethyl-1-phosphate (180)	4.20	39.0
(S)-1-phenylethyl-1-phosphate (181)	2.72	55.0
alkyl phosphate substrate ^{84a}	1.59	256.0

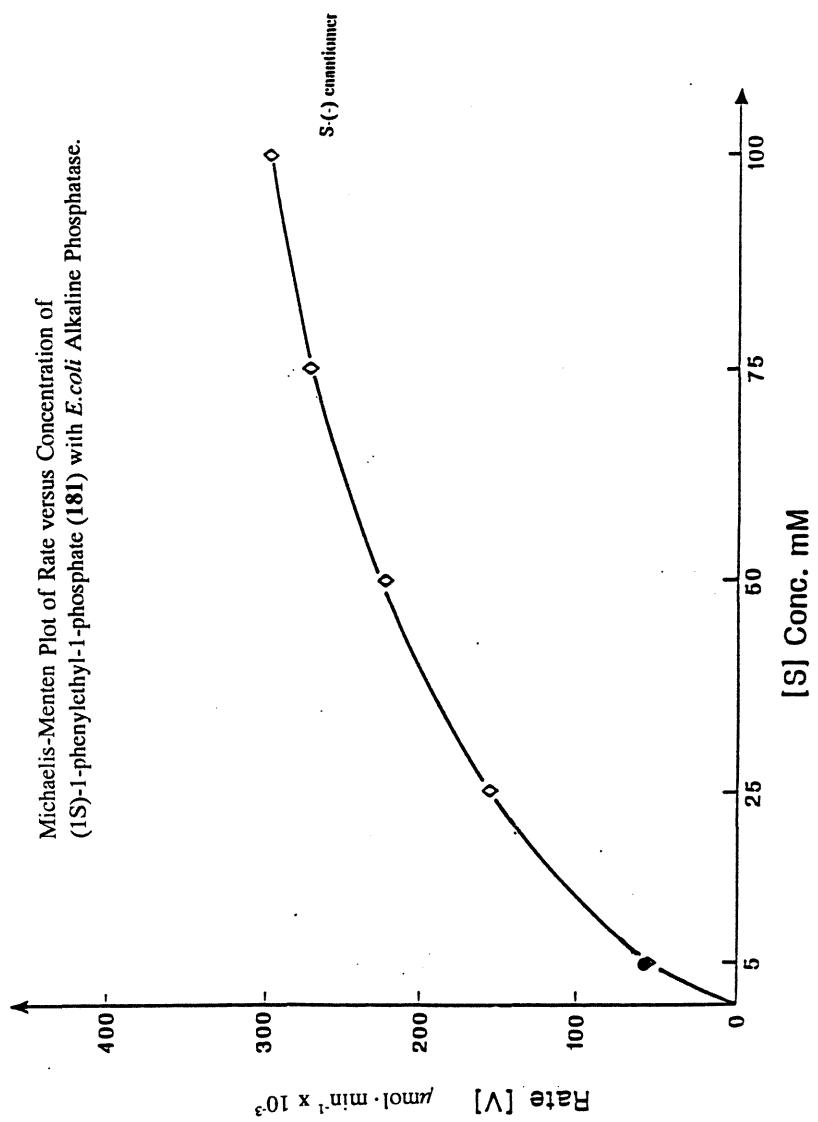
A comparison of the k_{cat} and K_m values illustrates that the altered secondary phosphate derivatives (180) and (181) lie between the kinetic values of the aryl phosphates^{84a,149} and those of the simple alkyl phosphate derivatives.^{84a} It is clear that the enantiomeric phosphates monoesters (180) and (181), are turned over faster than the simple alkyl derivatives investigated by Williams.^{84a} This is not unexpected since the substrates (R)-(180) and (S)-(181) are secondary benzyl phosphate which have a hydrophobic phenyl ring which will bind to the lipophilic binding site. Thereby, making them better substrates than the simple alkyl derivatives. Williams has suggested that the differences in rate between the alkyl and aryl substrates is associated with changes in the rate limiting steps, presumably reflecting the large differences in pKa of the leaving group.⁶⁶

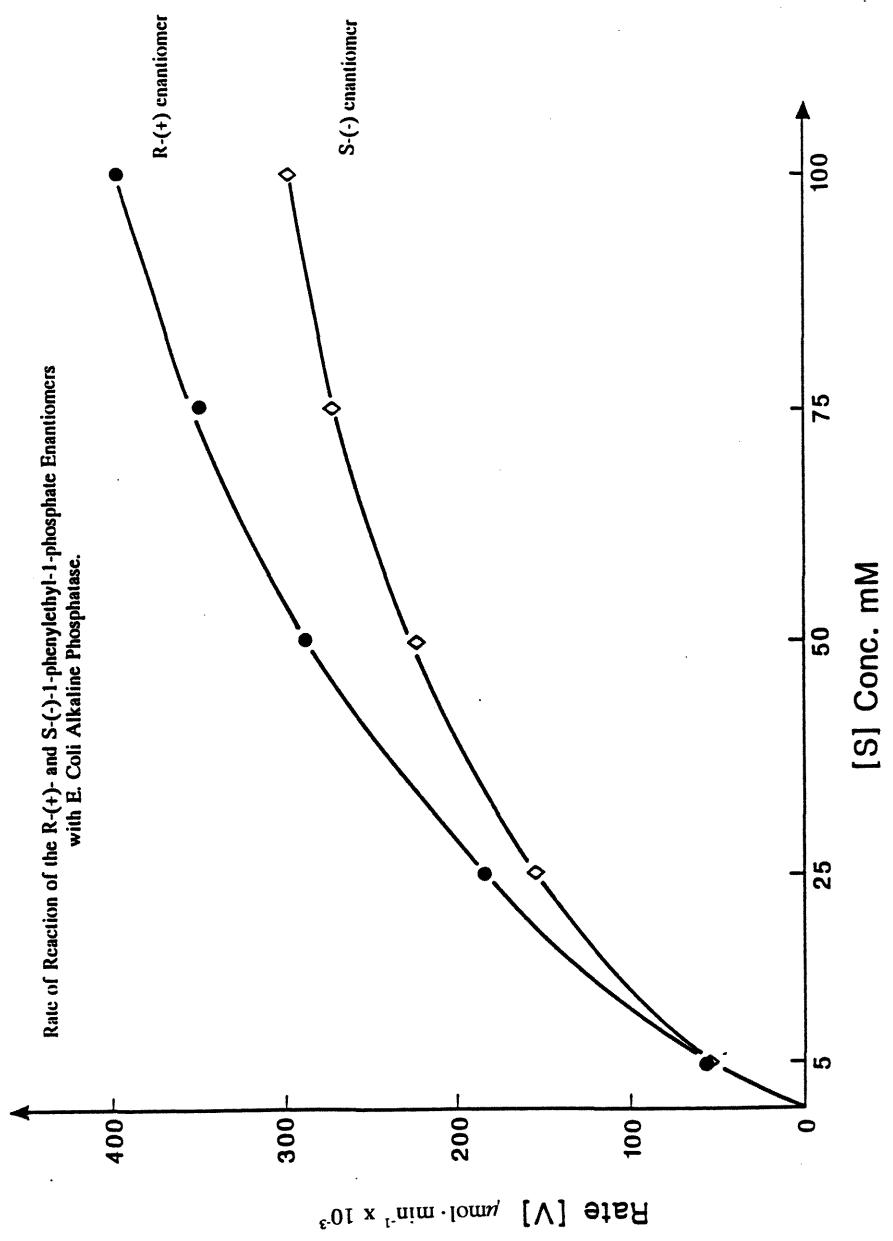
The kinetic data for the secondary substrates (180) and (181) fall between these aryl and simple alkyl monophosphates. A Michaelis-Menten plot incorporating both sets of results is shown on page 136, revealing the differences in the kinetic parameters of the two enantiomeric phosphates (180) and (181). An examination of this plot shows that the reaction rates of the two derivatives, (R)-(180) and (S)-(181), are very close throughout the substrate concentrations studied. Though if anything, they are still diverging at higher concentrations. These results indicate that it would be difficult to improve the ee by manipulating substrate concentration. The results predict that enantiodiscrimination of the *E.coli* phosphatase with the racemic 1-phenylethyl-1-phosphate (179) will be much lower than that observed with bovine phosphatase. From the kinetic data presented, the concentration of substrate (179) used to attain maximum selectivity with the *E.coli* was 75mM. The 75mM concentration of the monophosphate substrate (179) was used to maintain some consistency with the bovine phosphatase experiments. At 75mM the rate

of turnover of the (R)-phosphate monoester (**180**) is 1.3 times faster than (S)-substrate (**181**). At concentration below this the reaction rates of the two substrates (**180**) and (**181**) are too close to one another for any reasonable enantioselectivity to be observed.



Michaelis-Menten Plot of Rate versus Concentration of
(1S)-1-phenylethyl-1-phosphate (181) with *E.coli* Alkaline Phosphatase.





4.3.2. Conclusions:

From the k_{cat}/K_m values it was possible to calculate the differences in binding energies of the (R)-1-phenylethyl-1-phosphate (180) and (S)-1-phenylethyl-1-phosphate (181) with the respective bovine and *E.coli* alkaline phosphatases. The differences in the binding energies between the alkyl and aryl monophosphate esters has previously been discussed in Chapter 1.^{84a} The difference between the interactions of the two sets of leaving groups is said to constitute a ΔG of $2.5 \text{ kcal} \cdot \text{mol}^{-1}$ ($10.45 \text{ kJ} \cdot \text{mol}^{-1}$) in binding energy.^{84a} Using the Gibbs free energy equation, differences in the binding energies of the phosphate enantiomers (R)-(180) and (S)-(181), can be calculated. Calculations using k_{cat}/K_m is a better reflection of specificity differences than using K_m only, since it includes both the activation and binding energies and avoids possible under estimations.^{26,146}

Bovine alkaline phosphatase - calculation of binding energy difference

$$\Delta G = -R T (\ln K_R - \ln K_S)$$

$$\Delta G = -R T \ln K_R / K_S \quad K_R = (k_{cat}/K_m)_R \quad K_S = (k_{cat}/K_m)_S$$

$$\Delta G = -R T \ln (2.59 \times 10^{-3} / 9.0 \times 10^{-4}) \quad K_R = 2.59 \times 10^{-3} \quad K_S = 9.0 \times 10^{-4}$$

$$\Delta G = -2.7 \text{ kJ} \cdot \text{mol}^{-1} \quad \Delta G = 0.64 \text{ kcal} \cdot \text{mol}^{-1}$$

E.coli alkaline phosphatase - calculation of binding energy difference

$$K_R = 1.07 \times 10^{-4} \quad K_S = 4.95 \times 10^{-5}$$

$$\Delta G = -2.0 \text{ kJ} \cdot \text{mol}^{-1} \quad \Delta G = 0.48 \text{ kcal} \cdot \text{mol}^{-1}$$

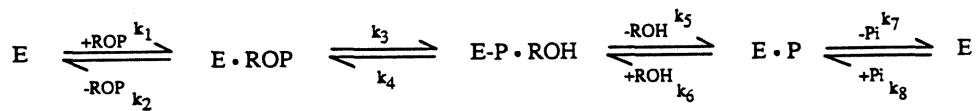
Since, the (R)-(180) and (S)-(181) phosphate substrates have the same structure, the inherent activation energy of bond making and bond breaking will be the same.¹⁴⁶ Therefore, the ΔG values calculated can be considered to be purely the difference in the *binding energy* between the two enantiomers. These differences in ΔG are smaller than was hoped, but are reasonable enough to allow both the bovine and *E.coli* phosphatases

to affect modest enantioselectivity in their reactions with the racemic 1-phenylethyl-1-phosphate (179).

The enantioselectivity value (*E*), and related work by Sih using the lipases in affecting enantioselective transformations has been discussed in **Section 3.3.2**. In this section, a correlation of the *E* value with the kinetic parameters k_{cat} , K_m and V_{max} , is illustrated. A description and relevant equations of how this correlation is achieved are explained below.

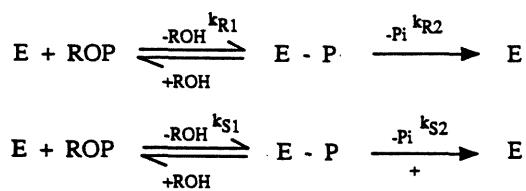
The kinetics of the phosphatase catalysed hydrolysis of the racemic phosphate substrate (179) may be represented by the sequential mechanism shown in **Scheme 4.2**.

Scheme 4.2:



During the initial stages of the reaction, the complex catalytic sequence can be reduced to a simpler mechanism shown in **Scheme 4.3**, consisting of two bond breaking steps for the two separate phosphate enantiomers, (R)-(180) and (S)-(181).

Scheme 4.3:



Kinetically, k_{R1} , k_{R2} , k_{S2} and k_{S1} are equivalent to k_{cat}/K_m for the (R)-(180) and (S)-(181) phosphate enantiomers and represent the overall binding and catalytic performance of each diastereomeric alcohol-enzyme complex. The phosphate hydrolysis of the (R)- and (S)-1-phenylethyl-1-phosphates are the fast and slow reacting enantiomeric substrates respectively. The ratio of the two partial rates of reaction can be shown by steady-state kinetics as follows.

$$v_R/v_S = V_{max}(R)/V_{max}(S) \cdot K_m(S)/K_m(R) \cdot [R]/[S]$$

In this equation, $V_{max}(R)$, $V_{max}(S)$, $K_m(R)$ and $K_m(S)$ denote the maximum velocities and Michaelis constants for the fast and slow enantiomers. This is true for all hydrolytic reactions in which the sole contribution to enantioselectivity is the catalytic sequence leading to and including the first irreversible step. The discrimination between the two competing enantiomers by the phosphatase is dictated by E (enantioselectivity value), the ratio of the specificity constants V_{max}/K_m is calculated as follows. This discrimination is also characterised by the ee of alcohol products detected.

$$\ln(R/R_o) / \ln(S/S_o) = V_{max}(R)/K_m(R) / V_{max}(S)/K_m(S) = E^{26,146}$$

From the Michaelis-Menten plots of the bovine and *E.coli* phosphatases it can be seen that the E value is dependent on the substrate concentration. Sih^{2e} has shown recently, that when this substrate concentration is taken into consideration the E value can be represented as follows.

$$(k_{cat}/K_m)_R / (k_{cat}/K_m)_S = E^{2e,26}$$

Using these two expressions it is possible to calculate the E values (enantiomeric ratio) from the kinetic parameters obtained from the reactions of the individual enantiomers (R)-(180) and (S)-(181) with the bovine and *E.coli* alkaline phosphatases.

Table 4.5:

Alkaline phosphatase	Enantioselectivity values (E)	
	V_{max}/K_m	k_{cat}/K_m
Bovine	2.96	2.83
<i>E.coli</i>	2.18	2.18

Enantioselectivity value calculated for *E.coli* alkaline phosphatase:

$$E = (k_{cat}/K_m)_R / (k_{cat}/K_m)_S^{2e}$$

$$E = 107.7 / 49.5 \quad E = 2.18$$

$$E = (V_{max}/K_m)_R / (V_{max}/K_m)_S^{2e}$$

$$E = (588/39) / (380/55)$$

$$E = 15.07 / 6.91 \quad E = 2.18$$

The E values calculated using the different kinetic parameters are essentially the same, verifying that the kinetic constants determined and the calculations made are consistent. The results shown in **Table 4.5** illustrate that under all conditions the maximum E values for the bovine phosphatase is around 3.00, and that of the *E.coli* enzyme is around 2.20. These low E values indicate that there is little room for manipulation of the phosphatase enzymes to improve their enantioselectivity. It has been reported by Sih *et.al.*^{26,28,146} that in order to obtain high enantiodiscrimination, E values of >10 are necessary. The E values obtained for the phosphatases were not as high as had been anticipated. By terminating the enzyme reaction after only a small amount of the monophosphate substrate had been turned over, it is possible to observe reasonably high enantiomeric enrichment of the monophosphate substrate with the (R)-1-phenylethyl-1-phosphate material.

The results show that with both sources of alkaline phosphatase the (R)-1-phenylethyl-1-phosphate (180) was the preferred substrate of the two enantiomeric derivatives. With both the *E.coli* and bovine phosphatases the k_{cat}/K_m values for the (R)-phosphate monoester (180) are larger than those of the (S)-(181) derivative. A tighter binding of the phosphate enantiomers (180) and (181) is seen with the *E.coli* enzyme compared to that of the bovine alkaline phosphatase. However, the reaction rates of the (R)-(180) and (S)-(181) phosphate monoesters with the bovine alkaline phosphatase are faster. This is not wholly unexpected since it has been reported by Kantrowitz,^{64b} that the mammalian alkaline phosphatases are more active than the *E.coli* enzyme. These investigations produced important kinetic data, including K_m , k_{cat} and V_{max} values, for the reactions of the (R)-phosphate (180) and (S)-phosphate (181) esters with the bovine and *E.coli* alkaline phosphatases. The results provide an analysis of the levels of enantioselectivity empirically observed for the racemic 1-phenylethyl-1-phosphate (179).

4.4. Study of alkaline phosphatases from various sources with racemic 1-phenylethyl-1-phosphate (179):

Further investigations were carried out to study the selectivity of the racemic phosphate substrate (179) with the other alkaline phosphatases. The progress of these enzyme reactions were monitored by ^{31}P NMR spectroscopy. The enantiomeric excesses (ee) of the alcohol products were measured at regular intervals by G.C. analysis as described in Chapter 7. The error in measuring the percentage completion of the enzyme reactions using ^{31}P NMR spectroscopy is approxiamtely $\pm 2.0\%$. The standard deviation in determining the enantiomeric ratio of (R) and (S)-1-phenylethanol from the peak areas of the G.C. chromatographs was calculated to be $\pm 0.38\%$. The enantiomeric ratios of the 1-phenylethanol were rounded up to the nearest whole number to give a maximum

deviation of \pm 0.5%. Consequently, the deviation in the ees calculated for the 1-phenylethanol is \pm 1.0%. If the ees obtained had been more significant the standard deviation of the peaks of the G.C. chromatographs would have been examined further. The ee obtained from an individual percentage of completion can be used to calculate an E value. This can be compared with the E values, determined from the kinetic parameters, to ensure consistency.

In order to investigate fully the enantioselectivity of the alkaline phosphatases with the phosphate monoester substrate (179), a wide range of enzyme sources were used. These included samples from mammals, birds, fish and bacteria, which were represented by the respective bovine, rabbit, pigeon, trout and *E.coli* enzyme, and were purchased from Sigma. All the highly purified sources of alkaline phosphatases have been shown to be zinc metalloenzymes existing as dimers of identical monomers. The amino acid sequences of alkaline phosphatase from *E.coli*^{51a}, rat^{151a} and human^{151b} have been determined directly.

It is known that the alkaline phosphatases show a degree of homology in their amino acid sequences (about 25-30%) and that they hydrolyse a wide variety of phosphate monoester derivatives.^{57,58,64} The extremely high degree of homology between the bacterial, bovine intestinal and human placental phosphatases around serine 102, indicates that the basic elements of the reaction mechanism have been conserved. However, there are subtle differences in the sequences of the active site of the various alkaline phosphatases which has been discussed previously in Chapter 3. The enantioselectivities of the various alkaline phosphatases with the racemic 1-phenylethyl-1-phosphate (179) substrate were used to investigate these differences within their active sites. The first part of this work involved evaluating the effect of these changes in amino acid sequences on the reaction

rates of the racemic substrate (179). The latter and more important part of these investigations was to study the implications of these sequence changes within the active site with respect to the enantioselectivity observed.

The binding orientations of the 1-phenylethyl-1-phosphate (179) can be interpreted using **Model 1** which has been described previously in **Chapter 3**. The differences in the catalytic activities of the mammalian and bacterial alkaline phosphatases are due to small changes in the amino acid sequences within their active sites. It is known that lysine 328 in the *E.coli* enzyme is replaced with a histidine residue in the mammalian alkaline phosphatase.^{64,151} Krantrowitz *et.al.*⁶⁴ has suggested that the presence of histidine residues, 153 and 328, increases the K_m values of the substrates of the mammalian phosphatases. This might be important, since the histidines are bound to the catalytic zinc ion centre. The zinc ion at site A is known to play a major role in stabilising the leaving group (ROH),¹⁵¹⁻¹⁵³ and this may affect the enantioselectivity of the enzyme reactions. In *E.coli* the lysine 328 is known to bind the phosphate substrate *via* a water molecule which is involved in a hydrogen bonding network to stabilise the zinc ion. These individual changes in amino acid residues *cannot* be assigned to the different enantioselectivities of the mammalian and bacterial phosphatases. Although the phosphatases only show 25-30% homology throughout their amino acid sequence their active sites are well conserved. The different methods by which the zinc ions are stabilised may make a minor contribution in determining the reactivity and selectivity of the mammalian and bacterial phosphatases. The investigations were extended to include sources of alkaline phosphatase from birds and fish, making it possible to monitor selectivity as a function of evolutionary change.

The selectivity of these enzyme reactions were monitored as they proceed from 10% completion through to 50% completion using 75mM concentrations of the phosphate

substrate at pH 8.0. Additional information correlating the enantioselectivity at a given percentage completion for a particular enzyme to the progress, turnover rate and E value, can be envisaged. From these studies it will be possible to compare the E values calculated from the kinetic parameters with those determined from a single ee measurement at a given percentage of completion. Finally, the alkaline phosphatase with the best enantioselectivity towards the 1-phenylethyl-1-phosphate (179), would be further investigated and the enantiodiscrimination evaluated by altering the reaction conditions. Shown in **Tables 4.6-4.12**, are the results of the reactions of the various alkaline phosphatases with the 1-phenylethyl-1-phosphate (179). The ratios of the (R) and (S)-1-phenylethanol products are expressed as well as the ees obtained and the E values calculated. On pages 147 to 151, graphical representations of this data are presented. These illustrate that as the enzyme reaction proceeds the percentage of (R)-1-phenylethanol product declines and that of the (S)-1-phenylethanol increases. This is evident from the ees determined.

Table 4.6: Bovine Alkaline Phosphatase

	<u>% Completion</u>				
	<u>10</u>	<u>20</u>	<u>35</u>	<u>40</u>	<u>50</u>
Ratio R:S	78:22	76:24	73:27	71:29	70:30
%ee	56	52	46	42	40
E value	3.77	3.59	3.41	3.18	3.38
Reaction Time	25min	---	---	---	2hr 10min

Table 4.7: Rabbit Alkaline Phosphatase

	<u>% Completion</u>				
	<u>10</u>	<u>25</u>	<u>35</u>	<u>40</u>	<u>50</u>
Ratio R:S	80:20	75:25	74:26	73:27	71:29
%ee	60	50	48	46	42
E value	4.27	3.52	3.63	3.60	3.61
Reaction Time	10min	---	---	---	1hr 20min

Table 4.8: E.coli Alkaline Phosphatase

	<u>% Completion</u>				
	<u>10</u>	<u>20</u>	<u>30</u>	<u>40</u>	<u>50</u>
Ratio R:S	68:32	66:34	65:35	63:37	62:38
%ee	36	32	30	26	24
E value	2.21	2.10	2.10	2.00	2.02
Reaction Time	55min	---	---	---	5hr 20min

Table 4.9: Pigeon Alkaline Phosphatase

	<u>% Completion</u>				
	<u>10</u>	<u>25</u>	<u>30</u>	<u>40</u>	<u>50</u>
Ratio R:S	69:31	68:32	67:33	65:35	62:38
%ee	38	36	34	30	24
E value	2.32	2.38	2.33	2.23	2.02
Reaction Time	2hr 40min	---	---	---	12hr 15min

Table 4.10: **Trout Alkaline Phosphatase**

	<u>% Completion</u>			
	<u>10</u>	<u>30</u>	<u>40</u>	<u>50</u>
Ratio R:S	58:42	57:43	55:45	53:47
%ee	16	14	10	6
E value	1.41	1.41	1.30	1.20
Reaction Time	1hr 15min	---	---	6hr 45min

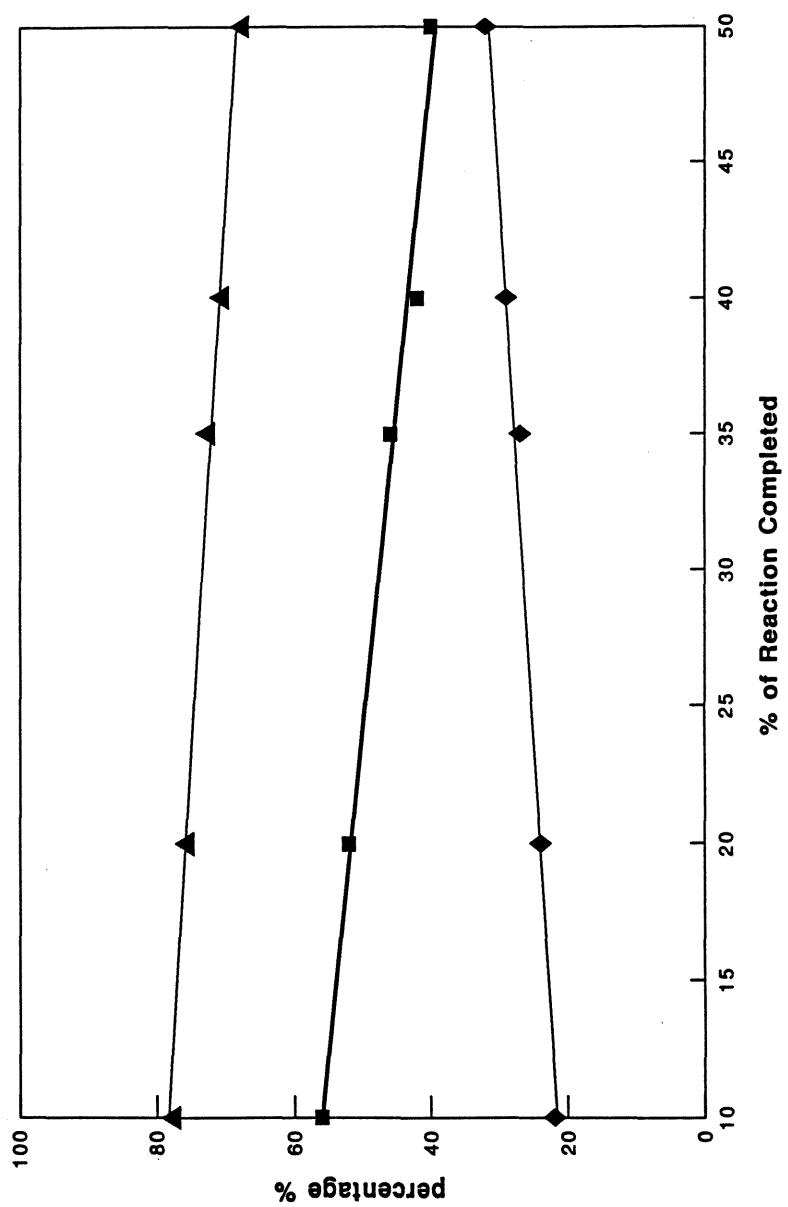
Table 4.11: Enantiomeric Excesses Determined upon 10% Completion of the Enzyme Reactions

<u>Alkaline Phosphatase Source</u>	<u>Ratio R:S</u>	<u>%ee</u>	<u>E value</u>	<u>Specific activity</u> ($\mu\text{mol}/\text{min}/\text{unit}$)
Bovine	78:22	56	3.77	1.2×10^{-5}
Rabbit	80:20	60	4.27	1.5×10^{-4}
<i>E.coli</i>	68:32	36	2.21	1.3×10^{-5}
Pigeon	69:31	38	2.32	2.3×10^{-6}
Trout	58:42	16	1.41	1.6×10^{-5}

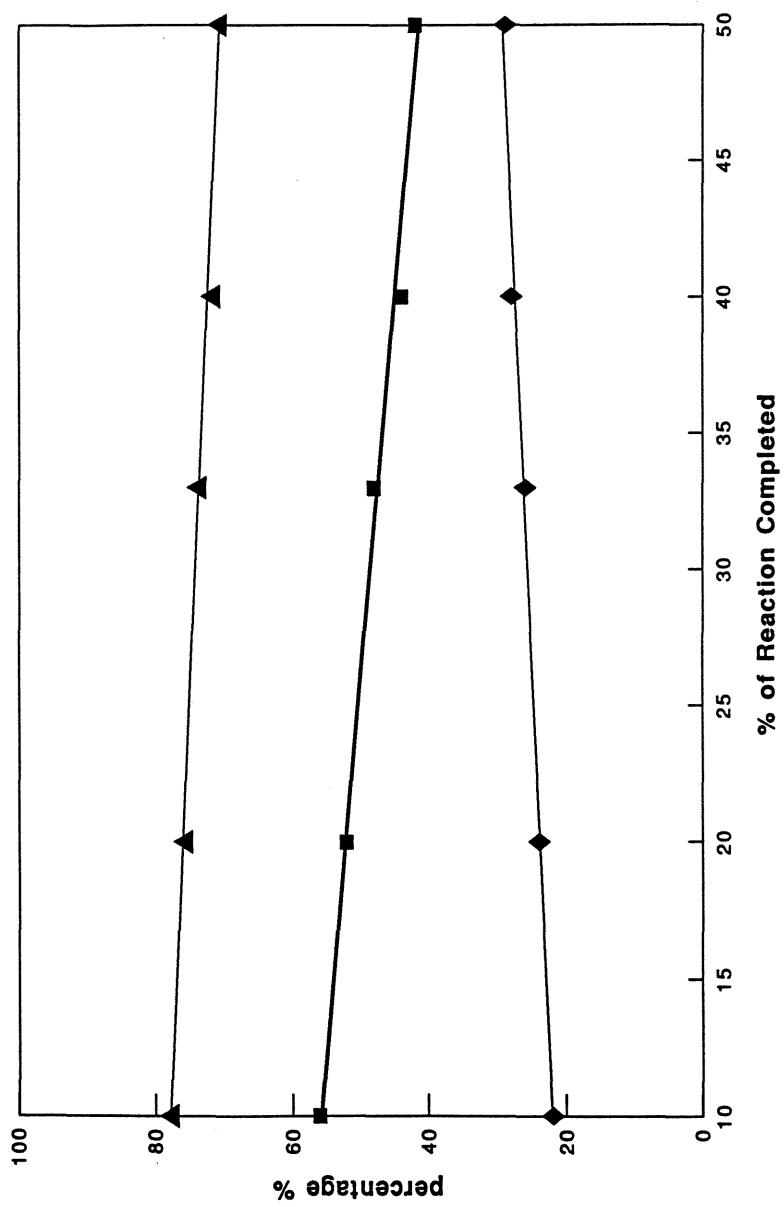
Table 4.12:Enantiomeric Excesses Determined upon 50% Completion of the Enzyme Reactions

<u>Alkaline Phosphatase Source</u>	<u>Ratio R:S</u>	<u>%ee</u>	<u>E value</u>	<u>Specific activity</u> ($\mu\text{mol}/\text{min}/\text{unit}$)
Bovine	70:30	40	3.38	1.1×10^{-5}
Rabbit	71:29	42	3.61	9.4×10^{-5}
<i>E.coli</i>	62:38	24	2.02	1.1×10^{-5}
Pigeon	62:38	24	2.02	2.6×10^{-6}
Trout	53:47	6	1.20	1.5×10^{-5}

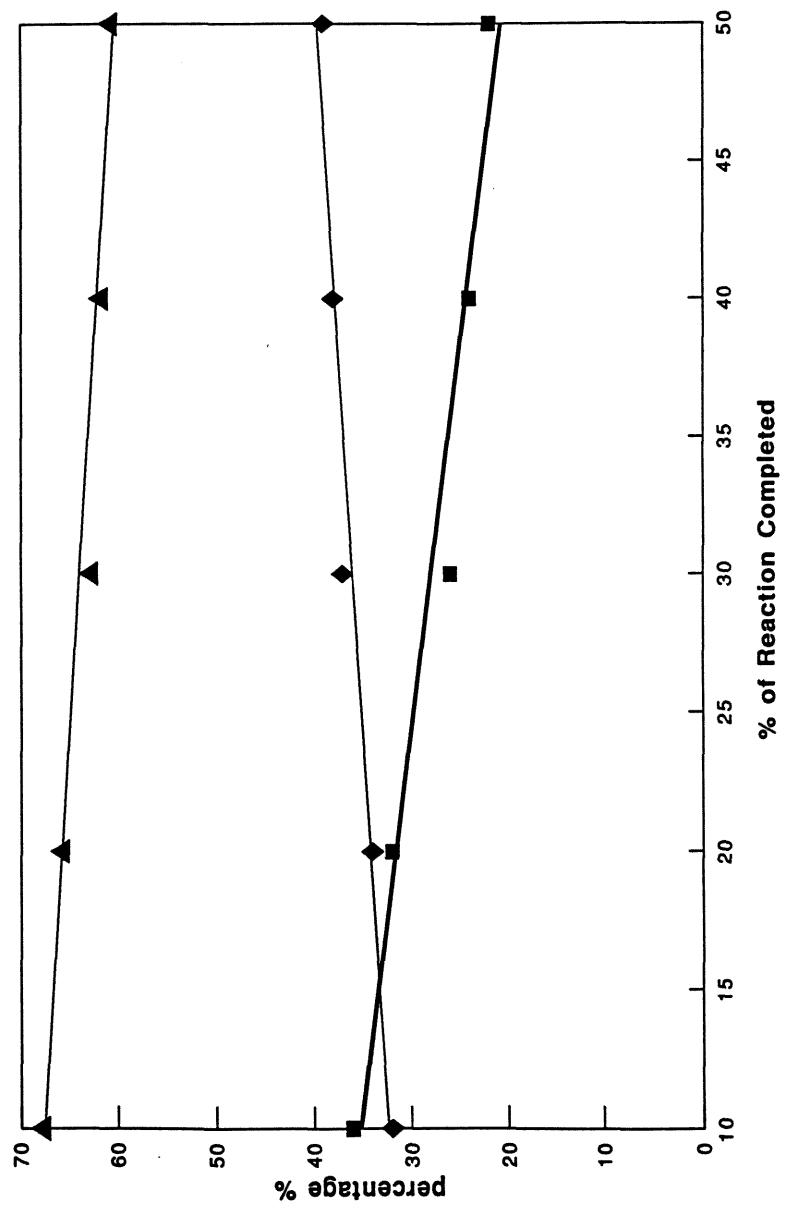
Graph to show the % Enantiomeric Excess obtained for BOVINE Alkaline Phosphatase.
100mM concentration of substrate. R-(+)(▲), S-(-)(◆), % ee (■)



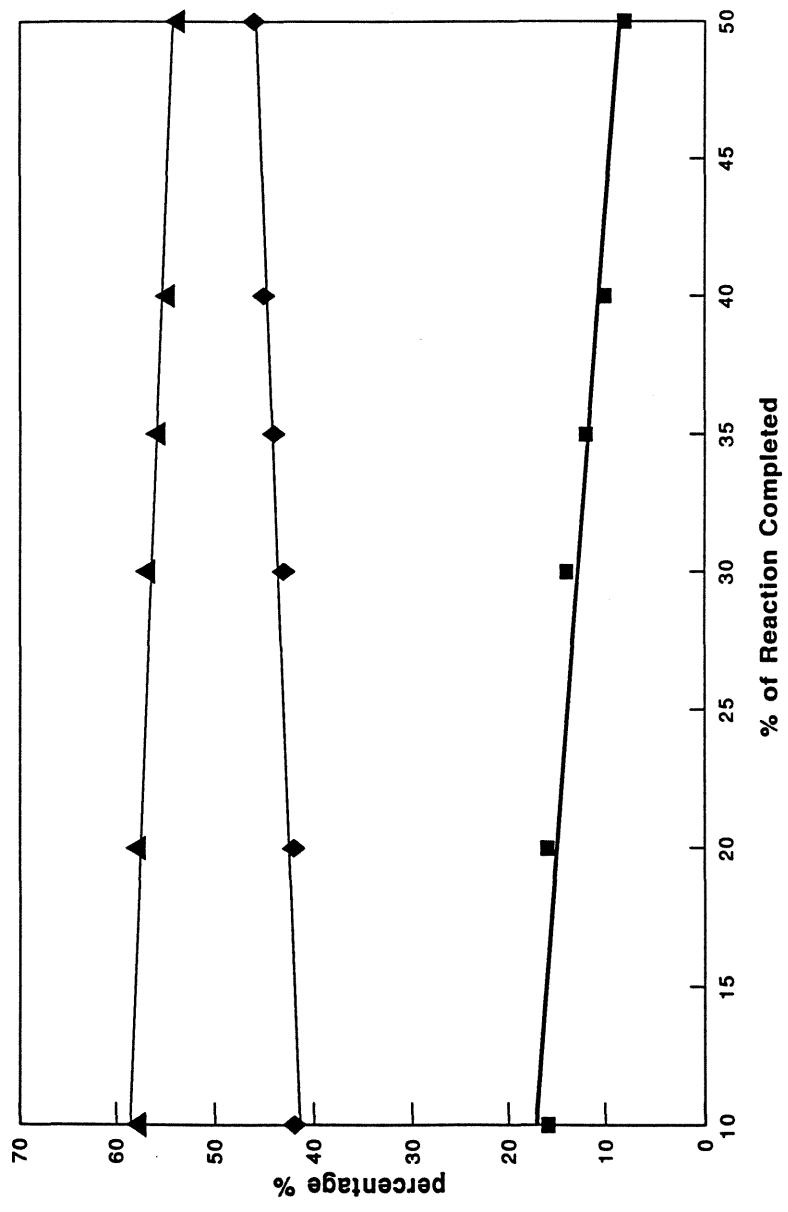
Graph to show the Enantiomeric Excess obtained for RABBIT Alkaline Phosphatase.
100mM concentration of substrate. R-(+)(\blacktriangle), S-(-)(\blacklozenge), % ee (\blacksquare)



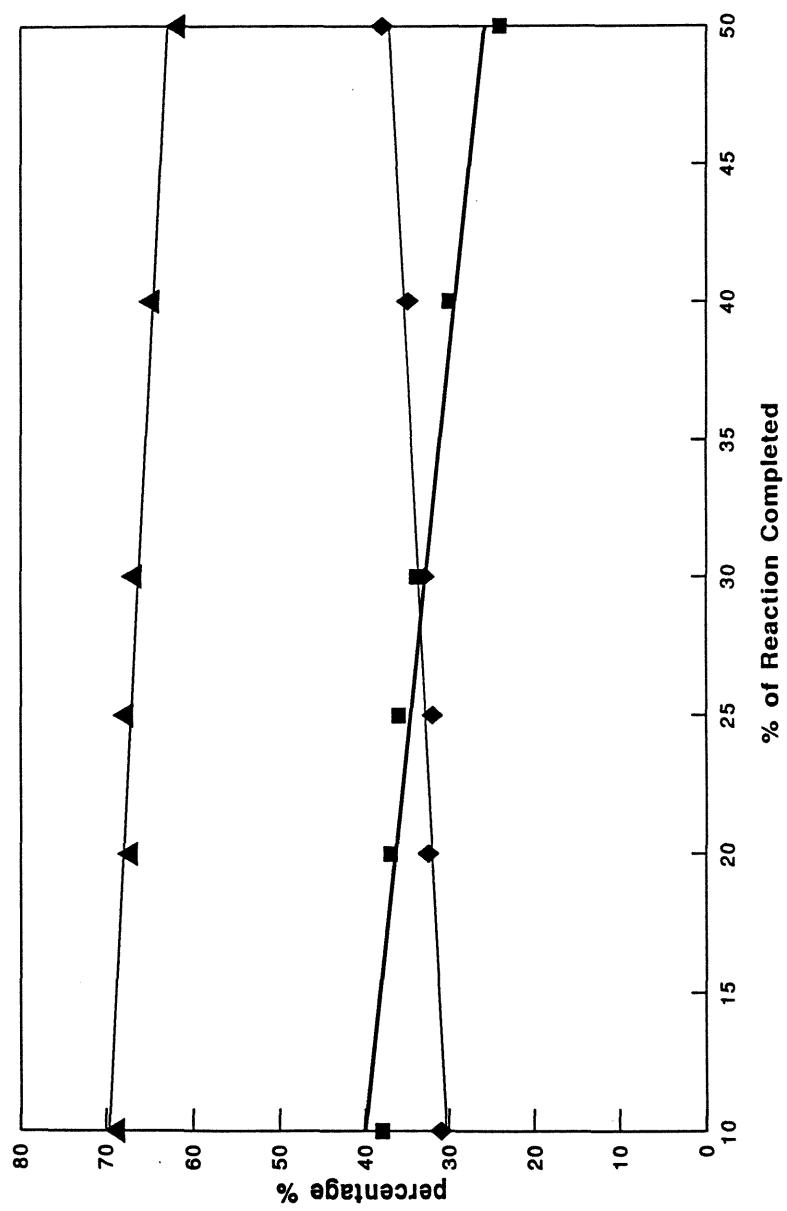
Graph to show the % Enantiomeric Excess obtained for E-COLI Alkaline Phosphatase.
100mM concentration of substrate. R-(+)(Δ), S-(-)(\blacklozenge), % ee (\blacksquare)



Graph to show the % Enantiomeric Excess obtained for TROUT Alkaline Phosphatase.
100mM concentration of substrate. R-(+)(\blacktriangle), S-(-)(\blacklozenge), % ee (\blacksquare)



**Graph to show the % Enantiomeric Excess obtained for PIGEON Alkaline Phosphatase.
100mM concentration of substrate. R-(+)(\blacktriangle), S-(-)(\blacklozenge), % ee (\blacksquare)**



4.4.1. Conclusions:

The plots of enantiomeric excesses determined for the various alkaline phosphatase sources, shows that there is a linear relationship between the extent of reaction and the percentage ee determined during the early stages of catalytic turnover. However it is known from the work of Sih *et.al.*^{2e,26} that as these enzyme reactions proceed beyond 50% completion the relationship becomes hyperbolic. The ee measured decreases as the enzyme reaction progresses. This is also reflected in the percentages of the (R)- and (S)-1-phenylethanol products detected. Any deviation from these linear plots are within acceptable experimental error.

With all of the alkaline phosphatase sources, the (R)-phosphate monoester (**180**) is turned over preferentially. These results suggest that the (R)-enantiomer orientation of **Model 1**, is the preferred binding mode within the active site of all the alkaline phosphatases studied. In this configuration, the more hydrophobic phenyl group is bound to the lipophilic binding site and the methyl group is located in the alternative binding site. It is evident that as the enzyme reaction progresses the ee of the (R) alcohol product gradually decreases. This is not unexpected since the enzyme observes a relative increase in the concentration of the (S)-phosphate substrate (**181**) with respect to the (R)-phosphate monoester (**180**). Consequently, more of the (S)-phosphate monoester (**181**) is turned over by the phosphatases, thereby reducing the ee. The enantioselectivity of biocatalytic reactions is often expressed as an enantioselectivity value (E)^{2e,146} An overall decline in E values is observed as the reaction proceed from 10% completion until the reactions are terminated after 50% completion. The decline in E values implies that the reaction is reversible. Reactions that are irreversible have E values that remain constant as the reaction progresses.²⁸ It is clear from the ees and E values that the reactions of the various

alkaline phosphatases show only modest enantioselectivity with the racemic phenylethyl-1-phosphate (179). After 10% completion, the highest E values obtained were for the mammalian enzymes and were in the region of 4.27-3.77. The lowest E value at 10% completion was 1.41 for the trout enzyme. All of these values continue to decrease as the reactions progress. These E values are uniformly lower than the reported values of the lipases.^{2e,26,146} In order to attain high ees of product with low E values, the product must be recycled two or more times.²⁶ This is a serious obstacle for the phosphatase reactions, since the phosphate substrates must be prepared by phosphorylating the enantiomerically enriched alcohol products. Shown in **Table 4.13**, is a correlation of the E values calculated from the K_m and V_{max} constants for the individual (R)-(180) and (S)-(181) phosphate enantiomers and those determined from an ee derived from a single percentage of completion.

Table 4.13:

<u>Alkaline phosphatase</u>	<u>Calculated from K_m/V_{max}</u>	<u>Enantioselectivity value (E)</u>	<u>% Completion Determination</u>
<i>E.coli</i>	2.18		2.20-2.10
Bovine	3.12		3.77-3.30

The results in **Table 4.13** illustrate that there is high degree of consistency in the E values calculated using the two methods. This verifies that the E values determined for the individual phosphate enantiomers (R)-(180) and (S)-(181) are valid for all the enantiomeric excesses measured at any single percentage completion. At 50% completion, the mammalian enzymes, bovine and rabbit have the highest ees of 56% and 60% respectively. This is followed by the pigeon phosphatase which achieves an ee of 38%.

The trout alkaline phosphatase has a low ee of 16%. However, the *E.coli* phosphatase fall between the pigeon and trout enzymes with a relatively high ee of 36% at 50% completion. The results show that bovine, trout and *E.coli* alkaline phosphatase have very similar specific activities for the 1-phenylethyl-1-phosphate substrate. The rabbit enzyme appears to turnover (179) eight times faster than this, with a specific activity of $1.2 \times 10^4 \mu\text{mol}/\text{min}/\text{unit}$; whereas, the specific activity of the pigeon alkaline phosphatase is four times smaller.

The mammalian enzymes show far greater selectivity with the 1-phenylethyl-1-phosphate substrate (179) at 10% completion through to 50% completion compared to the *E.coli* alkaline phosphatase. These enzymes are known to stabilise the zinc ion by different methods. The *E.coli* enzyme stabilises this ion centre through a hydrogen bonding pattern involving lysine 328 and phosphate substrate.^{64a} In contrast, the mammalian phosphatases stabilise the catalytic zinc ion centre through direct binding to histidine 153.^{64b} A greater selectivity of the (R)-phosphate monoester (180) is seen with the mammalian phosphatases compared to the *E.coli* enzyme. There is no evidence from these experiments to suggest that the different enantioselectivities of the mammalian and bacterial enzymes is due to the different ways they stabilise the zinc ion centre.

The moderate selectivity common to all the alkaline phosphatases utilised in this study, suggests that the enzymes are a result of recent evolutionary divergence. The slight variations in the amino acid sequences of the active sites play only a minor role in affecting the enantioselectivity. The observation that the (R)-phosphate monoester (180) is the preferred substrate indicates that the basic elements of reaction have been conserved from bacterial to mammalian sources. This is in agreement with the work of Coleman *et.al.*^{44b} The results suggests that the enzymes derived from the more complex species may

have been exposed to a wider variety of phosphate substrates, and so are able to handle the altered secondary substrate 1-phenylethyl-1-phosphate (179). This is evident from the higher selectivity and reactivity detected with the mammalian phosphatases. Clearly the reactions of the bovine phosphatase with the 1-phenylethyl-1-phosphate (179) hold the greatest promise. This system needs to be investigated further to see if the enantioselectivity can be improved. Studies concerned with altering the reaction conditions and their implications are discussed in Chapter 5.

Chapter 5

***Studies of Bovine Alkaline Phosphatase with (R,S)-1-Phenylethyl-1-
Phosphate under Various Reaction Conditions.***

5.1. Introduction:

The results from Section 4.4., show that the bovine alkaline phosphatase produced the best enantiomeric excess when reacted with the 1-phenylethyl-1-phosphate (179). In terms of Model 1, the enantioselectivity may reflect the location of either the methyl or phenyl groups in the hydrophobic binding site. Assuming that the phosphoryl group and hydrogen atom for both enantiomers occupy similar regions in the active site, for one enantiomer, the phenyl moiety will occupy the putative lipophilic site; whereas in the other, the methyl group will occupy this site. Further investigations were undertaken to see if the selectivity of the bovine enzyme with the 1-phenylethyl-1-phosphate (179) could be improved. These investigations involved varying the reaction conditions of the enzyme assays; for example, changes in pH, which are known to be important in formation of the Michaelis complex (E· ROP).^{44b,56,152} Changes were made to the buffer solutions by adding organic solvents, and by altering the concentrations of the essential zinc and magnesium ions.^{57a,58,64} Varying the concentrations of NaCl in the solutions were investigated to study the effect of ionic strength.¹⁵³ In addition to attempting to improve the enantiomeric excesses observed, these investigations would provide further insight into the nature of the binding regions within the active site. The final investigations described in this chapter, concern the selectivity of the phosphatases from mammalian and bacterial sources with the 1-(*para*-nitrophenyl)ethyl-1-phosphate (189) and 1-(*para*-methoxyphenyl)ethyl-1-phosphate (190) substrates.

5.2. pH changes:

The original bovine phosphatase experiments were carried out at pH 8.0, close to the pH-rate maximum of 7.5, for mammalian alkaline phosphatase.¹⁴⁵ The pH of the buffer solution was changed to 7.0, 9.0, 10.0 and 11.0. The selectivity of the bovine phosphatase with the 1-phenylethyl-1-phosphate (179), under these various pH conditions was monitored. Neutral and alkaline pH conditions were chosen, as in this range the rate-limiting step is the same as that at pH=8.0. At pH values above 7.0, the rate-determining step is the release of phosphate from the non-covalent (E· P) complex;^{44b,154} whereas at acid pH, the rate-limiting step is the hydrolysis of the phosphoserine intermediate (E-P).^{44b} The purpose of this was to see if changes in the pH would affect the ee's observed. It may be that a residue in the active site, or on the surface of the protein, has an ionisable group within this pH range. This may differentially affect the binding of the two phosphate enantiomers, resulting in improved selectivity. The results of these experiments are shown in Tables 5.1-5.5. The extent of reaction was monitored by ³¹P NMR spectroscopy. The enantiomeric ratios of the (R)- and (S)-1-phenylethanols were determined by G.C. The standard deviation in measuring the ratio of the peaks was calculated to be \pm 0.4%. The ratios were rounded up to the nearest whole number to give a maximum deviation of \pm 0.5%. Consequently, the deviation in %ee determined for the 1-phenylethanol products is \pm 1.0%.

Table 5.1: Buffer Solution pH 7.0

	<u>% Completion</u>			
	<u>10</u>	<u>20</u>	<u>30</u>	<u>50</u>
Ratio R:S	73:27	72:28	70:30	66:34
%ee	46	44	40	32
E value	2.85	2.85	2.75	2.60
Reaction Time	---	---	---	2hr

Table 5.2: Buffer Solution pH 9.0

	<u>% Completion</u>			
	<u>10</u>	<u>20</u>	<u>30</u>	<u>50</u>
Ratio R:S	72:28	71:29	70:30	67:33
%ee	44	42	40	34
E value	2.70	2.70	2.75	2.75
Reaction Time	---	---	---	1hr 40min

Table 5.3: Buffer Solution pH 10.0

	<u>% Completion</u>				
	<u>10</u>	<u>20</u>	<u>30</u>	<u>40</u>	<u>50</u>
Ratio R:S	71:29	69:31	67:33	66:34	65:35
%ee	42	38	36	32	30
E value	2.51	2.41	2.30	2.35	2.44
Reaction Time	---	---	---	---	1hr 15min

Table 5.4: **Buffer Solution pH 11.0**

	<u>% Completion</u>				
	<u>10</u>	<u>20</u>	<u>30</u>	<u>40</u>	<u>50</u>
Ratio R:S	72:28	71:29	70:30	69:31	67:33
%ee	44	42	40	38	34
E value	2.71	2.71	2.75	2.80	2.76
Reaction Time	---	---	---	---	1hr 10min

Table 5.5:**Enantiomeric Excesses Determined for the Enzyme Reactions at Various pH Values.**

<u>10% Completion</u>	<u>pH 7.0</u>	<u>pH 8.0</u>	<u>pH 9.0</u>	<u>pH 10.0</u>	<u>pH 11.0</u>
Ratio R:S	73:27	75:25	72:28	70:30	72:28
%ee	46	56	44	40	44
E value	2.85	3.17	2.71	2.41	2.71
<u>50% Completion</u>					
Ratio R:S	66:34	72:28	67:33	65:35	67:33
%ee	32	44	34	30	34
E Value	2.60	3.32	2.76	2.44	2.76
Reaction Time	2hr	2hr 20min	1hr 40min	1hr 15min	1hr 10min

The results in **Tables 5.1-5.5** show that the enantioselectivity of the bovine phosphatase with the 1-phenylethyl-1-phosphate (**179**) under the various pH conditions, is reduced compared to the selectivity seen under the standard conditions at pH 8.0. At 10% completion, under standard conditions, the ee observed is 56% and this falls to 44%

at 50% completion of the reaction. The E values calculated for the various pH solutions at 10% completion, never rise above 3.0 and are consistent in the range 2.8-2.4 until the reaction is terminated at 50% completion. At pH 10.0 and 11.0, the rate of turnover of substrate (179) is faster than under standard conditions; achieving 50% completion in two thirds of the time. The enzyme reactions at pH 7.0 and 9.0 have the same reaction rates. Under these altered pH conditions, the enantiomeric excesses detected are within experimental error, the same for a given percentage of completion. These results suggest that the changes in pH have little effect on the environment within the binding site. It has been reported by Coleman et.al,^{44b} that the hydroxide ions of alkaline solutions bind to the zinc centres of the active pocket. The binding of these ions competes with the binding of the alcoholic group of the substrate. The result of this is to reduce the binding of the substrate molecule which is reflected in the larger K_m values reported at high pH values.^{44b} It appears that the pH changes affect the two diastereomeric enzyme-substrate complexes to the same extent. The result of this is that the turnover of the (R)- and (S)-enantiomers is the same. As the pH is increased, the rate of turnover of the 1-phenylethyl-1-phosphate substrate (179) increases, reaching a maximum at pH 11.0. This is remarkable since the natural substrate shows a rate-maximum at pH 8.0. There is no explanation for this and it appears that the pH-rate increase merely increases the reaction rate of both enantiomers.

5.2.1. Changes in zinc and magnesium ion concentration:

It is known that the alkaline phosphatases contain two zinc ions and one magnesium ion per monomer.^{51a,68,153} The catalytic zinc ions are known to play an important role in activating the serine 102 residue involved in binding the phosphate moiety, and in stabilising the water molecules involved in the nucleophilic attack of the covalent phosphoserine complex (E-P).^{53a,155} The magnesium ion is involved in providing

a conformationally stabilised enzyme structure and an electrostatic environment around the active site.^{145b} Because these functional metal ions are found in the active site, they have the potential to affect the enantioselectivity of the bovine phosphatase in their reactions with the 1-phenylethyl-1-phosphate substrate (**179**). The reactions of the bovine phosphatase with the monophosphate ester (**179**), were studied by increasing the concentrations of zinc acetate and magnesium acetate in the buffer solutions. Each metal ion was increased 50 fold above its original concentration. It was hoped that the increased magnesium ion concentration would provide improved stability, inducing conformational constraints within the active site which has been suggested by Wilson *et.al.*¹⁵⁶

The zinc ion concentrations were altered as they play a vital role in stabilising the non-covalent Michaelis complex (E· ROP), in which the departing alcohol group is coordinated to the zinc ion centre (site A).^{44b} Changes to the zinc ion levels may affect the loss of the alcohol group, thereby influencing the selectivity of the bovine phosphatase. The results of these experiments of altering the concentrations of zinc and magnesium ions in the buffer solutions, are shown in **Tables 5.6-5.8**.

Table 5.6:

Buffer Solution with 50 fold Increase in Zinc Ion Concentration.

	<u>% Completion</u>			
	<u>10</u>	<u>20</u>	<u>40</u>	<u>50</u>
Ratio R:S	71:29	70:30	68:32	66:34
%ee	42	40	36	32
E value	2.56	2.56	2.65	2.60
Reaction Time	10min	---	---	55min

Table 5.7:**Buffer Solution with 50 fold Increase in Magnesium Ion Concentration.**

	<u>% Completion</u>			
	<u>10</u>	<u>20</u>	<u>35</u>	<u>50</u>
Ratio R:S	71:29	70:30	66:34	65:35
%ee	42	40	32	30
E value	2.56	2.56	2.28	2.44
Reaction Time	15min	---	---	1hr 40min

Table 5.8:**Enantiomeric Excesses Determined for the Enzyme Reactions with Increased Concentrations of Zinc and Magnesium Ions.****10% Completion**

	<u>Zn(OAc)₂, 187 μmol</u>	<u>Mg(OAc)₂, 18.7 mmol</u>
Ratio R:S	71:29	71:29
%ee	42	42
E value	2.56	2.56
Reaction Time	10min	15min

50% Completion

Ratio R:S	66:34	65:35
%ee	32	30
E Value	2.60	2.44
Reaction Time	55min	1hr 40min

The 50 fold increase in zinc ion concentration has a dramatic effect on the enzyme's reaction rate with the 1-phenylethyl-1-phosphate (179). The rate of reaction is seen to be halved and 50% completion is achieved in only 55 minutes. However, accompanying this increased reaction rate is a slight reduction in selectivity. The 42% ee observed after 10% completion is lower than the 56% ee seen under the standard conditions. After 50% completion, these ee values are lower than those determined under standard conditions. The binding of the (R) and (S) enantiomers of 1-phenylethyl-1-phosphate (179) are similar and this is reflected in the enantiomeric excesses determined under these different conditions.

After 50% completion, the enantiomeric excess detected for the 50 fold increase in magnesium ion concentration is 30%. This is lower than that seen under standard conditions which is 44% ee. The results obtained are the same as those seen with a 50 fold increase in zinc ion concentration. Like the zinc ions, the magnesium ions appear to have little effect on the conformational constraints of the active site, suggested by Wilson *et.al.*¹⁵⁶ The increase in magnesium ions, appears to affect the diastereomeric enzyme-substrate complexes of the two enantiomers to the same extent. Consequently, their turnover rates are the same and no enhanced selectivity is observed.

5.2.2. Changes in sodium chloride concentration:

In order to investigate the affect of ionic strength on the selectivity of the bovine phosphatases with the 1-phenylethyl-1-phosphate substrate (179), various concentrations of NaCl were added to the standard buffer solution. Sodium chloride has previously been used to probe the hydrophobic binding sites of various enzyme systems. The addition of NaCl may enhance the hydrophobic effects. This may accentuate the differences in binding of the two enantiomers, resulting in increased ee's observed. The results of these

experiments, using 10mM, 100mM, 300mM and 500mM concentrations of sodium chloride are shown in **Tables 5.9-5.13**.

Table 5.9: Buffer Solution with 10mM NaCl.

	<u>% Completion</u>			
	<u>10</u>	<u>20</u>	<u>35</u>	<u>50</u>
Ratio R:S	73:27	72:28	69:31	67:33
%ee	46	44	38	34
E value	2.86	2.85	2.70	2.76
Reaction Time	18min	---	---	1hr 55min

Table 5.10: Buffer Solution with 100mM NaCl.

	<u>% Completion</u>			
	<u>10</u>	<u>25</u>	<u>35</u>	<u>50</u>
Ratio R:S	72:28	71:29	70:30	68:32
%ee	44	42	40	36
E value	2.71	2.70	2.85	2.95
Reaction Time	15min	---	---	1hr 50min

Table 5.11: Buffer Solution with 300mM NaCl

	<u>% Completion</u>				
	<u>10</u>	<u>20</u>	<u>30</u>	<u>40</u>	<u>50</u>
Ratio R:S	72:28	71:29	70:30	69:31	68:32
%ee	44	42	40	38	36
E value	2.71	2.70	2.75	2.80	2.95
Reaction Time	20min	---	---	---	1hr 48min

Table 5.12: Buffer Solution with 500mM NaCl

	<u>% Completion</u>				
	<u>10</u>	<u>20</u>	<u>30</u>	<u>40</u>	<u>50</u>
Ratio R:S	73:27	72:28	70:30	68:32	66:34
%ee	46	44	40	36	32
E value	2.86	2.86	2.75	2.66	2.60
Reaction Time	22min	---	---	---	1hr 45min

Table 5.13:

Enantiomeric Excesses Determined for the Enzyme Reactions at Various NaCl Concentrations.

10% Completion

	<u>10mM</u>	<u>100mM</u>	<u>300mM</u>	<u>500mM</u>
Ratio R:S	73:27	72:28	72:28	73:27
%ee	46	44	44	46
E value	2.86	2.71	2.71	2.86
Reaction Time	18min	15min	20min	22min

50% Completion

Ratio R:S	67:33	68:32	68:32	66:34
%ee	34	36	36	32
E Value	2.76	2.95	2.95	2.60
Reaction Time	1hr 55min	1hr 50min	1hr 48min	1hr 45min

The results in **Tables 5.9-5.13** illustrate that the addition of sodium chloride to the buffer solutions does not improve the selectivity of the bovine enzyme with the 1-phenylethyl-1-phosphate (179). After 10% completion, 46% ee was seen, which

decreased to 36% after 50% completion of the reaction. For all of the sodium chloride concentrations used, the ee's observed, within experimental error, were the same for a given percentage of completion. The addition of sodium chloride to the buffer solutions has no effect on the reactivity and selectivity of the bovine alkaline phosphatase. The NaCl solutions have little effect on the interactions of the binding sites with the 1-phenylethyl-1-phosphate (179). Higher concentrations of NaCl, have no effect on selectivity, indicating that the chloride ions do not displace the water molecules of the active site as had been anticipated. Coleman and Gettins,¹⁵⁷ have reported that some chloride ions can occupy space within the active site and aid in loss of inorganic phosphate from the non-covalent complex (E· P). They have proposed that the chloride ions can do this *via* a 5 coordinate zinc form, involving the phosphate group, zinc ion and chloride ions. The negatively charged chloride ion adjacent to the phosphate group, is coordinated to the zinc ion (A site) in the (E· P) complex enabling phosphate dissociation.¹⁵⁷ This may explain why further increases in the sodium chloride concentration have no effect on the reaction rate or selectivity, as only a certain number of chloride ions can occupy the active site as essential ligands to enhance the enzyme's activity. With little displacement of the water molecules by the chloride ions, the hydrophilic nature of the active site, remains intact. The result of this is that the reactivity of the two diastereomeric enzyme-substrate complexes are affected to a similar extent by the NaCl concentrations.

5.2.3. Changes in solvent conditions:

It is well known that the selectivity of a biocatalytic reaction can be dramatically affected by changing the solvent in which the reaction is carried out. The lipases have been shown to be uniquely stable in non-polar organic solvents and this has a dramatic

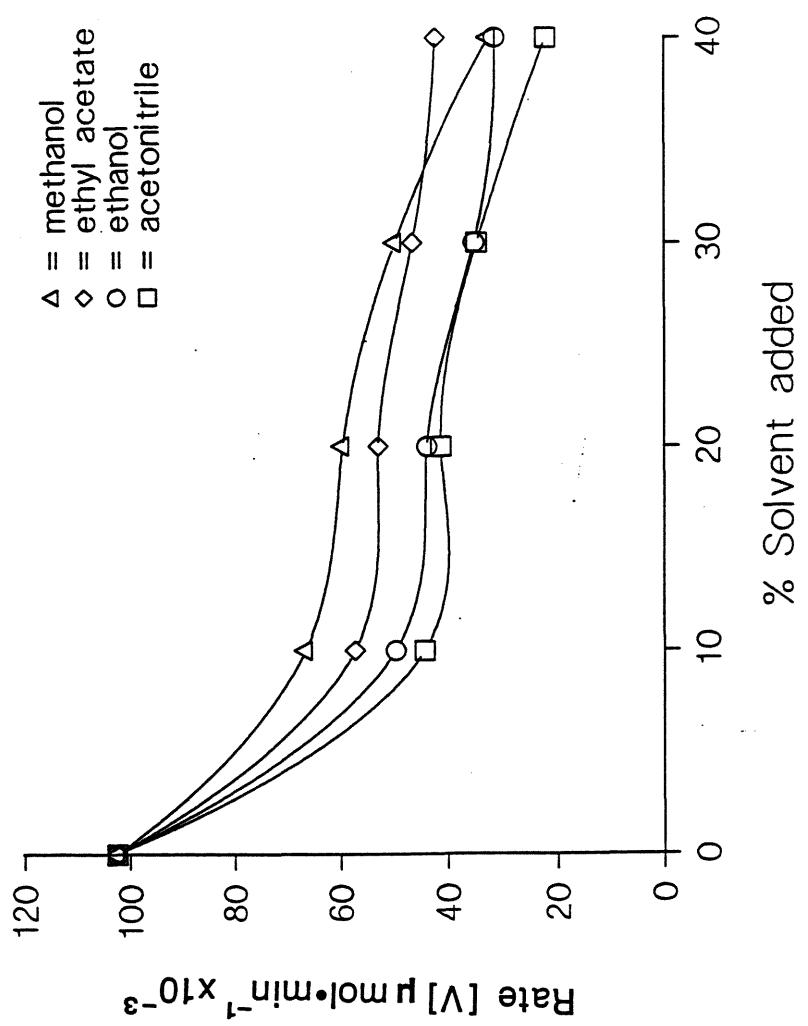
affect on their reactivity and selectivity.^{28b,152} The potential advantages of conducting enzyme reactions in organic solvents are that; reactions catalysed by hydrolytic enzymes may be shifted towards the synthetic direction, undesirable side reactions and product inhibition can be reduced and the enzymes could be more stable in anhydrous organic media.²⁷ When water is replaced with an apolar solvent, the interior hydrophobic residues tend to disperse resulting in a reorganization of the enzymes tertiary structure. The conformation of the enzyme is altered and consequently the catalytic behaviour of the enzyme should be altered with respect to the enzyme in aqueous media. It has become apparent that many biocatalytic reactions in non-aqueous media proceed with improved selectivity over those carried out in aqueous solutions.¹⁵²

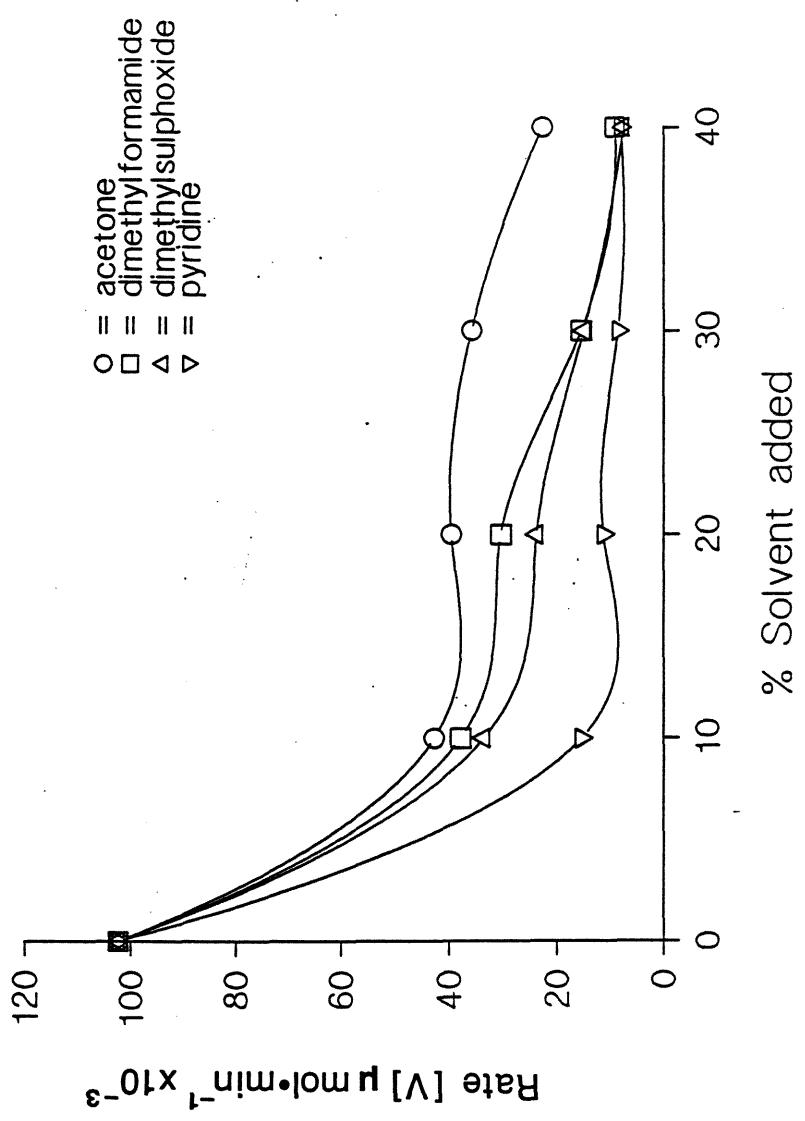
In most of the examples known, proteases and lipases have been used in apolar organic solvents, such as cyclohexane, toluene and diethyl ether.^{28,152} In general, these hydrophobic solvents are preferable to the polar hydrophilic solvents, since the catalytic efficiency of these enzymes is known to diminish as the polarity of the solvent is increased.^{28a,151} Hydrophilic solvents can denature enzymes by penetrating into the hydrophobic core of proteins and disrupting their structure.

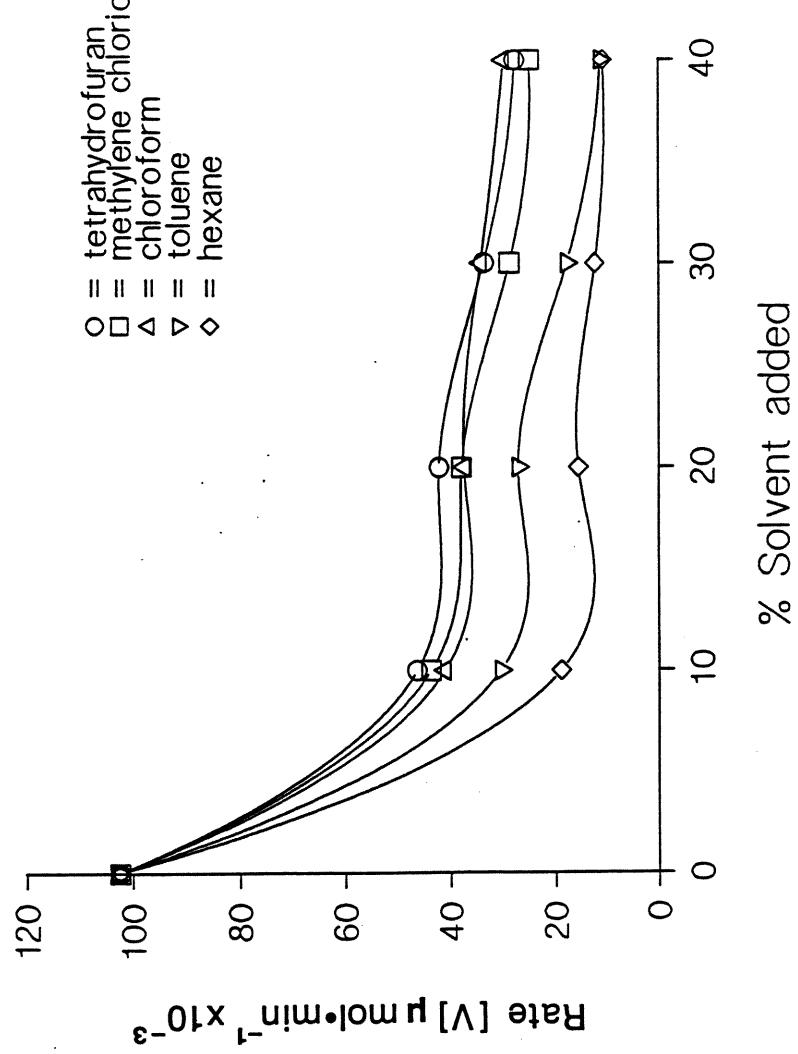
An initial study was carried out using the spectrophotometric assay reaction of *para*-nitrophenyl phosphate ($500\mu\text{M}$) with bovine alkaline phosphatase ($20\mu\text{l}$, 0.5units) in various concentrations of water solvent mixtures. The results of these experiments are shown in Figures 5.1-5.3. These plots illustrate the relationship of the enzymes activity (rate $\mu\text{mol}/\text{min}$) with the percentage of organic solvent present in each assay mixture. The results show that solutions containing only 10% organic solvent severely reduce the activity of bovine alkaline phosphatase with the *para*-nitrophenyl phosphate. This is illustrated by the addition of acetone to the enzyme assay, in which the rate of turnover

was reduced by half to $4.3 \times 10^{-2} \mu\text{mol}/\text{min}$, compared to an initial reaction rate of $10.2 \times 10^{-2} \mu\text{mol}/\text{min}$ seen under standard conditions. The chlorinated and apolar solvents including, chloroform, dichloromethane, toluene and hexane caused a significant reduction in activity of the bovine phosphatase to reaction rates of between $4.2-2.0 \times 10^{-2} \mu\text{mol}/\text{min}$. The highly polar solvents, dimethyl sulphoxide and dimethyl formamide, also caused the rates to fall to around $4.0 \times 10^{-2} \mu\text{mol}/\text{min}$. The moderately polar solvents; acetone, acetonitrile and tetrahydrofuran, also showed reduced turnover rates in this region of around $4.2-3.5 \times 10^{-2} \mu\text{mol}/\text{min}$. The rate of reaction in the pyridine-water solution was greatly reduced to a rate of $1.5 \times 10^{-2} \mu\text{mol}/\text{min}$ at 10% solvent volume; this continued to fall rapidly as additional pyridine was added. Clearly, pyridine inhibits the bovine phosphatase or denatures the protein. This is not unusual as it has been reported by Van Belle¹⁵⁸ that cyclic amines can inhibit the activity of the alkaline phosphatases. The solvents found to cause the least inactivation of the enzyme were methanol, ethanol and ethyl acetate. Their activity at 10% addition of the solvent was in the region of $6.7-5.0 \times 10^{-2} \mu\text{mol}/\text{min}$.

These differences in the reactivity of the phosphatases and the lipases in organic solvents are explained by the fact that most mammalian phosphatases are extracellular enzymes located on the cell surface^{44b,82a,145b} and the brush borders of the intestinal tract, regions which are highly hydrophilic in nature.^{44b} These differ from the lipases and esterases which are intracellular, membrane bound enzymes, exposed to a hydrophobic environment. The monophosphate substrates are readily soluble in water and the use of organic co-solvents has been shown to affect their solubilities and rates of solvolysis.¹⁵⁹ Kirby and Varvoglou have reported that the percentage and rate of solvolysis are important in determining the reaction rate of the phosphatases.¹⁵⁹







Further investigations were carried out to see whether the selectivity of the phosphatases could be improved using some of these organic solvents in the buffer solutions. The results of the initial investigations of the reactivity of bovine alkaline phosphatases with the various organic solvents produced useful information. The selectivity of bovine alkaline phosphatase with 1-phenylethyl-1-phosphate (179) was studied using buffer solutions containing 10% volumes of MeOH, EtOH, EtOAc and MeCN. The MeOH, EtOH and EtOAc were chosen since at 10% composition the enzyme maintained a reasonable amount of activity. Acetonitrile was selected from the remaining solvents that showed activity in the range $4.5\text{--}4.0 \times 10^{-2}$ $\mu\text{mol}/\text{min}$ at 10% composition. Higher solvent concentrations were not used as these progressively lead to protein denaturation and loss of enzyme activity.

The purpose of this was to see if changes in the solvent conditions would affect the enantioselectivity observed. The addition of the moderately hydrophobic acetonitrile and ethyl acetate to the buffer solutions may affect the aqueous environment of the active site. It was anticipated that these solvents would increase the hydrophobic effects of the binding region. The result of this would be to differentially affect the binding of the two enantiomers. Concentrations of the polar solvents (10%), ethanol and methanol, were used since alkaline phosphatase maintains a reasonable activity in these solutions. Being alcohols, both of these solvents act as acceptors of the phosphate leaving group. Therefore, they may be present in the active site, thereby affecting the enantioselectivity seen with the phosphate substrate (179). The results of these experiments are shown in **Tables 5.14-5.18**.

Table 5.14: Buffer Solution with 10% EtOAc

	<u>% Completion</u>				
	<u>10</u>	<u>20</u>	<u>30</u>	<u>40</u>	<u>50</u>
Ratio R:S	72:28	71:29	70:30	68:32	66:34
%ee	44	42	40	36	32
E value	2.71	2.70	2.75	2.66	2.60
Reaction Time	24min	---	---	---	2hr 25min

Table 5.15: Buffer Solution with 10% MeCN

	<u>% Completion</u>				
	<u>10</u>	<u>20</u>	<u>30</u>	<u>40</u>	<u>50</u>
Ratio R:S	73:27	72:28	70:30	68:32	66:34
%ee	46	44	40	36	32
E value	2.86	2.85	2.75	2.66	2.60
Reaction Time	35min	---	---	---	4hr 10min

Table 5.16: Buffer Solution with 10% MeOH

	<u>% Completion</u>			
	<u>10</u>	<u>20</u>	<u>40</u>	<u>50</u>
Ratio R:S	72:28	70:30	68:32	65:35
%ee	44	40	36	30
E value	2.71	2.56	2.66	2.44
Reaction Time	15min	---	---	1hr 50min

Table 5.17: **Buffer Solution with 10% EtOH**

	<u>% Completion</u>			
	<u>10</u>	<u>20</u>	<u>35</u>	<u>50</u>
Ratio R:S	72:28	71:29	70:30	68:32
% ee	44	42	40	36
E value	2.71	2.70	2.85	2.95
Reaction Time	15min	---	---	1hr 40min

Table 5.18:**Enantiomeric Excesses Determined for the Enzyme Reactions Carried out in Various Organic Solvents (10%).****10% Completion**

	<u>MeCN</u>	<u>EtOH</u>	<u>MeOH</u>	<u>EtOAc</u>
Ratio R:S	73:27	72:28	72:28	72:28
% ee	46	44	44	44
E value	2.86	2.71	2.71	2.71
Reaction Time	35min	15min	15min	24min

50% Completion

Ratio R:S	66:34	68:32	65:35	66:34
% ee	32	36	30	32
E Value	2.60	2.95	2.44	2.60
Reaction Time	4hr 10min	1hr 40min	1hr 50min	2hr 25min

The addition of organic co-solvent has an effect on the enantioselectivities observed. At 50% completion, the observed ee's are around 34%, lower than the 44% seen under standard conditions. They also lead to a reduction in the rate of turnover. This can arise either as a result of denaturation, or the effect of organic co-solvent on the K_m for the substrate. For any given percentage of completion, the observed ee's are the same, within experimental error, for the various co-solvents. Solvent polarity can be quantitatively measured in terms of a partition coefficient, P .¹⁶⁰ This function has been used by Sih,^{28a} to explain the reactivity of the lipases in various mixed solvent systems. The logarithmic partition coefficient, P , values for acetonitrile and ethyl acetate are shown below in Table 5.19.

Table 5.19:	<u>solvent</u>	<u>logP</u>
	ethyl acetate	0.68
	acetonitrile	-0.33

Table of values reported by Sih and coworkers.^{28a}

It has been reported that catalytic activity is reduced with increased solvent polarity.^{28a} The parameters shown in Table 5.19, illustrate that acetonitrile is more polar than ethyl acetate and has a lower logP value. Consistent with this it has been observed that the reaction rate of the bovine alkaline phosphatase in 10% acetonitrile is slower than in the 10% ethyl acetate solution.

The addition of methanol and ethanol to the buffer solutions does not enhance the phosphatase's selectivity. The addition of these solvents increases the rate of turnover of the 1-phenylethyl-1-phosphate substrate (179). Clearly, these alcohol acceptors must accelerate the loss of the phosphoryl group from the (E-P) complex.

Evidently, changes in the solvent conditions cannot be used to enhance the selectivity of bovine alkaline phosphatases with the 1-phenylethyl-1-phosphate (**179**). This is because the phosphate group of the substrate is polar and hydrophilic; consequently, the hydrophilic catalytic site plays a pivotal role in the activity of the phosphatases. Any changes made to this binding region only serve to reduce its enantioselectivity with racemic substrates. It appears that changes in solvent conditions affect the binding of the (R)- and (S)-enantiomers to the same extent. Consequently, the organic co-solvents have no significant effect on the selectivity on bovine alkaline phosphatase. This is in contrast to the lipases which show increased selectivity when their reactions are carried out in apolar solvents.^{28,150,152} The substrates of these enzyme reactions commonly contain large hydrophobic groups, and a change to hydrophobic solvents will serve only to increase particular interactions of the binding site with these groups, enhancing the selectivity.

5.2.4. Conclusions:

The results obtained in Sections 5.2.-5.2.3, demonstrate that the observed enantioselectivity is not significantly affected by many different variations to the assay conditions. Any perturbations to the rate of turnover must be the same for both diastereomeric enzyme-substrate complexes. In all of these experiments, the observed ee's and E values calculated, were lower at both 10% and 50% completion, compared to those determined under standard conditions. The binding of the 1-phenylethyl-1-phosphate (**179**) can be envisaged as predominantly **Model 1**. In this model, the enantioselectivity reflects the location of either the methyl group or phenyl group in the lipophilic binding site. The methyl group and phenyl group are both hydrophobic and consequently, either group might be readily be bound into this site. It is this potential for the phosphate enantiomers to bind in either orientation discussed in **Model 1**, that results in the unchanged

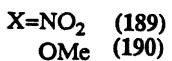
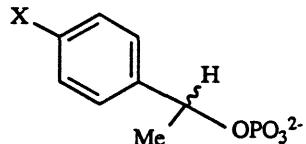
enantioselectivity of the bovine alkaline phosphatase. The selectivity remains unaffected by the various changes in the reaction conditions.

5.3. Study of alkaline phosphatases from various sources with *para*-substituted racemic 1-phenylethyl-1-phosphate derivatives:

A series of *para*-substituted derivatives of 1-phenylethyl-1-phosphate were used to investigate further the nature of the putative lipophilic binding region. The two derivatives chosen for this study were 1-(*para*-nitrophenyl)ethyl-1-phosphate (**189**) and 1-(*para*-methoxyphenyl)ethyl-1-phosphate (**190**). These derivatives were chosen since they have different effects on the electronic nature of the phenyl ring. The nitro group is electron withdrawing and in the case of the aryl phosphates,^{84a} a *para*-nitro substituent activates the substrate by reducing the pKa of the leaving group. In contrast to this, the methoxy is electron donating. These *para*-substituted derivatives are shown below in

Figure 5.4.

Figure 5.4:



Hall and Williams, have reported that the reaction rates of the benzylic and alkyl phosphates with *E.coli* alkaline phosphatase are very different from those of the aryl substituted phosphates.^{84a} They have suggested that this is associated with a change in the rate limiting step for the reactions involving these two different classes of phosphate ester. The studies of the alkaline phosphatases with the 1-phenylethyl-1-phosphate (**179**)

substrate have already provided insight into the binding interactions of the active site. Work by Hall and Williams has shown that there is a correlation between the type and position of the substituent of the aryl monophosphates and their reactivities. It was envisaged that a similar comparison could be drawn from the reactions of the *para*-substituted benzyl phosphates (189) and (190) with the alkaline phosphatases. Both substituents cause electron dispersions across the phenyl rings to which they are attached. The nitro group will reduce the electron density on the phenyl ring, since it is electron withdrawing. Whereas, the methoxy group is electron donating, and will make the phenyl ring electron rich. They possess the common 1-phenylethyl-1-phosphate template, but clearly the electronic nature of their phenyl rings are different. It should be possible to correlate the electronic effects of the *para*-substituents with the enantioselectivities observed.

It is known that close to the active site, are positioned two phenylalanine residues 317 and 318 that are thought to be in the putative lipophilic binding site. The interactions of these residues with the phenyl rings of the 1-phenylethyl-1-phosphate derivatives (189) and (190) will be important in determining the reactivity and selectivity of the alkaline phosphatases. Highly favourable π - π stacking interactions are only seen when the phenyl rings have a donor-acceptor interaction (i.e. electron rich with electron deficient). In the absence of this, edge to face interactions between the phenyl rings are preferred. By introducing *para*-substituents, the electronic nature of the phenyl substrates can be altered. The *para*-substituted derivatives (189) and (190) were used to investigate the interactions of the phenyl rings with the phenylalanine residues 317 and 318. Whether these substituents enhance or reduce the π - π interactions will be reflected in the observed ee's.

From the initial results of the reactions of the alkaline phosphatases with the 1-phenylethyl-1-phosphate derivatives it might be possible to correlate the electronegativity of the *para*-substituent with the enantioselectivity observed. This type of analysis is based on work by Topliss,¹⁶¹ who has shown that models correlating the reactivity of various aromatic derivatives, to the position and type of aryl substituent can be formulated. The model produced from these results can be used to correlate the reactivity and selectivity of the phosphatases with the electronegativity of the *para*-substituents. From such results it may be possible to select other substituents which would afford greater selectivity. The results of these enzyme reactions were determined as enantiomeric excesses (ee). These were determined by reacting the alcohol products with MTPA-Cl (209) as described in Chapter 2. The respective diastereoisomers produced were analysed by ¹⁹F NMR spectroscopy and their relative amounts determined by measurement of the peak areas. The (R) and (S) alcohol assignments were made by comparing them with authentic samples of the racemic and chiral alcohol derivatives shown in Table 2.1. These results are shown under the various sections of the alkaline phosphatases.

5.3.1. Bovine alkaline phosphatase:

Table 5.20 shows the results of the reactions of the bovine alkaline phosphatase with the 1-phenylethyl-1-phosphate (179), 1-(*para*-nitrophenyl)ethyl-1-phosphate (189) and 1-(*para*-methoxymethyl)benzyl-1-phosphate (190) substrates.

Table 5.20:

Substrate	Rate($\mu\text{mol}/\text{min}$)	% R-R	% S-R	% e.e.
25% REACTION COMPLETION				
1-phenylethyl-1-phosphate	----	75	25	50(R)
1-(<i>para</i> -nitrophenyl)ethyl-1-phosphate	----	42	53	16(S)
1-(<i>para</i> -methoxyphenyl)ethyl-1-phosphate	----	57	43	14(R)
50% REACTION COMPLETION				
1-phenylethyl-1-phosphate	2.6×10^{-4}	72	28	44(R)
1-(<i>para</i> -nitrophenyl)ethyl-1-phosphate	3.4×10^{-4}	47	53	6(S)
1-(<i>para</i> -methoxyphenyl)ethyl-1-phosphate	2.9×10^{-4}	56	44	12(R)

At both 25% and 50% completion the bovine phosphatase selectivity towards the *para*-substituted derivatives (**189**) and (**190**) is much lower than that of the 1-phenylethyl-1-phosphate (**179**). What is more significant is that the selectivity of the bovine phosphatase with the 1-(*para*-nitrophenyl)ethyl-1-phosphate (**189**), is reversed. This suggests that the electron withdrawing nitro group, by reducing the electron density on the phenyl ring, reduces its interactions with the hydrophobic region. If this is the case, preferential binding of the methyl group to the hydrophobic region will switch the selectivity to the (S)-enantiomer. In contrast to this the turnover of the 1-(*para*-

methoxyphenyl)ethyl-1-phosphate (**190**) is reduced with respect to (**179**) but the (R)-enantiomer is still turned over preferentially. For bovine alkaline phosphatase the order of reactivity of the *para*-substituents of 1-phenylethyl-1-phosphate is NO₂ > OMe > H.

Clearly, in terms of enantioselectivity, there is not a simple relationship between the electronic effects of the substituents on the aryl ring and the observed enantioselectivity.

5.3.2. Rabbit and *E.coli* alkaline phosphatase:

Table 5.21: Rabbit Alkaline Phosphatase

<u>Mosher's ester derivatives</u>				
<u>Substrate</u>	<u>Rate(μmol/min)</u>	<u>% R-R</u>	<u>% S-R</u>	<u>% e.e.</u>
50% REACTION COMPLETION				
1-phenylethyl-1-phosphate	5.7x10 ⁻⁴	71	29	41(R)
1-(<i>para</i> -nitrophenyl)ethyl-1-phosphate	9.3x10 ⁻⁴	52	48	4(R)
1-(<i>para</i> -methoxyphenyl)ethyl-1-phosphate	8.3x10 ⁻⁴	64	36	28(R)

Table 5.22: *E.coli* Alkaline Phosphatase

<u>Mosher's ester derivatives</u>				
<u>Substrate</u>	<u>Rate(μmol/min)</u>	<u>% R-R</u>	<u>% S-R</u>	<u>% e.e.</u>
50% REACTION COMPLETION				
1-phenylethyl-1-phosphate	1.2x10 ⁻⁴	62	38	24(R)
1-(<i>para</i> -nitrophenyl)ethyl-1-phosphate	1.5x10 ⁻⁴	53	47	6(R)
1-(<i>para</i> -methoxyphenyl)ethyl-1-phosphate	1.3x10 ⁻⁴	56	44	12(R)

The results of the reactions of both the rabbit and *E.coli* alkaline phosphatases show that the (R)-enantiomers of the 1-phenylethyl-1-phosphate derivatives are turned over preferentially. For both enzymes, the reaction rates of the substrates is: *para*-nitro-(189) > *para*-methoxy-(190) > 1-phenylethyl-1-phosphate (179). With both the bovine and *E.coli* phosphatases, there is no trend in selectivity with changes in the electronic nature of the aryl rings.

Complete results of all the alkaline phosphatase reactions with the racemic 1-(*para*-nitrophenyl)ethyl-1-phosphate (189) are shown in **Table 5.23**. The results of the 1-(*para*-methoxyphenyl)ethyl-1-phosphate (190) are displayed in **Table 5.24**.

Table 5.23:

Enzyme reactions with the 1-(*para*-nitrophenyl)ethyl-1-phosphate substrate-[100mM]

Enzymes	<u>Mosher's ester products</u>				
	<u>%Completion of reaction</u>	<u>Specific activity (μmol/min/unit)</u>	<u>% R-R</u>	<u>%S-R</u>	<u>%ee (enantiomer)</u>
Bovine	10%	---	34	66	32(S)
Bovine	25%	---	42	53	16(S)
Bovine	50%	1.4×10^5	47	58	6(S)
Rabbit	50%	1.9×10^4	52	48	4(R)
<i>E.coli</i>	50%	1.4×10^5	53	47	6(R)

Table 5.24:**Enzyme reactions with the 1-(*para*-methoxyphenyl)ethyl-1-phosphate substrate**

Enzymes	Mosher's ester products				
	%Completion of reaction	Specific activity ($\mu\text{mol}/\text{min}/\text{unit}$)	% R-R	%S-R	%ee (enantiomer)
Bovine	25%	---	57	43	14(R)
Bovine	50%	1.1×10^{-5}	56	44	12(R)
Rabbit	50%	1.7×10^{-4}	64	36	28(R)
E.coli	50%	1.3×10^{-5}	56	44	12(R)

5.3.3. Conclusions:

The (S)-enantiomer of 1-(*para*-nitrophenyl)ethyl-1-phosphate (**189**) is preferentially turned over when it is incubated with the bovine alkaline phosphatase. When (**189**) is reacted with rabbit and *E.coli* alkaline phosphatase, turnover of the (R)-enantiomer is favoured. In all of these experiments, the observed ee's are considerably less than those of the 1-phenylethyl-1-phosphate (**179**). There are no obvious simple relationships between selectivity and the electronic effects of the substituents on the aryl group.

Chapter 6

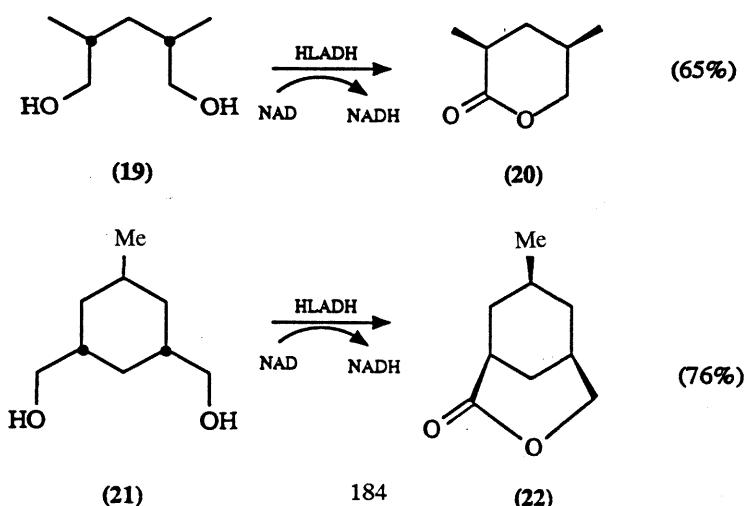
*Studies of Bovine and E.coli Alkaline Phosphatase with the
2-Alkylpropyl-1,3-Bisphosphate Derivatives*

6.1. Introduction:

Discrimination between enantiotopic atoms or groups is another valuable aspect of enzyme specificity that enables asymmetric transformations to be achieved on symmetrical substrates. Using racemic substrates in asymmetric synthesis is not ideal. This type of enzymatic reaction suffers from the disadvantage that with all kinetic resolutions at best only 50% of the substrate is converted to the desired chiral product. The advantages of exploiting the prochiral specificity of enzymes is that all of the symmetrical substrate can be converted into the desired chiral product.

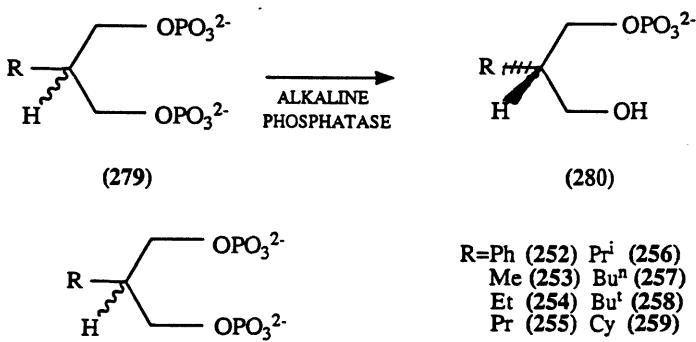
Enzymatic discriminations of enantiotopic groups of *meso*-compounds have been widely studied in asymmetric synthesis.^{19,20} An example of this is the work of Jones *et.al.*²⁰ shown in **Scheme 6.1**. This illustrates the stereospecific oxidation of the 1,3-disubstituted diols (**19**) and (**21**) to the corresponding lactones (**20**) and (**22**), using horse liver alcohol dehydrogenase (HLADH). The lactone products are attractive intermediates in the synthesis of prostaglandins and macrolides.

Scheme 6.1:



Clearly, similar strategies can be envisaged with the alkaline phosphatases. The alkaline phosphatases could be used to discriminate between the two enantiotopic phosphate monoester groups of a series of prochiral 2-alkylpropyl-1,3-bisphosphates (**252**-**259**). The alkaline phosphatases could be expected to carry out enantioselective hydrolysis of the symmetrical 1,3-bisphosphate (**279**) to produce the chiral monophosphate ester (**280**) as shown in **Scheme 6.2**. The advantage of this over the reactions with the racemic monophosphates is that all of the prochiral 1,3-bisphosphate can be converted into the desired chiral 2-alkylpropyl-monophosphate product. In these prochiral 1,3-bisphosphate substrates, the phosphates are attached to primary carbon centres and enzymatic cleavage of these is well documented. Earlier work discussed in **Chapters 3 and 4**, has shown that the phosphatases have a broad specificity in hydrolysing various monophosphate esters, even compared to aryl phosphates, primary alkyl phosphates are readily turned over.

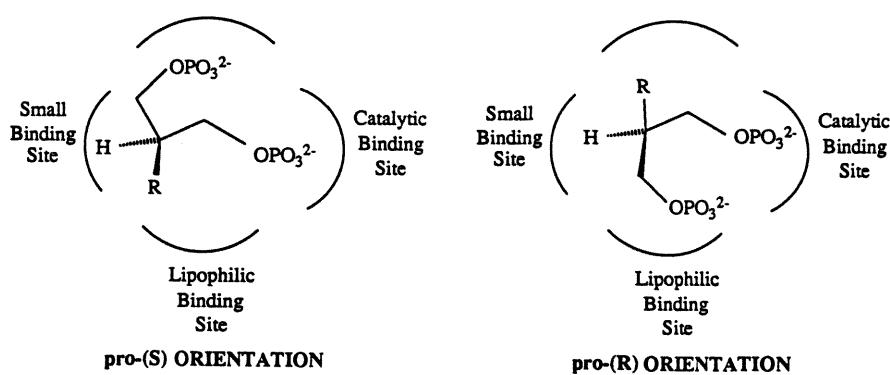
Scheme 6.2:



6.2. Reactions of bovine alkaline phosphatase with the 2-alkylpropyl-1,3-bisphosphates (252-259):

Discussed here are the reactions of the 2-alkylpropyl-1,3-bisphosphate substrates with the bovine phosphatase. In addition to potentially producing enantiomerically pure monophosphates, the reactions of the prochiral 1,3-bisphosphate derivatives (252-259) with the bovine enzyme were used to evaluate further the significance of the different binding sites within the active pocket. These investigations would confirm the importance of the lipophilic binding site and its affinity for large hydrophobic alkyl groups and aromatic substituents. Using **Model 1**, the binding orientations of the 1,3-bisphosphate derivatives (252-259) within the active site would be as shown in **Figure 6.1**. With the pro-(S) phosphoryl group in the catalytic site, the second phosphate moiety is located in the alternative binding site and the phenyl group is bound to the lipophilic binding site. Hydrolysis of this orientation produces the chiral (R)-monophosphate product. With the pro-(R) phosphoryl group in the catalytic site, the second phosphate group is located in the lipophilic binding region, and this produces the (S)-monophosphate product.

Figure 6.1:



The series of 2-alkyl derivatives, (252-259) were used in these investigations. The enantiotopic group discrimination of the phosphate moieties by bovine alkaline phosphatase would depend upon which binding orientation is preferred within the active site. The size and hydrophobic nature of the alkyl group at the prochiral centre C-2 would be important in affecting the selectivity of the bovine phosphatase with the 1,3-bisphosphates (252-259). The interactions of these alkyl groups with the lipophilic binding site would be important, as well as the binding of the second phosphate group not in the catalytic site. Hall and Williams have reported that the lipophilic binding site shows preferential binding of large alkyl groups and aromatic substituents.^{66a,84a} Consequently, the larger 2-alkyl derivatives; 2-phenyl-(252), 2-butyl-(257), 2-*tert*-butyl-(258) and 2-cyclohexyl-(259), were evaluated as substrates. Work with the monophosphate substrates discussed in Chapter 3, illustrates how important the size and length of the alkyl group is in contributing to the selectivity of the phosphatases.

It was envisaged that the large bulky groups of these 1,3-bisphosphate derivatives would bind strongly to the lipophilic binding site in the preferred pro-(S) orientation, in which the second phosphate group is located in the less hydrophobic alternative binding site. These two groups are very different. The alkyl moieties are hydrophobic whereas the phosphate group being negatively charged, is highly hydrophilic; as well as being sterically demanding. Therefore, how well the lipophilic and alternative binding sites can accommodate these different functional groups will be critical in determining the selectivity. This large discrimination by the binding regions, between the negatively charged phosphate group and large hydrophobic alkyl group would potentially result in an enhanced selectivity. With these substrates it would also be possible to compare the ring systems of the phenyl and cyclohexyl derivatives, which is significant as much of the

initial work of the lipases¹⁹ and esterases²⁰ involved cyclic 1,2- and 1,3-substrates.

6.2.1. Reaction conditions:

The 1,3-bisphosphates (252-259), were incubated at 37°C in standard buffer solution and the reactions were initiated by the addition of bovine alkaline phosphatase. Their progress was monitored by ³¹P NMR spectroscopy over several days.¹⁶³ The substrate peak around $\delta_p = 3.00$ ppm was seen to decrease slowly as the product peak of inorganic phosphate was identified at $\delta_p = 2.69$ ppm.

The progress of the reactions were monitored by measuring the peak areas of the phosphate substrate and inorganic phosphate. Consequently, they were terminated when 50% of the phosphate had been liberated as inorganic phosphate.

Analysis of the organic extracts of these reactions by G.C. and TLC against authentic samples of the 2-alkyl-1,3-propanediols was carried out to confirm that these undesired products had been produced from the reactions. After scaling up these reactions, the organic extracts were analysed by ¹H and ¹³C NMR spectroscopy to confirm unequivocally that the by-products were the 2-alkyl-1,3-propanediol derivatives. The aqueous layer was taken up in distilled water and the phosphate materials were isolated by ion exchange chromatography. The buffer eluent, TEAB, of 50mM-700mM concentrations were run through the DEAE-sephadex column. The only phosphate materials to be isolated from these columns were the free inorganic phosphate and unreacted 1,3-bisphosphates. The fractions close to and including the inorganic phosphate fractions were analysed by ¹H and ¹³C NMR spectroscopy to see if there was any monophosphate product present; none was ever identified. Only the single peak of inorganic phosphate was identified in the ³¹P NMR spectra. The bisphosphate derivatives were identified by ¹H and ¹³C NMR spectroscopy, as well as by their longer elution times

from the columns and comparisons with authentic samples. These reactions were completed and terminated at 20% and 30% completion, but with all of these experiments none of the desired 2-alkylpropyl-monophosphate product was detected. The only compounds to be identified from the reactions were the 2-alkyl-1,3-propanediols, inorganic phosphate and unreacted 1,3-bisphosphate derivatives.

6.2.2 Results:

The results of these experiments are shown below in **Table 6.1**.

Table 6.1: Reactions with the Bovine Alkaline Phosphatase

<u>2-Alkylpropyl-1,3-bisphosphates</u>	<u>Reaction times</u> <u>50% Completion</u>	<u>Organic product</u>	<u>Aqueous product</u>
phenyl (252)	14 hours	1,3-diol	bisphosphate
methyl (253)	3 days 8 hours	1,3-diol	bisphosphate
ethyl (254)	3 days 14 hours	1,3-diol	bisphosphate
propyl (255)	2 days 20 hours	1,3-diol	bisphosphate
<i>iso</i> -propyl (256)	2 days 16 hours	1,3-diol	bisphosphate
butyl (257)	3 days 16 hours	1,3-diol	bisphosphate
<i>tert</i> -butyl (258)	N/S 10 days	-----	-----
cyclohexyl (259)	N/S 10 days	-----	-----

N/S - not a substrate

No appreciable turnover of the 2-*tert*-butyl-(258) and 2-cyclohexyl-(259)-1,3-bisphosphates was detected after being incubated with bovine alkaline phosphatase for 10 days. Turnover of the 2-phenylpropyl-1,3-bisphosphate (252) was observed and 50% completion was achieved in 14 hours. The reactions of the remaining 1,3-bisphosphates with bovine phosphatase were very slow. A similarity in the reaction times suggests that the size and length of the alkyl chain is not important. This clearly indicates that the second negatively charged phosphate group of these substrates has a significant adverse

affect on the reaction rates.

The reason for the slow kinetics is thought to be due to poor binding of these 1,3-bisphosphates within the active site of the bovine phosphatase. Consequently their turnover is very poor. The results suggest that a combination of the large alkyl group and the second phosphate group prevent the substrate from being bound strongly to the enzyme. Besides being sterically demanding, these two groups are very different in character. The alkyl groups are hydrophobic, while the phosphate groups are hydrophilic and negatively charged. These deductions concerning the binding of the 1,3-bisphosphates however are somewhat speculative. In order to evaluate these suggestions, the reactivities of the 2-alkyl-1,3-bisphosphates (253-259) with bovine and *E.coli* alkaline phosphatases needed to be studied further. Their effects as inhibitors of the alkaline phosphatases in the presence of the natural substrate, *para*-nitrophenyl phosphate, were examined.

6.3. Inhibition studies:

The reactivity of the bovine phosphatases with the 2-alkylpropyl-1,3-bisphosphates (253-259) needed to be examined further. Using the usual substrate, *para*-nitrophenyl phosphate which is rapidly turned over, it is possible to evaluate the inhibitory activity of these 1,3-bisphosphate derivatives. It was hoped that this work would be extended to determine the type of inhibition that the 1,3-bisphosphates show towards the bovine and *E.coli* phosphatases; competitive, noncompetitive or uncompetitive.

Kinetic studies of the bovine and *E.coli* alkaline phosphatases with *para*-nitrophenyl phosphate had been previously studied and the results are shown in Sections 3.3.2 and 3.3.3, including a brief definition of the K_m , V_{max} and k_{cat} values. The results of these experiments are summarised in Table 6.2.

Table 6.2:

<u>Kinetic Parameters</u>	<u>Enzyme Sources</u>	
	<i>Bovine</i>	<i>E.coli</i>
K_m (μM)	13.6	5.6 5.2 ¹⁴⁸
V_{max} ($\mu M \cdot min^{-1}$)	28.0	18.0
k_{cat} (sec^{-1})	208 200 ¹⁴⁹	26.0 25 ^{148,149}
k_{cat}/K_m ($sec^{-1} \cdot \mu M^{-1}$)	14.7	4.64

6.3.1: Types of inhibition:

Inhibitors are described as substances which reversibly or irreversibly bind to the enzyme through non-covalent interactions and prevent the normal action of the enzyme. There are 4 main types of inhibition; competitive, noncompetitive, uncompetitive and mixed.

Competitive inhibitors: These are substances that bind to the active site of the enzyme and prevent the binding and reaction of the substrate. They also cause an apparent increase in the K_m value.

Noncompetitive inhibitors: These are substances that bind to regions other than the active site of the enzymes and affect the rate of binding of the substrate.

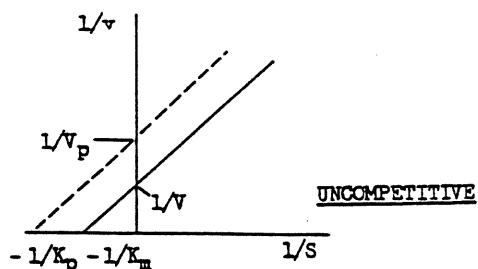
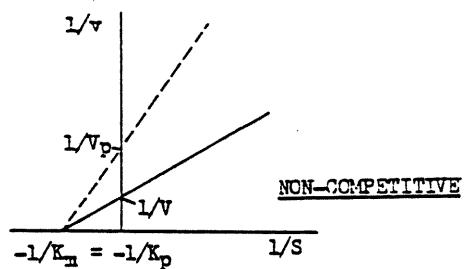
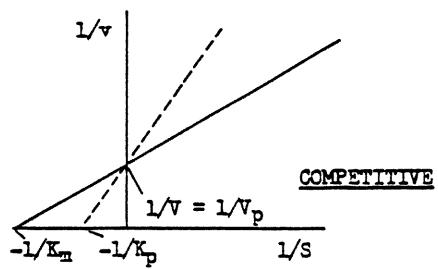
Uncompetitive inhibitors: These are substances that interact with the intermediate enzyme-substrate complex. They cause a decrease in K_m and the rate of reaction.

Mixed inhibitors: These are substance that show a combination of characteristics of competitive and noncompetitive inhibitors. The rate of reaction is decreased but the K_m can be either increased or decreased.

Shown in **Figure 6.2** are Lineweaver-Burk plots,^{83,147} widely used to distinguish the different types of inhibition. With competitive inhibitors, the lines are seen to intersect

horizontal axis. Mixed inhibitors can have points of intersection either above or below the horizontal axis, depending on the contributions from the competitive and noncompetitive effects. Finally, uncompetitive inhibitors have lines which run parallel with one another. The inhibition constant, K_i , can be determined from the values of the appropriate intercepts of the double reciprocal plots (1/rate vs 1/concentration). K_i is the apparent dissociation constant for the enzyme-inhibitor complex.

Figure 6.2:



6.3.2. Inhibition studies of the 2-alkylpropyl-1,3-bisphosphates (253-259) with bovine alkaline phosphatase:

The inhibition studies of the prochiral 1,3-bisphosphates with the bovine phosphatase were studied as described in the **Chapter 7**. The various concentrations of the 2-alkylpropyl-1,3-bisphosphates were incubated with *para*-nitrophenyl phosphate in Tris-buffer, and the reactions were initiated by the addition of the bovine phosphatase. The reactions were monitored spectrophotometrically by the increased absorbance of the yellow colour of *para*-nitrophenolate product (405nm). In order to determine the K_i more accurately, the reactions were repeated using a series of 1,3-bisphosphate concentrations. From these experiments a series of reciprocal plots were produced to determine K_i . The results of these studies are illustrated in the Lineweaver-Burk plots on pages 196-197. The inhibitory affect of the 2-alkylpropyl-1,3-bisphosphates (253-259) with the bovine phosphatase are presented in **Table 6.3**.

An examination of these double reciprocal plots reveals that most of the 1,3-bisphosphate derivatives are competitive inhibitors of the bovine alkaline phosphatase. Using the intercept on the horizontal axis ($-1/K_p$), the inhibition constant, K_i can be calculated from the following expression.

$$K_i = i / (K_p / K_m - 1)$$

$$K_i = (K_m \times i) / (K_p - K_m)$$

K_i = inhibition constant

i = inhibitor concentration

$-1/K_p$ = intercept on horizontal axis

$1/V$ = intercept on vertical axis

e.g. bovine alkaline phosphatase $K_m = 13\mu M$ $V_{max} = 28.0\mu M \cdot min^{-1}$

2-propylpropyl-1,3-bisphosphate: $500\mu M$ concentration

$$1/K_p = 0.019\mu M^{-1}$$

$$K_p = 52\mu M$$

$$i = 500\mu M$$

$$K_i = (K_m \times i) / (K_p - K_m)$$

$$K_i = 6800\mu M / 39\mu M$$

$$K_i = 174\mu M$$

Similarly, for the other concentrations:

$$\begin{array}{ll} i = 1000\mu M & K_i = 175\mu M \\ i = 2000\mu M & K_i = 177\mu M \\ i = 500\mu M & K_i = 174\mu M \quad \underline{\text{average } K_i = 176\mu M} \end{array}$$

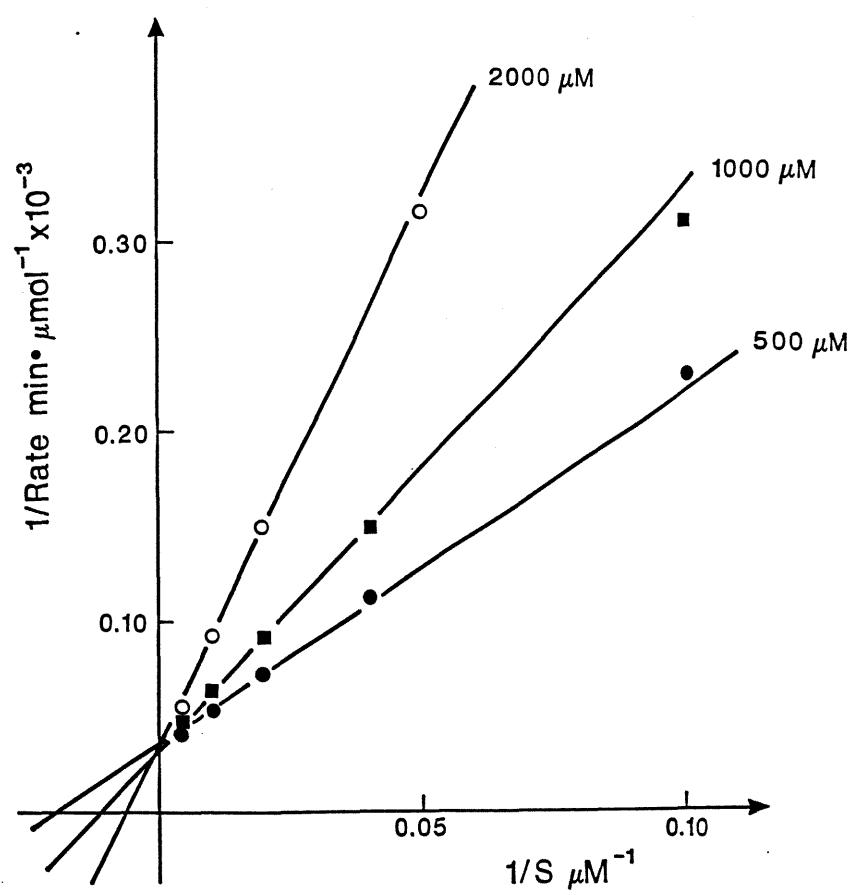
The results of these calculations are summarised in **Table 6.3**. The K_i values for the various 2-alkylpropyl-1,3-bisphosphate derivatives are all within a narrow range. These K_i values are about 10^3 times smaller than the K_m values calculated for the 1-phenylethyl-1-phosphates. They appear to be approximately 10 greater than the K_m values of *para*-nitrophenyl phosphate. These low K_i values suggest that the 2-alkylpropyl-1,3-bisphosphates, contrary to initial suggestions, are bound strongly to the bovine phosphatase, but turnover is low. It appears that the binding of these derivatives to the bovine phosphatase is very strong and therefore, poor binding is not a factor in the slow turnover rates of the 1,3-bisphosphates. One possibility would be that the slow turnover arises from the slow "off-rate" of the phosphate monoester and/or inorganic phosphate products.

Table 6.3:**Bovine Alkaline Phosphatase Competitive Inhibition Studies**

<u>2-alkylpropyl-1,3-bisphosphates</u>	<u>1/K_i(μM⁻¹)</u>	<u>K_p(μM)</u> (± 0.4)	<u>i(μM)</u>	<u>K_i(μM)</u> (± 0.3)
methyl (253)	0.018	55	500	162
methyl (253)	0.010	100	1000	157
mean K _i = 160				
ethyl (254)	0.090	111	1000	139
propyl (255)	0.019	52	500	174
propyl (255)	0.011	90	1000	176
propyl (255)	0.006	167	2000	178
mean K _i = 176				
iso-propyl (256)	0.010	100	1000	157
iso-propyl (256)	0.005	200	2000	146
mean K _i = 152				
butyl (257)	0.008	125	1000	122
cyclohexyl (259)	0.004	250	2000	115

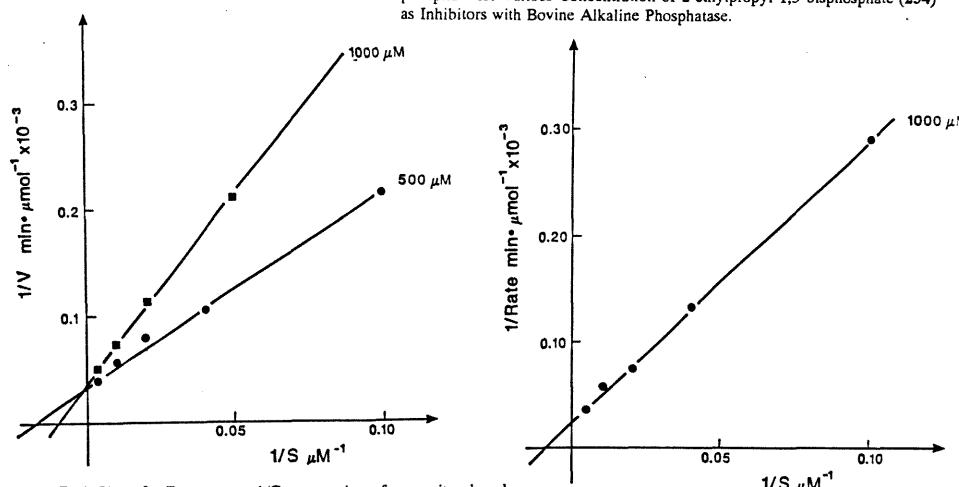
The Lineweaver-Burk plot of the *2-tert-butyl-1,3-bisphosphate (258)* derivative indicate that it is a mixed inhibitor. The general considerations for mixed inhibitors are as follows:

- i) K_i = K'_i this system can be reduced down to true noncompetitive inhibition.
- ii) K'_i > K_i the apparent K_m is seen to be increased and the line intersect above the horizontal axis (1/concentration). This represents a significant competitive inhibitor contribution.
- iii) K'_i < K_i the apparent K_m is decreased and the lines intersect below the horizontal axis (1/concentration).



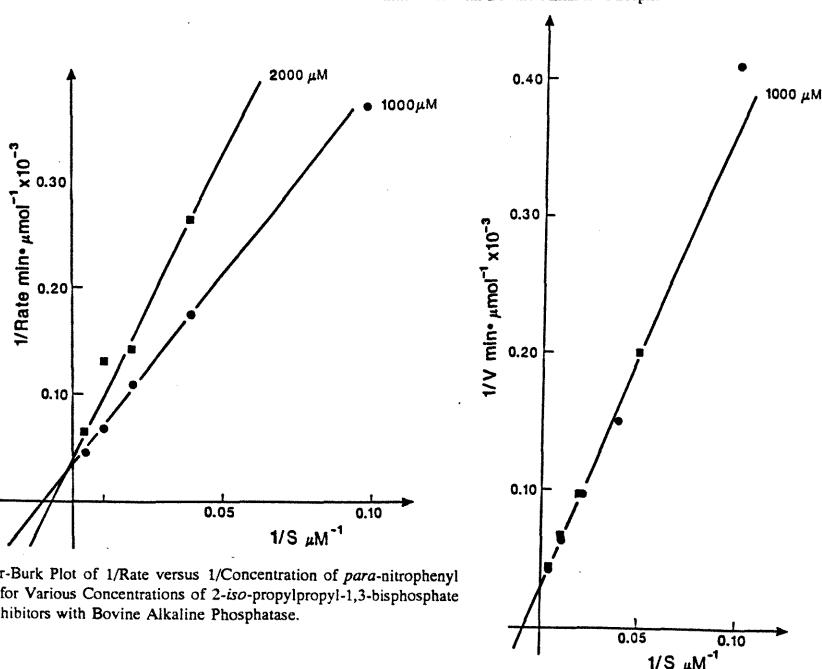
Lineweaver-Burk Plot of 1/Rate versus 1/Concentration of *para*-nitrophenyl phosphate for Various Concentrations of 2-propylpropyl-1,3-bisphosphate (255) as Inhibitors with Bovine Alkaline Phosphatase.

Lineweaver-Burk Plot of 1/Rate versus 1/Concentration of *para*-nitrophenyl phosphate for Various Concentration of 2-ethylpropyl-1,3-bisphosphate (254) as Inhibitors with Bovine Alkaline Phosphatase.



Lineweaver-Burk Plot of 1/Rate versus 1/Concentration of *para*-nitrophenyl phosphate for Various Concentrations of 2-methylpropyl-1,3-bisphosphate (253) as Inhibitors with Bovine Alkaline Phosphatase.

Lineweaver-Burk Plot of 1/Rate versus 1/Concentration of *para*-nitrophenyl phosphate for Various Concentrations of 2-butylpropyl-1,3-bisphosphate (257) as Inhibitors with Bovine Alkaline Phosphatase.



Lineweaver-Burk Plot of 1/Rate versus 1/Concentration of *para*-nitrophenyl phosphate for Various Concentrations of 2-*iso*-propylpropyl-1,3-bisphosphate (256) as Inhibitors with Bovine Alkaline Phosphatase.

With both of these derivatives it can be seen that the lines of inhibition intersect with the *para*-nitrophenyl phosphate plot above the baseline axis. This implies that the K_m is increased and that the 2-*tert*-butyl-1,3-bisphosphate (258) derivative has a high competitive inhibitor contribution. The increase in K_m slows down the rate of reaction and inhibits the enzyme's reactivity. Using the slope of the plot, the inhibition constants, K_i , for these mixed inhibitors can be calculated using the following expression.

$$g = K_m (1 + i/K_i) / V$$

K_i = inhibition constant (μM)

$$g \cdot V = K_m + (K_m \times i) / K_i$$

i = inhibitor concentration (μM)

$$K_i = (g \cdot V - K_m) = (K_m \times i)$$

K_m = Michaelis constant (μM)

$V = V_{\max}$ = maximum reaction rate

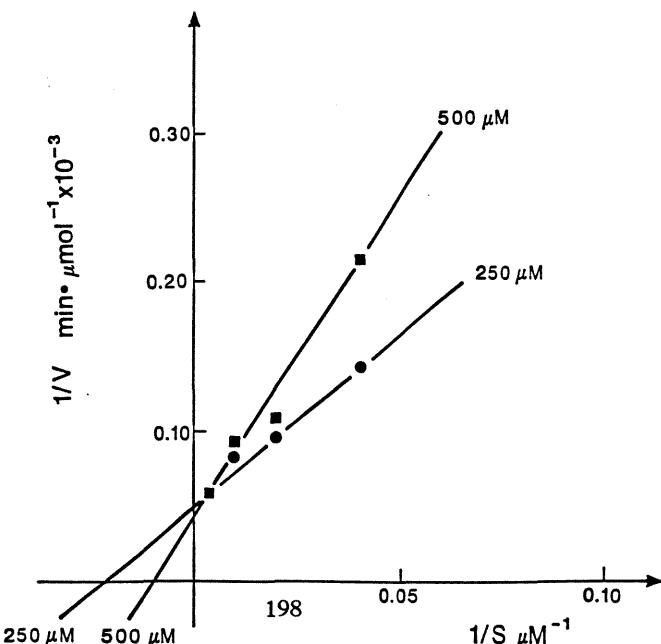
g = gradient of plot

$$K_m = 13 \mu\text{M} \quad i = 500 \mu\text{M} \quad V = 28.0 \mu\text{M} \cdot \text{min}^{-1} \quad g = 4.3 \text{ min} \cdot \mu\text{M}^{-1}$$

$$K_i = (13 \times 5000) / [(4.3 \times 28) - 13]$$

$$\underline{K_i = 50 \mu\text{M}}$$

Lineweaver-Burk Plot of 1/Rate versus 1/Concentration of *para*-nitrophenyl phosphate for Various Concentrations of 2-*tert*-butylpropyl-1,3-bisphosphate (258) as Inhibitors with Bovine Alkaline Phosphatase.



The results of the mixed inhibitor is summarised in **Table 6.4** shown below.

Table 6.4:

Bovine Alkaline Phosphatase Mixed Inhibition Study

<u>2-alkylpropyl-1,3 bisphosphate</u>	<u>gradient</u>	<u>i(μM)</u>	<u>K_i(μM)</u> (± 0.3)
<i>tert</i> -butyl (258)	4.3	500	63

The K_i value is about 5 times greater than the K_m value of the *para*-nitrophenyl phosphate. It is also 10^3 times smaller than the K_m value of the 1-phenylethyl-1-phosphate. These comparisons indicate that the 2-*tert*-butyl-1,3-bisphosphate (**258**) is bound strongly to the bovine enzyme. Having completed the investigations of the bovine alkaline phosphatase with the 1,3-bisphosphatase derivatives, the next step was to evaluate their inhibitory effects with *E.coli* alkaline phosphatase.

6.3.3. Inhibition studies of the 2-alkylpropyl-1,3-bisphosphate (253-259) with *E.coli* alkaline phosphatase:

The inhibition studies of the prochiral 1,3-bisphosphate derivatives with the *E.coli* phosphatase were performed using the methods described for the bovine phosphatase. Similarly, a series of reciprocal plots were drawn to determine K_i and deduce the type of inhibition. The results of these experiments are illustrated by the Lineweaver-Burk plots shown. A survey of these plots shows that these results are similar to those for bovine alkaline phosphatase. The 2-alkylpropyl-1,3-bisphosphates are competitive inhibitors of the *E.coli* phosphatase. The inhibition constants, K_i values were calculated using the equations described previously. The results of these inhibition studies shown in **Table 6.5**. These values fall within a narrow range and show a 25 fold increase over the K_m calculated for the *para*-nitrophenyl phosphate. These K_i values are 400 times smaller than

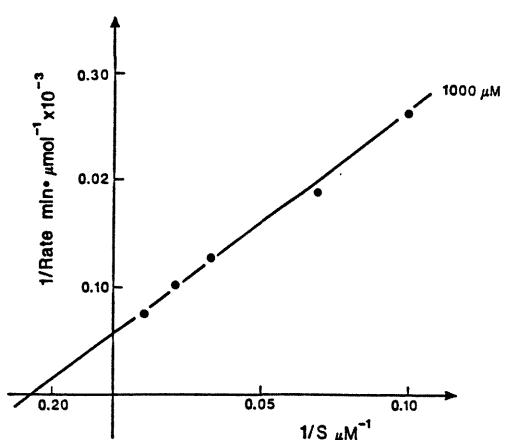
the K_m values calculated for the 1-phenylethyl-1-phosphates. These low K_i values, like those determined for the bovine enzyme, indicate that the 1,3-bisphosphates are strongly bound to the *E.coli* phosphatase compared to the monophosphate esters. This suggests that poor binding does not contribute to the poor turnover of these substrates.

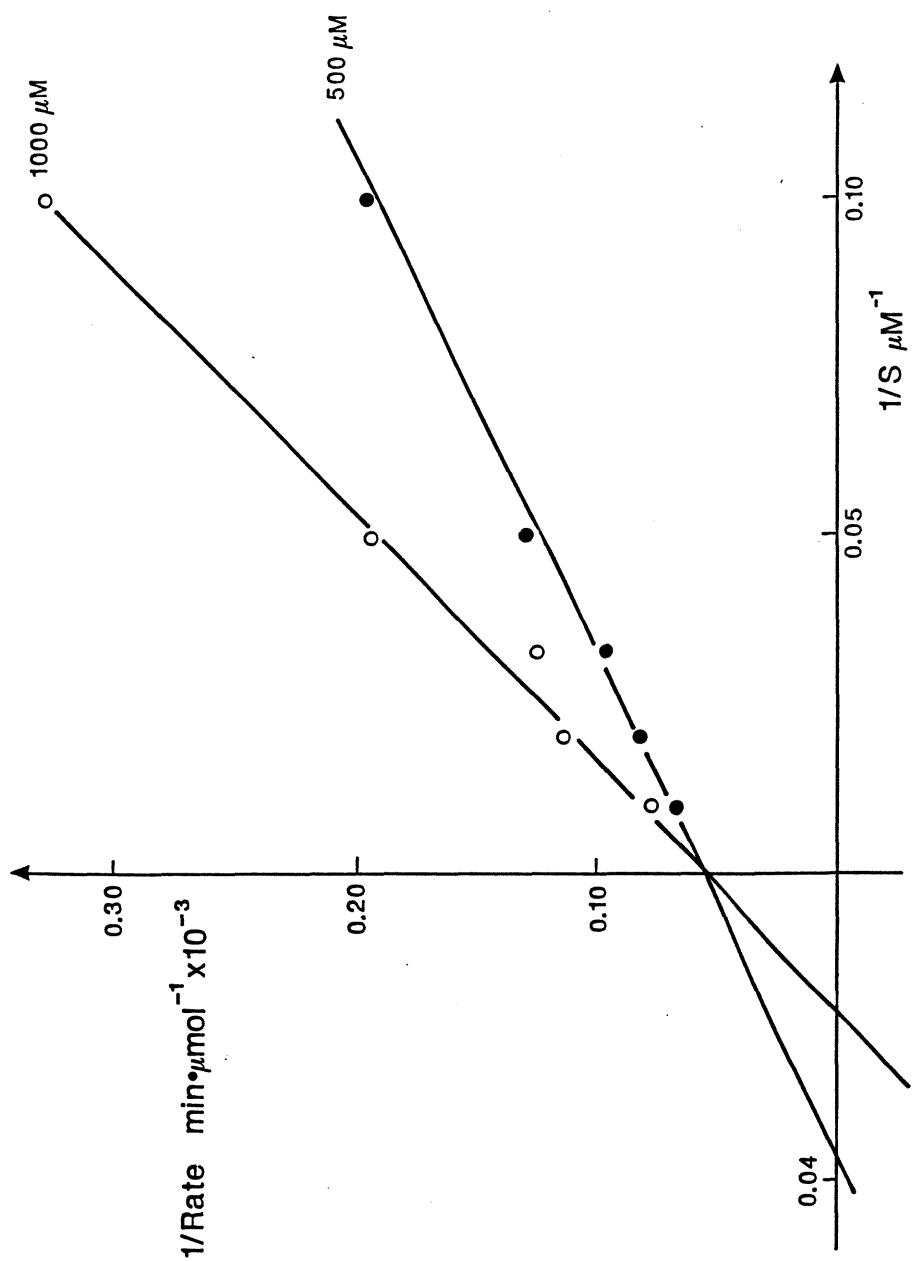
Table 6.5:

E.coli Alkaline Phosphatase Competitive Inhibition Studies

<u>2-alkylpropyl-1,3-bisphosphates</u>	<u>$1/K_p(\mu M^{-1})$</u>	<u>$K_p(\mu M)$ (± 0.5)</u>	<u>$i(\mu M)$</u>	<u>$K_i(\mu M)$ (± 0.4)</u>
methyl (253)	0.021	46	1000	139
ethyl (254)	0.037	27	500	132
propyl (255)	0.022	45	1000	141
<i>iso</i> -propyl (256)	0.020	50	1000	126
<i>iso</i> -propyl (256)	0.036	28	500	126
mean $K_i = 126$				
cyclohexyl (259)	0.040	25	500	144

Lineweaver-Burk Plot of 1/Rate versus 1/Concentration of *para*-nitrophenyl phosphate for Various Concentrations of 2-propylpropyl-1,3-bisphosphate (255) as an Inhibitors with *E.coli* Alkaline Phosphatase.





Lineweaver-Burk Plot of 1/Rate versus 1/Concentration of *para*-nitrophenyl phosphate for Various Concentrations of 2-*iso*-propylpropyl-1,3-bisphosphate (256) as Inhibitors with *E.coli* Alkaline Phosphatase.

6.4. Conclusions:

The initial reaction rates show that the 2-alkylpropyl-1,3-bisphosphates are only turned over to products at extremely slow rates, and the monophosphate intermediates are readily converted into the 1,3-propanediol products. The inhibition studies illustrate that the 1,3-bisphosphates are competitive inhibitors of *E.coli* and bovine alkaline phosphatase. Therefore, like the monophosphate ester substrates the 1,3-bisphosphates must be bound to the active site, interacting with the zinc ions and catalytic binding site.

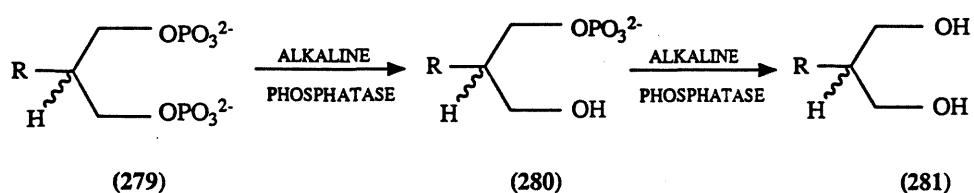
With bovine phosphatase, the inhibition constants K_i , calculated for each of the 1,3-bisphosphates are all within the same order of magnitude and are 10 times greater than the K_m value of *para*-nitrophenyl phosphate. The K_i values are approximately 10^3 times smaller than the K_m of the 1-phenylethyl-1-phosphate substrates. A similar pattern is seen for the K_i for the 1,3-bisphosphates in their reactions with *E.coli* alkaline phosphatase. The K_i values are 20 times greater than the K_m values of *para*-nitrophenyl phosphate, whereas, the K_m values of the monophosphate substrates are 10^3 times greater. These results indicate that the 1,3-bisphosphates are bound far more tightly to the active site of the *E.coli* and bovine phosphatases. It is possible that the slow reaction rate may be due to the slow release of phosphate monoester and/or inorganic phosphate products from the active site. Alternatively, the binding of the phosphate in the wrong orientation (non-productively) may result in the slow reaction rates. Progressive hydrolysis of both phosphate groups of the 1,3-bisphosphates is possible, in which the monophosphate intermediate twists within the active site to allow cleavage of the second phosphate moiety. This is highly speculative and there is no clear evidence to support this. An alternative suggestion is that both phosphate groups bind to the zinc ion centres of the catalytic region. The consequence of this is to reduce the K_i values and extend the

reaction times. This again is highly speculative.

Changing the size and length of the alkyl group appears to have little effect on the inhibition constants determined for the 2-alkylpropyl-1,3-bisphosphates or their reaction times. This implies that alkyl groups attached to the prochiral C-2 centre do not contribute significantly to the inhibition of the alkaline phosphatases, nor to the slow turnover rate. The 2-phenylpropyl-1,3-bisphosphate (**252**) is readily turned over, indicating that its binding with the lipophilic site is better than that of the alkyl derivatives. This is not unexpected as previous work described in **Chapter 3**, illustrates how important an aromatic substituent is in determining the reactivity of the phosphate substrates.

The size and hydrophobic nature of the alkyl group maybe significant in determining the prochiral selectivity of the alkaline phosphatases towards the *meso*-1,3-bisphosphates. However, because the monophosphate intermediates are apparently readily turned over this was impossible to determine. Once the monophosphate intermediate (**280**) is produced, the bovine alkaline phosphatase appears to readily turn it over to yield the 1,3-propanediol product (**281**) and free inorganic phosphate. This reaction sequence is illustrated below in **Scheme 6.3**. This observation suggests that the intermediate monoester product (**280**) is a better substrate of the bovine phosphatase than its parent 1,3-bisphosphate (**279**).

Scheme 6.3:



To confirm that these monophosphates are turned over faster than the 1,3-bisphosphates, several 2-alkylpropyl-monophosphates needed to be synthesised and their properties as substrates determined independently.

6.5. Reactions of 2-alkyl-3-hydroxypropyl-1-phosphates (262-263) with bovine alkaline phosphatase:

To investigate the proposal that the 2-alkylpropyl-monophosphate intermediates are reacting faster than the parent 2-alkylpropyl-1,3-bisphosphates, two monophosphates (**282**) of the type shown in **Figure 6.3**, were prepared. The 2-phenyl- (**262**) and 2-propyl-3-hydroxypropyl-1-phosphates (**263**) were prepared according to the synthetic sequence detailed in **Chapter 2**. These monophosphate materials were incubated at 37°C with the bovine phosphatase and their progress was monitored by ^{31}P NMR spectroscopy. The results of these experiments are shown in **Table 6.6**.

Figure 6.3:

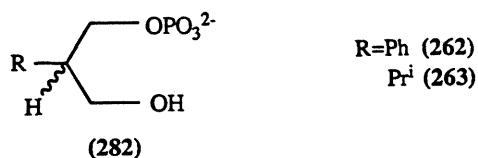


Table 6.6: Reactions of the Bovine Alkaline Phosphatase

<u>Monophosphate substrate</u>	<u>Reaction time</u>	<u>Product</u>
2-phenyl (262)	6 hours	1,3-diol
2-propyl (263)	14 hours	1,3-diol

These results clearly show that the 2-alkyl monophosphate substrates (**262**) and (**263**) are turned over by the bovine phosphatase more rapidly than the 2-alkylpropyl-1,3-bisphosphate derivatives (**252**) and (**255**) respectively. The simple change from the non-

reacting negatively charge phosphate group to a polar hydroxy group has a profound effect on the reaction rates of the 2-phenyl and 2-propyl phosphate derivatives.

6.5.1: Conclusions:

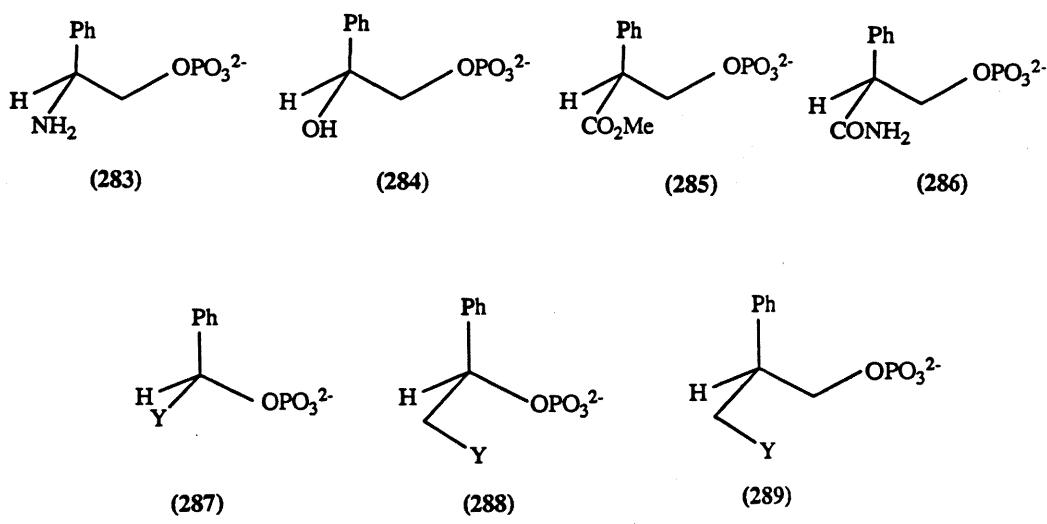
The turnover of the hydroxy-monophosphate substrates (262) and (263) are much faster than the respective 1,3-bisphosphate derivatives (252) and (255). Clearly, the second phosphate group makes a significant contribution to the extended reaction times of the 1,3-bisphosphates. Comparable *monophosphate* substrates attain 50% completion in around one third the time. It would appear that bovine alkaline phosphatase will preferentially turnover substrates containing polar hydroxy groups as opposed to a second negatively charged phosphate moiety. However, compared to the reaction times of the simple secondary alkyl monophosphates, the hydroxy-monophosphates (262) and (263) appear to react slower.

6.6. Further work:

This work has concentrated on stereoselectivities arising from differing steric demand and/or hydrophobic interactions with a putative hydrophobic binding site proposed by Williams *et.al.*^{66,84} The possible existence of other potential interactions (hydrogen bonding, electrostatic) that could be exploited to push the observed enantioselectivities higher is clearly something that should be explored. The enantioselectivity of the alkaline phosphatases with the phosphate derivatives possessing various functional groups is an obvious progression for this research. In particular, substituting functional groups into the benzyl-1-phosphate template, replacing the alkyl groups used in Chapter 3. These can also be used to probe further and evaluate the relationships of the lipophilic and alternative binding sites. Using monophosphate esters with functional groups bound to a benzylic carbon centre would be of interest, to evaluate the selectivity of the various

alkaline phosphatases. These groups are able to participate in hydrogen bonding. It would be possible for these groups to bind to amino acid residues within the less hydrophobic alternative binding site. The result of enhanced binding of these functional groups and the binding of the phenyl ring to the lipophilic binding site will be to fix the substrate into the well defined orientation (as seen with **Models 1 and 2**), which would serve to enhance the stereoselectivity. Shown below in **Figure 6.4**, are the four racemic substrates of the benzyl-1-phosphate template possessing an amine (**283**), alcohol (**284**), ester (**285**) and amide (**286**) groups. Removing the methylene linker between the chiral centre and the phosphate group would lead to the racemic substrates (**287**) and (**288**) shown in **Figure 6.4**. In these racemic derivatives, the functional groups (Y) are either attached directly to the chiral centre or made more remote by the inclusion of a methylene group. Finally, racemic substrates with both the phosphate group and functional groups (Y) remote from the chiral centre can be investigated.

Figure 6.4:



Future avenues of research might include the evaluation of *ortho*- and *meta*-substituents on the phenyl ring. Included in this work would be the study of multi-substituted phenyl ring derivatives. It is important to retain the phenyl ring component since results in Chapters 3-6 show that an aromatic group greatly contributes to favourable binding. The exploitation of the phosphatases to synthesise chiral building blocks also needs to be addressed, since many resultant phosphate functionalities can be chemically exploited. Their successful use would avoid the need for tedious protection and deprotection steps common to many synthetic strategies. The phosphate group can be readily converted into a phosphate triester leaving group.

Further work with the alkaline phosphatases using various altered monophosphate substrates, not only offers an opportunity for a better understanding of the binding regions of these alkaline phosphatases, but can also be used to evaluate their potential as reagents in asymmetric synthesis. Regrettably, the opportunity to prepare and investigate the selectivity of these monophosphate esters (283-289) with the alkaline phosphatases was precluded by a shortage of time.

Chapter 7

Experimental

REAGENTS AND SOLVENTS:

All the reactions described in the experimental section were performed in oven dried glassware. Moisture sensitive reactions were performed under dry nitrogen. Benzene, diethyl ether and tetrahydrofuran were distilled immediately before use, under dry nitrogen from sodium and benzophenone. Dichloromethane, ethyl acetate and petroleum ether were distilled from powdered calcium hydride under nitrogen. Pyridine was distilled from potassium hydroxide under nitrogen. Triethylamine was distilled prior to use. Methanol and ethanol were distilled from magnesium and iodine under dry nitrogen. The distilled water used throughout these experiments was of high purity, obtained from a milliqui-purification system. All other reagents and solvents were purified as necessary following the published procedures.^{164,165}

APPARATUS, INSTRUMENTATION AND TECHNIQUES:

Melting points were determined as covered microscope slides, with a Kofler hot-stage melting point apparatus and are uncorrected. Elemental analyses were performed by Butterworth Laboratories, Teddington, Middlesex. Infra-red (IR) spectra were obtained as suspensions in nujol or as neat films, using a Perkin-Elmer 298 spectrophotometer, in which the following descriptions are used: br=broad, m=medium, s=strong, vs=very strong and w=weak. All are reported in cm⁻¹.

All 90MHz ¹H NMR spectra were recorded on a Varian EM-390 spectrometer. High field ¹H NMR (300MHz), ¹³C NMR (75MHz), ³¹P NMR (121MHz) and ¹⁹F NMR (282MHz) spectra were recorded on a Bruker AM-300 spectrometer at the University of Leicester. Spectra were recorded in CDCl₃ using tetramethylsilane (TMS) as the internal standard (δ =0.0ppm), unless otherwise stated. The following descriptions are used to

describe the ^1H NMR spectra: br=broad, d=doublet, m=multiplet, q=quartet, quin=quintet, s=singlet and t=triplet. Coupling constants (J) are reported in hertz (Hz). For the ^{13}C NMR peaks the following descriptions are used: q=methyl (CH_3), t=methylene (CH_2), d=methine (CH) and s=carbon with no hydrogens attached (C). The ^{19}F NMR chemical shifts were reported relative to trichlorofluoromethane (CFCl_3) as the internal standard ($\delta=0.0\text{ppm}$). The ^{31}P NMR were recorded on JEOL FX90Q (36.24MHz) spectrometer, and were all completely decoupled unless otherwise indicated. They were all run at a sweep width of 10.0K unless stated differently. The chemical shifts are reported relative to the tetrahydroxyphosphonium ion ($\text{P}(\text{OH})_4$)¹⁴³ standard ($\delta=0.0\text{ppm}$) in which downfield shifts are reported as positive. Ultraviolet (UV) spectra were performed on a Shimadzu UV-240 ultraviolet-visible spectrophotometer. Optical rotations were recorded on a Perkin-Elmer 141 polarimeter. High resolution mass spectra and accurate mass analysis were performed on a Kratos Concept 1H double-focusing mass spectrometer at the University of Leicester.

Flash chromatography was performed according to Still¹⁶⁶ using Merck and Co Kiesel 60 silica gel 230-400 mesh. Analytical thin layer chromatography (TLC) was performed on Merck and Co. aluminium sheets precoated with silica 60-254. Preparative layer chromatography was performed on Machery-Nagel silica preparative plates G-200. Ultraviolet light, iodine, potassium permanganate solution and phosphomolybdic acid in 95% ethanol were used to visualize the compounds. Reactions requiring heat were placed in thermostated oil baths or heating mantles. Reactions conducted at reduced temperatures were achieved by immersion in an appropriate bath; dry ice/acetone for -78°C, ice/ $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ for -20°C and ice/ H_2O for 0°C. Solutions and liquids were delivered by syringe and cannula through rubber septa or by pressure equalizing dropping funnels where appropriate.

The ion exchange chromatography was performed using DEAE-sephadex A-25 resin from Pharmacia. Before use, it was left to swell for 24 hours in 1M ammonium bicarbonate solution. It was purified and contaminants were removed by subsequent washings with excess volumes of 1M NH_4HCO_3 solution and distilled water. Triethylammonium bicarbonate (TEAB) buffer, the eluent, was prepared as a 1M stock solution. Initially the triethylamine (101g, 139ml) was dissolved in distilled water and made to a 1M standard solution in 1 litre. Gaseous carbon dioxide was then bubbled through the mixture at 25°C until the pH of the solution approached pH 7.0. The necessary concentrations of 700mM, 400mM, 100mM, and 50mM were prepared as required by the appropriate dilutions of volumes of the stock solution with distilled water. Fractions were collected in test tubes. UV absorbing materials were directly observed as they eluted by use of a Cecil UV-detector and recorded on a chart recorder. Non-UV active phosphate derivatives were detected using the Briggs phosphate test.^{134a} Evaporation of fractions from this type of column gave solid TEAB which was broken down and lost by co-evaporation with methanol several times.

PHOSPHATE ASSAY:^{134a}

Column eluents were assayed for phosphate in the following manner. Ammonium molybdate (2-5g) was dissolved in a mixture of water (20ml) and concentrated sulphuric acid (8ml). Quinol was dissolved in water (20ml-acidified with one drop of concentrated sulphuric acid). Finally sodium sulphite (4g) was dissolved in water (20ml). Samples (250 μ l) from the fractions were put in clean, dry test tubes and evaporated to dryness at 180°C for 30 minutes. Four drops of concentrated sulphuric acid were added to the residue and the mixture was again heated at 180°C for 30 minutes. When the mixture had cooled, water (250 μ l) was added. The molybdate (500 μ l), the quinol (250 μ l) and the sodium

sulphite ($250\mu\text{l}$) solutions were added to each assay tube and the mixture boiled for 10 seconds, by which time samples containing phosphate produced a blue colouration. Phosphate was quantified by measuring the absorbance at 820nm in a UV-spectrophotometer and comparisions made with a standard curve produced by performing the assay on known concentrations of KH_2PO_4 .

(R,S)-2-Methyl-1-phenyl-1-propanol (94):

Lithium aluminium hydride (5.12g, 30mmol) was added to dry ether (35ml) and left stirring for ten minutes. To this stirred solution was slowly added 2-methyl-1-phenyl-1-propanone (**93**) (2.96g, 3.0ml, 20mmol) in dry ether (40ml) over a fifteen minute period. After the addition had been completed the reaction mixture was refluxed for ninety minutes. On cooling the solution was transferred to a large beaker and the flask rinsed with ether (3x10ml). The combined solutions were cooled to 0°C and the excess lithium aluminium hydride was quenched by careful addition of ether (30ml) and water (10ml). To this stirred mixture was added anhydrous sodium sulphate to produce a crystalline by-product of the slurry. The ether layer from the solution was then decanted off and further dried over anhydrous sodium sulphate. The organic layer was concentrated and dried under vacuum, before the remaining residue was distilled under reduced pressure 111-113°C, 12mmHg (lit.⁹¹ 110-111°C, 12mmHg) using a Kugelrohr apparatus to yield the title compound as a clear liquid (2.3g, 76%).

ν_{max} (thin film) 3360brs (OH), 2950s, 2920s and 2870s (C-H aryl), 1490m, 1450s, 1360m, 1200m, 1180m, 1070m, 1020vs, 910m, 760s.

δ_{H} (300MHz:CDCl₃) 7.23 (5H, m, C₆H₅), 4.23 (1H, d, J 6.8Hz, CH-OH), 2.46 (1H, brs, CH-OH), 1.87 (1H, dq, J 13.0, 6.8Hz, CH(Me)₂), 0.94 (3H, d, J 6.8Hz, CH(CH₃)₂), 0.73 (3H, d, J 6.8Hz, CH(CH₃)₂).

δ_{C} (75MHz:CDCl₃) 143.60(s), 127.99(d), 127.19(d), 126.54(d), 79.81(d), 35.13(d), 18.90(q), 18.30(q).

(R,S)-(para-Nitrophenyl)ethanol (98):

This lithium aluminium hydride reduction procedure was also used in the preparation of 1-(*para*-nitrophenyl)ethanol (**98**) (878mg, 62%) from aceto-*para*-nitrophenone.

B.p. 137-139°C, 4mmHg (lit.¹⁶⁷ 138-139°C, 4mmHg).

ν_{max} (thin film) 3400brs (OH), 2980s, 2820m, 1620vs (benzene ring), 1580s, (conjugated benzene ring), 1500s, 1360s, 1300s (conjugated NO₂ group), 1190s, 1110m, 1080vs, 1020m, 1000vs, 900s, 830s, 800s.

δ_{H} (300MHz:CDCl₃) 8.11 (2H, d, J 8.8Hz, C₆H₄), 7.50 (2H, d, J 8.5Hz, C₆H₄), 4.97 (1H, q, J 6.5Hz, MeCH-), 3.22 (1H, brs, MeCH-OH), 1.48 (3H, d, J 6.5Hz, CH₃CH-).

δ_{C} (75MHz:CDCl₃) 153.40(s), 146.92(s), 126.13(d), 123.62(d), 69.31(d), 25.37(q).

The general procedure for the preparation of the phenyl alcohol derivatives is by the reaction of benzaldehyde with the corresponding Grignard reagents (**81-86**). This is illustrated by the preparation of 1-phenyl-1-propanol (**87**).

(R,S)-1-Phenyl-1-propanol (87):

To a 250ml round bottom flask at 0°C charged with nitrogen was carefully added the solution of ethylmagnesium bromide (**81**) (3.0M, 14.67ml, 40mmol) in ether and left stirring with a small amount of dry ether (10ml). To this was slowly added the benzaldehyde (2.95ml, 3.07g, 29mmol) in dry ether (25ml) over a thirty minute period. After the addition had been completed the reaction mixture was refluxed for thirty five minutes. On cooling the mixture was poured into a large beaker containing 10% sulphuric acid (20ml) and crushed ice (30g). The reaction flask was rinsed with a further amount of ether (15ml) and water (10ml). This mixture was stirred vigorously to promote complete hydrolysis of the addition product. The solution was transferred to a separatory funnel and the aqueous layer removed. The organic layer was washed successively with 10% sulphuric acid (20ml) and saturated sodium chloride solution (20ml), before being separated and dried over anhydrous sodium sulphate. The solution was filtered and concentrated before being dried under vacuum. The remaining residue was distilled under

reduced pressure 102-103°C, 11mmHg, (lit.⁸⁵ 100-101°C, 11mmHg) to yield the 1-phenyl-1-propanol product (**87**) (2.38g, 60%).

ν_{\max} (thin film) 3360brs (OH), 2950s, 2920s and 2870s (C-H aryl), 1490m, 1450s, 1200m, 1100s, 1050m, 1010s, 970s, 890m, 750s.

δ_{H} (300MHz:CDCl₃) 7.24 (5H, m, C₆H₅), 4.43 (1H, t, J 6.5Hz, PhCHEt), 2.92 (1H, brs, CH-OH), 1.76-1.59 (2H, m, CH₂CH₃), 0.83 (3H, t, J 7.4Hz, CH₂CH₃).

δ_{C} (75MHz:CDCl₃) 144.60(s), 128.20(d), 127.23(d), 125.97(d), 75.74(d), 31.78(t), 10.09(q).

(R,S)-1-Phenyl-1-butanol (88):

(R,S)-1-Phenyl-1-butanol (**88**) (3.39g, 83%) was prepared using a similar method to the preparation of (**87**).

B.p. 119-120°C, 15mmHg (lit.⁸⁶ 119-120°C, 15mmHg).

ν_{\max} (thin film) 3360brs (OH), 2950s, 2920s, 2870s, 1490m, 1440s, 1200m, 1110m, 1060s, 1020s, 960m, 760s.

δ_{H} (300MHz:CDCl₃) 7.21 (5H, m, C₆H₅), 4.47 (1H, t, J 6.3Hz, CH-OH), 3.39 (1H, brs, CH-OH), 1.72-1.52 (2H, m, CH₂-CH), 1.30-1.10 (2H, m, CH₂CH₂Me), 0.85 (3H, t, J 7.4Hz, CH₂CH₂CH₃).

δ_{C} (75MHz:CDCl₃) 145.04(s), 128.17(d), 127.14(d), 125.94(d), 74.07(d), 41.20(t), 18.98(t), 13.96(q).

(R,S)-1-Phenyl-1-pentanol (89):

(R,S)-1-Phenyl-1-pentanol (**89**) (3.42g, 72%) was prepared using a similar method to the preparation of (**87**).

B.p. 137-139°C, 21mmHg (lit.⁸⁷ 136-138°C, 21mmHg).

ν_{\max} (thin film) 3250brs (OH), 2960s, 2920s, 2860s, 1490m, 1450s, 1200m, 1040s, 1020s, 960s, 700s.

δ_{H} (300MHz:CDCl₃) 7.25 (5H, m, C₆H₅), 4.51 (1H, dd, J 7.2, 6.2Hz, CH-OH), 2.79 (1H, brs, CH-OH), 1.66 (2H, m, CH-CH₂Et), 1.26 (4H, m, CH₂-(CH₂)₂Me), 0.85 (3H, t, J 7.0Hz, CH₂CH₃).
 δ_{C} (75MHz:CDCl₃) 144.97(s), 128.23(d), 127.22(d), 125.89(d), 74.43(d), 38.76(t), 27.95(t), 22.60(t), 14.00(q).

(R,S)-2,2-Dimethyl-1-phenyl-1-propanol (90):

(R,S)-2,2-Dimethyl-1-phenyl-1-propanol (**90**) (2.11g, 70%) was prepared using a similar method to the preparation of (**87**).

B.p. 89-90°C, 5mmHg (lit.,⁸⁸ 90°C, 5mmHg).

M.p. 42-44°C (lit.,⁸⁸ 43-44°C).

ν_{max} (nujol mull) 3400brs (OH), 2950s, 2920s and 2870s (C-H aryl), 1480m, 1450s, 1390m, 1360s, 1250m, 1170m, 1090m, 1050s, 1000s, 900s, 790m, 700s.

δ_{H} (300MHz:CDCl₃) 7.28 (5H, m, C₆H₅), 4.37 (1H, s, CHPh), 1.95 (1H, s, CH-OH), 0.91 (9H, s, C(CH₃)₃).

δ_{C} (75MHz:CDCl₃) 142.13(s), 128.48(d), 127.55(d), 127.21(d), 82.34(d), 35.59(s), 25.91(q).

(R,S)-1-Phenyl-2-propen-1-ol (91):

(R,S)-1-Phenyl-2-propen-1-ol (**91**) (2.14g, 72%) was prepared using a similar method to the preparation of (**87**).

B.p. 89-90°C, 11mmg (lit.,⁸⁹ 90-92°C, 11mmHg).

ν_{max} (thin film) 3300brs (OH), 3100m, 2870s, 1490s, 1200s, 1080m, 1030s.

δ_{H} (300MHz:CDCl₃) 7.30 (5H, m, C₆H₅), 5.99 (1H, ddd, J 16.6, 10.6, 6.0Hz, H₂C=CH), 5.27 (1H, dt, J 16.6, 1.3Hz, H_AH_BC=CH), 5.13 (1H, dt, J 10.6, 1.3Hz, H_AH_BC=CH), 4.56 (1H, d, J 6.6Hz, PhCH), 2.62 (1H, brs, CH-OH).

δ_{C} (75MHz:CDCl₃) 142.56(s), 140.18(d), 128.43(d), 127.59(d), 126.29(d), 114.97(t),

75.18(d).

(R,S)-1-Phenyl-2-propyn-1-ol (92):

(R,S)-1-Phenyl-2-propyn-1-ol (**91**) (1.08g, 82%) was prepared using a similar method to the preparation of (**87**).

B.p. 112-114°C, 12mmHg (lit.⁹⁰ 114°C, 12mmHg).

ν_{max} (thin film) 3400brs (OH), 3300s (C≡C-H stretching), 3020w, 2120w, 1490s, 1450s, 1200s, 940s, 740s, 650s (C≡C-H deformation).

δ_{H} (300MHz:CDCl₃) 7.46 (2H, m, C₆H₅), 7.28 (3H, m, C₆H₅), 5.35 (1H, d, J 2.2Hz, CHPh), 3.41 (1H, brs, CH-OH), 2.59 (1H, d, J 2.3Hz, HC≡C).

δ_{C} (75MHz:CDCl₃) 139.96(s), 128.50(d), 128.32(d), 126.57(d), 83.54(s), 74.75(d), 64.07(d).

(R,S)-(para-Methoxyphenyl)ethanol (100):

The same method was also used for the preparation of the *para*-methoxy derivative from the reaction of *para*-methoxybenzaldehyde (**99**) (anisaldehyde) with methylmagnesium bromide to yield (**100**) (4.58g, 82%).

ν_{max} (thin film) 3360brs (OH), 2980s (OMe), 1600s and 1520vs (benzene ring), 1350vs (OMe), 1200m, 1090vs, 1050s, 850vs, 750m, 700s.

δ_{H} (300MHz:CDCl₃) 7.18 (2H, d, J 8.5Hz, MeOC₆H₄), 6.78 (2H, d, J 8.7Hz, C₆H₄), 4.70 (1H, q, J 6.4Hz, MeCH), 3.69 (3H, s, CH₃OC₆H₄), 3.47 (1H, brs, CH-OH), 1.37 (2H, d, J 6.5Hz, CH₃CH).

δ_{C} (75MHz:CDCl₃) 158.63(s), 138.24(s), 126.64(d), 113.64(d), 69.50(d), 55.12(q), 25.06(q).

(R,S)-2-Phenyl-1-propanol (103):

To a stirred solution of borane-tetrahydrofuran complex (1.0M, 105ml, 105mmol), under nitrogen and cooled to 0°C was slowly added 2-phenylpropene (**101**, α -methylstyrene) (11.26g, 95mmol) and left stirring for a further two hours. After this time

the reaction was cooled to -78°C and ice water (20ml) was added to the flask. The mixture was allowed to warm to 0°C and 3N sodium hydroxide (40ml) and 20% hydrogen peroxide (40ml) were added to the mixture. The reaction was slowly warmed to room temperature and left stirring for a further thirty minutes. The reaction was quenched by the addition of (30ml) of ice water and the organic material was extracted with ether (3x20ml). The resultant solution was dried over anhydrous sodium sulphate, concentrated and dried under vacuum. The title compound (**103**), was isolated as a clear liquid by distillation under reduced pressure 109-111°C, 10mmHg, (lit.¹⁶⁸ 110-111°C, 10mmHg), (11.02g, 85%).

ν_{\max} (thin film) 3335brs (OH), 3010s, 2960s, 2860s, 1490m, 1370m, 1070m, 1030s, 1010s, 760s.

δ_{H} (300MHz:CDCl₃) 7.17 (5H, m, C₆H₅), 3.50 (2H, dd, J 13.5, 6.5Hz, CH₂OH), 3.19 (1H, s, CH₂OH), 2.79 (1H, q, J=7.0Hz, CHMe), 1.18 (3H, d, J 9.0Hz, CHCH₃).

δ_{C} (75MHz:CDCl₃) 143.74(d), 128.05(d), 127.08(d), 126.03(d), 67.9(t), 41.98(d), 17.40(q).

(R,S)-2,3-Dimethyl-1-butanol (104):

(R,S)-2,3-Dimethyl-1-butanol (**104**) (1.02g, 83%) was prepared using a similar method to the preparation of (**103**).

B.p. 56-58°C, 18mmHg (lit.^{95a,169} 58-59°C, 18mmHg).

ν_{\max} (thin film) 3550brs (OH), 2960s, 2910s, 2880s, 1510m, 1390m, 1130w, 1030vs, 1000w.

δ_{H} (300MHz:CDCl₃) 3.54 (1H, dd, J 10.5, 5.9Hz, CH₂OH), 3.37 (1H, dd, J 10.5, 7.2Hz, CH₂OH), 3.32 (1H, brs, CH₂OH), 1.69 (1H, m, CH(Me)₂), 1.49 (1H, dq, J 13.0, 6.7Hz, CHMe), 0.91 (6H, d, J 6.7Hz, CH(CH₃)₂), 0.84 (3H, d, J 6.6Hz, CH-CH₃).

δ_{C} (75MHz:CDCl₃) 66.21(t), 41.41(d), 28.87(d), 20.71(q), 17.91(q), 12.50(q).

2-Cyanoethylphosphorodichloridite (147):

(Improved method based on work by Sinha *et.al.*^{105,106})

To a three necked 250ml flask was added phosphorus trichloride (145) (27.46g, 200mmol) and this was refluxed for two hours to remove all the hydrogen chloride impurities. After allowing to cool for thirty minutes the flask was charged with a nitrogen delivery system and cooled to -78°C. To the stirring solution of phosphorus trichloride (145) was added dry pyridine (15.82g, 200mmol) in dry ether (40ml) over a thirty minute period and then left stirring for a further twenty minutes. Freshly distilled 2-cyanoethanol (146) (14.2g, 200mmol) in dry ether (40ml) was added to the stirring solution over a 50 minute period. Once the addition had been completed the mixture was warmed to room temperature and stirred for a further thirty minutes. The pyridine hydrochloride by-product was filtered off and the flask rinsed with dry ether (2x20ml). The filtrate was then concentrated to a dark yellow viscous liquid and subsequently distilled under reduced pressure 70°C, 0.5mmHg (lit.,^{99a,105} 70-72°C, 0.5mmHg), to yield the title compound (147) (34.6g, 50%).

δ_p (36MHz:CDCl₃) 179.23ppm (lit.,^{95a,105,106} 178.49ppm).

δ_H (300MHz:CDCl₃) 4.43 (2H, dt, $^3J_{P-H}$ 6.3Hz, J 6.1Hz, P-O-CH₂), 2.81 (2H, dt, $^4J_{P-H}$ 2.1Hz, J 6.0Hz, CH₂CN).

δ_C (75MHz:CDCl₃) 116.70(s), 61.86(dt, $^2J_{P-C}$ 10.5Hz), 19.31(dt, $^2J_{P-C}$ 3.0Hz).

2-Cyanoethyl N,N-diisopropylchlorophosphoramidite (132):

A solution of 2-cyanoethyl phosphorodichloridite (147) (18.5g, 107mmol) in dry ether (25ml) at -78°C under nitrogen was stirred for fifteen minutes. To this was added 2 equivalents of *N,N*-diisopropylamine (21.7g, 30.1ml, 215mmol) in dry ether (25ml) over a fifty minute period. After the addition had been completed the flask was warmed to

-20°C and stirred for two hours, before being allowed to warm to room temperature and left overnight. The white precipitate of *N,N*-diisopropylamine hydrochloride was filtered off and the flask rinsed with dry ether (2x25ml). The resultant filtrate was concentrated to a dark yellow solution and distilled under reduced pressure to yield the title compound (**132**) 110-112°C, 1mmHg, (lit.,^{105,106} 110-112°C, 1mmHg), (18.6g, 65%). The clear liquid was stored under dry conditions at -4°C until used.

δ_p (36MHz:CDCl₃) 179.89ppm (lit.,^{105,106} 179.82ppm).

δ_h (300MHz:CDCl₃) 4.20 (1H, t, J 6.2Hz, P-O-CH₂), 4.04 (1H, t, J 6.1Hz, P-O-CH₂), 3.80 (2H, m, N-(CH(Me)₂)₂), 2.75 (2H, t, J 6.2Hz, CH₂CN), 1.26 (12H, d, J 6.8Hz, N-(CH(CH₃)₂)₂).

δ_c (75MHz:CDCl₃) 116.97(s), 60.43(dt, ²J_{P-C} 11.5Hz), 46.11(dd, ²J_{P-C} 13.0Hz), 23.64(brq), 19.88(dt, ³J_{P-C} 6.7Hz).

The general procedure for the preparation of the di-2-cyanoethyl phosphates (**156-169**) from the corresponding alcohols can be illustrated by the synthesis of (R,S)-1-phenylethyl-1-(di-2-cyanoethyl)-phosphate (**156**).

(R,S)-1-Phenylethyl-1-(di-2-cyanoethyl)phosphate (156):

The (R,S)-1-phenylethanol (**80**) (1g, 0.987ml, 8.2mmol) was dissolved in dry dichloromethane (8ml) and left stirring for ten minutes, before the *N,N*-diisopropylethylamine (1.26g, 1.71ml, 9.83mmol) was added and the mixture cooled to 0°C. 2-Cyanoethyl *N,N*-diisopropylchlorophosphoramidite (**132**) (2.42g, 2.28ml, 9.0mmol) was carefully added to the mixture and left stirring for one hour at room temperature to yield the four phosphoramidite diastereoisomer derivatives, identified in the ³¹P NMR spectrum as δ_p (36.2MHz:CH₂Cl₂) = 148.37, 148.89ppm. After this time, 2-cyanoethanol (64mg, 0.62ml, 9.0mmol) and tetrazole (1.15g, 16.4mmol) were added to the mixture

which was again left stirring for one hour at room temperature. This yielded the di-2-cyanoethyl phosphite triester derivative which is identified by the chemical shift at δ_p (36.2MHz:CH₂Cl₂) = 139.12ppm. The phosphite triester moiety was oxidized in the same reaction flask by cooling the mixture to -78°C and slowly adding *tert*-butylhydroperoxide (1.5g, 1.57ml, 16.4mmol). The mixture was stirred for a further 30 minutes before being allowed to warm to room temperature and stirred for an additional twenty minutes. The phosphate triester product was identified in the ³¹P NMR spectrum as δ_p (36.2MHz:CH₂Cl₂) = -3.22ppm. The reaction mixture was taken up in dichloromethane (3x20ml) and carefully washed with distilled water (2x10ml). The organic layer was separated and dried over anhydrous sodium sulphate, before being concentrated to a yellow oil. The yellow oil was then purified by flash chromatography, (EtOAc:CH₂Cl₂ (4:1) R_f=0.32) to yield the title compound (**156**) (1.24g, 52%).

ν_{max} (thin film) 2980s, 2820m, 2875s (O-CH), 2295w (CN), 1620s, 1520s (benzene ring), 1270s (P=O), 1042m (P-O-CH).

δ_H (300MHz:CDCl₃) 7.35-7.21 (5H, m, C₆H₅), 5.24 (1H, dq, ³J_{P,H} 14.1Hz, J 7.0Hz, PhCH-O), 4.13-4.04 (2H, m, CH₂C(H_A)₂-O), 4.02-3.91 (2H, m, C(H_B)₂-O), 2.62 (4H, dt, J 12, 6.2Hz, (CH₂CN)₂), 1.31 (3H, d, J 7.0Hz, CHCH₃).

δ_C (75MHz:CDCl₃) 142.20(s), 128.58(d), 127.43(d), 126.97(d), 116.65(s, CN), 116.54(s, CN), 80.13(dd, ²J_{P,C} 7.2Hz), 62.14(dt, ²J_{P,C} 5.7Hz), 62.07(dt, ²J_{P,C} 5.4Hz), 10.52(dt, ³J_{P,C} 7.3Hz), 19.42(dt, ³J_{P,C} 7.1Hz), 17.38(q).

m/z(EI): M⁺ 308(10%), (ROPO₃CH₂CH₂CN)⁺ 254(100%), (ROPO₂CH₂CH₂CN)⁺ 237(20%), (ROPO₃H)⁺ 201(25%), (ROPO₂)⁺ 185(10%), (ROP)⁺ 152(60%), (RO)⁺ 120(80%), (C₆H₅C≡O)⁺ 105(100%), (C₇H₇)⁺ 91(10%), (C₆H₅)⁺ 77(60%).

(R)-1-Phenylethyl-1-(di-2-cyanoethyl)phosphate (157):

(R)-1-Phenylethyl-1-(di-2-cyanoethyl)phosphate (**157**) (1.70g, 67%) was prepared using a similar method to the preparation of (**157**).

(EtOAc:CH₂Cl₂ (4:1) R_f=0.36).

δ_p(36MHz:CH₂Cl₂) -3.22ppm. [α]²³₅₈₉ = +4.60 (neat material).

(R)-1-phenylethyl-1-phosphoramidite δ_p(CH₂Cl₂) 148.34ppm.

(R)-1-phenylethyl-1-(di-2-cyanoethyl)phosphite δ_p(CH₂Cl₂) 139.21ppm.

(S)-1-Phenylethyl-1-(di-2-cyanoethyl)phosphate (158):

(S)-1-Phenylethyl-1-(di-2-cyanoethyl)phosphate (**158**) (1.50g, 68%) was prepared using a similar method to the preparation of (**156**).

(EtOAC:CH₂Cl₂ (4:1) R_f=0.36).

δ_p(36MHz:CH₂Cl₂) -3.32ppm. [α]²³₅₈₉ = -5.09 (neat material).

(S)-1-phenylethyl-1-phosphoramidite δ_p(CH₂Cl₂) 148.86ppm.

(S)-1-phenylethyl-1-(di-2-cyanoethyl)phosphite δ_p(CH₂Cl₂) 138.51ppm.

(R,S)-1-(para-Nitrophenyl)ethyl-1-(di-2-cyanoethyl)phosphate (166):

(R,S)-1-(para-Nitrophenyl)ethyl-1-(di-2-cyanoethyl)phosphate (**166**) (570g, 54%) was prepared using a similar method to the preparation of (**156**).

(EtOAc:CH₂Cl₂ (4:1) R_f=0.25).

δ_p(36MHz:CH₂Cl₂) -3.36ppm.

ν_{max}(thin film) 2985s, 2820m, 2260m (CN), 1580s, 1460s (conjugated NO₂), 1290s (P=O),

1062m (P-O-CH).

δ_H(300MHz:CDCl₃) 8.15 (2H, d, J 8.5Hz, C₆H₄), 7.65 (2H, d, J 8.5Hz, C₆H₄), 5.24 (1H, dq, ³J_{P-H} 14.6Hz, J 7.0Hz, PhCH-O), 4.20-4.07 (2H, m, C(H_A)₂-O), 4.04-3.92 (2H, m, C(H_B)₂-O), 2.75 (4H, dt, J 12, 6.4Hz, (CH₂CN)₂), 1.61 (3H, d, J 7.2Hz, CHCH₃).

δ_{C} (75MHz:CDCl₃) 153.41(s), 147.36(s), 127.14(d), 123.27(d), 116.72(s, CN), 116.66(s, CN), 78.23(dd, ²J_{P-C} 7.1Hz), 62.17(dt, ²J_{P-C} 5.7Hz), 62.10(dt, ²J_{P-C} 5.4Hz), 19.72(dt, ³J_{P-C} 7.1Hz), 19.62(dt, ³J_{P-C} 6.9Hz), 17.58(q).

(R,S)-1-(*para*-nitrophenyl)ethyl-1-phosphoramidite δ_{P} (CH₂Cl₂) 148.89, 148.54ppm.

(R,S)-1-(*para*-nitrophenyl)ethyl-1-(di-2-cyanoethyl)phosphite δ_{P} (CH₂Cl₂) 139.30ppm.

(R,S)-1-(*para*-Methoxyphenyl)ethyl-1-(di-2-cyanoethyl)phosphate (167):

(R,S)-1-(*para*-Methoxyphenyl)ethyl-1-(di-2-cyanoethyl)phosphate (167) (680g, 60%) was prepared using a similar method to the preparation of (156).

(EtOAc:CH₂Cl₂ (4:1) R_f=0.33).

δ_{P} (36MHz:CH₂Cl₂) -3.23ppm.

ν_{max} (thin film) 2980vs (OMe), 2790m (O-CH), 2245m (CN), 1520s, 1300s (P=O), 1020s (P-O-CH).

δ_{H} (300MHz:CDCl₃) 7.38 (2H, d, J 8.6Hz, C₆H₄), 7.28 (2H, d, J 8.5Hz, C₆H₄), 5.36 (1H, m, PhCH-O), 4.31-4.23 (2H, m, C(H_A)₂-O), 4.17-4.06 (2H, m, C(H_B)₂-O), 3.52 (3H, s, CH₃O-), 2.81 (4H, dt, J 12.6, 6.2Hz, (CH₂CN)₂), 1.41 (3H, d, J 6.4Hz, CHCH₃).

δ_{C} (75MHz:CDCl₃) 145.21(s), 133.62(s), 128.79(d), 127.62(d), 116.67(s, CN), 116.56(s, CN), 77.26(dd, ²J_{P-C} 6.5Hz), 62.21(dt, ²J_{P-C} 5.4Hz), 62.15(dt, ²J_{P-C} 5.7Hz), 52.12(q), 19.79(dt, ³J_{P-C} 6.9Hz), 19.57(dt, ³J_{P-C} 7.1Hz), 18.12(q).

(R,S)-1-(*para*-methoxyphenyl)ethyl-1-phosphoramidite δ_{P} (CH₂Cl₂) 147.96, 147.58ppm.

(R,S)-1-(*para*-methoxyphenyl)ethyl-1-(di-2-cyanoethyl)phosphite δ_{P} (CH₂Cl₂) 138.52ppm.

(R,S)-1-Phenylpropyl-1-(di-2-cyanoethyl)phosphate (159):

(R,S)-1-Phenylpropyl-1-(di-2-cyanoethyl)phosphate (159) (2.08g, 68%) was prepared using a similar method to the preparation of (156).

(EtOAc:CH₂Cl₂ (4:1) R_f=0.32).

δ_p (36MHz:CH₂Cl₂) -3.29ppm.

ν_{max} (thin film) 2972s, 2840m (O-CH), 2256m (CN), 1450s, 1085s (P-O-CH), 762m, 701s.

δ_H (300MHz:CDCl₃) 7.43-7.29 (5H, m, C₆H₅), 5.24 (1H, dt, ³J_{P-H} 9.1Hz, J 7.0Hz, PhCH-O), 4.19-4.06 (2H, m, O-C(H_A)₂), 4.04-3.91 (2H, m, O-C(H_B)₂), 2.61 (2H, dt, J 7.4, 6.6Hz, CH₂CN), 2.47 (2H, dt, J 7.8, 6.7Hz, CH₂CN), 2.01 (2H, dquin, J 22, 7.4Hz, CH₂Me), 0.93 (3H, t, J 7.4Hz, CH₃).

δ_C (75MHz:CDCl₃) 139.44(s), 128.70(d), 128.63(d), 126.55(d), 116.55(s,CN), 116.49(s,CN), 83.65(dd, ²J_{P-C} 5.4Hz), 62.00(dt, ²J_{P-C} 5.8Hz), 61.92(dt, ²J_{P-C} 6.1Hz), 30.66(dt, ³J_{P-C} 7.3Hz), 19.48(dt, ³J_{P-C} 7.0Hz), 19.34(dt, ³J_{P-C} 7.2Hz), 9.78(q).

m/z(EI): M⁺ 322(5%), (ROPO₃CH₂CH₂CN)⁺ 268(100%), (ROPO₃H)⁺ 215(30%), (ROP)⁺ 166(40%), (RO)⁺ 134(70%), (C₆H₅CH(Et))⁺ 119(80%), (C₆H₅C≡O)⁺ 105(100%), (C₇H₇)⁺ 91(10%), (C₆H₅)⁺ 77(60%).

(R,S)-1-phenylpropyl-1-phosphoramidite δ_p (CH₂Cl₂) 147.46, 146.92ppm.

(R,S)-1-phenylpropyl-1-(di-2-cyanoethyl)phosphite δ_p (CH₂Cl₂) 138.64ppm.

(R,S)-1-Phenylbutyl-1-(di-2-cyanoethyl)phosphate (160):

(R,S)-1-Phenylbutyl-1-(di-2-cyanoethyl)phosphate (**160**) (1.89g, 84%) was prepared using a similar method to the preparation of (**156**).

(EtOAc:CH₂Cl₂ (4:1) R_f=0.36).

δ_p (36MHz:CH₂Cl₂) -3.16ppm.

ν_{max} (thin film) 2960s, 2870s (O-CH), 2235w (CN), 1495s, 1255vs (P=O), 1078vs (P-O-alkyl), 840s.

δ_H (300MHz:CDCl₃) 7.41-7.30 (5H, m, C₆H₅), 5.30 (1H, dt, ³J_{P-H} 8.6Hz, J 6.7Hz, PhCH), 4.09-3.90 (4H, m, (CH_AH_B-O)₂), 2.66 (2H, dt, J 10.4, 6.2Hz, CH₂CN), 2.51 (2H, dt, J 8.9, 6.2Hz, CH₂CN), 2.03 (1H, m, CH₂CH-O), 1.80 (1H, m, CH₂CH-O), 1.39 (2H, m, CH₂Me),

0.94 (3H, t, J 7.3Hz, CH₃).

δ_C(75MHz:CDCl₃) 139.74(s), 127.72(d), 128.66(d), 126.54(d), 116.46(s,CN), 116.41(s,CN), 82.26(dd, ²J_{P-C} 6.4Hz), 61.97(dt, ²J_{P-C} 5.7Hz), 61.89(dt, ²J_{P-C} 6.2Hz), 39.63(dt, ³J_{P-C} 7.4Hz), 19.48(dt, ³J_{P-C}=7.7Hz), 19.41(dt, ³J_{P-C} 7.7Hz), 18.63(t), 13.61(q).

m/z(EI): M⁺ 336(5%), (ROPO₃)⁺ 239(25%), (PrOPO₃)⁺ 149(10%), (C₆H₅CH(Pr))⁺ 133(75%), (C₇H₇O)⁺ 117(15%), (C₆H₅C≡O)⁺ 105(100%), (C₇H₇)⁺ 91(10%), (C₆H₅)⁺ 77(60%).

(R,S)-1-phenylbutyl-1-phosphoramidite δ_P(CH₂Cl₂) 147.66, 146.86ppm.

(R,S)-1-phenylbutyl-1-(di-2-cyanoethyl)phosphite δ_P(CH₂Cl₂) 138.78ppm.

(R,S)-2-Methyl-1-phenylpropyl-1-(di-2-cyanoethyl)phosphate (161):

(R,S)-2-Methyl-1-phenylpropyl-1-(di-2-cyanoethyl)phosphate (161) (1.54g, 68%) was prepared using a similar method to the preparation of (156).

(EtOAc:CH₂Cl₂ (4:1) Rf 0.35).

δ_P(36MHz:CH₂Cl₂) -3.02ppm.

ν_{max}(thin film) 2980s, 2840m (O-CH), 2240m (CN), 1450w, 1260s (P=O), 1050vs (P-O-alkyl), 770s, 700s.

δ_H(300MHz:CDCl₃) 7.40-7.27 (5H, m, C₆H₅), 5.02 (1H, dd, ³J_{P-H} 7.5Hz, J 7.0Hz, PhCH), 4.22-4.10 (2H, m, O-C(H_A)₂), 4.03-3.90 (2H, m, O-C(H_B)₂), 2.63 (2H, dt, J 9.8, 6.7Hz, CH₂CN), 2.49 (2H, dt, J 11.6, 6.2Hz, CH₂CN), 2.15 (1H, dq, J 13.2, 6.4Hz, CH(Me)₂), 1.06 (3H, d, J 6.6Hz, CH-CH₃), 0.79 (3H, d, J 6.7Hz, CH-CH₃).

δ_C(75MHz:CDCl₃) 138.83(s), 128.44(d), 128.38(d), 126.98(d), 116.95(s,CN), 116.88(s,CN), 86.99(dd, ²J_{P-C} 6.7Hz), 62.14(dt, ²J_{P-C} 4.9Hz), 62.03(dt, ²J_{P-C} 5.1Hz), 34.61(dd, ³J_{P-C} 7.4Hz), 19.38(dt, ³J_{P-C} 6.8Hz), 19.24(dt, ³J_{P-C} 7.0Hz), 18.48(q), 18.33(q).

m/z(EI): M⁺ 336(10%), (ROPO₃)⁺ 239(35%), (PrⁱOPO₃)⁺ 149(15%), (C₆H₅CH(Pr))⁺

133(75%), (C₇H₇O)⁺ 117(15%), (C₆H₅C≡O)⁺ 105(90%), (C₇H₇)⁺ 91(60%), (C₆H₅)⁺ 77(25%).

(R,S)-2-methyl-1-phenylpropyl-1-phosphoramidite δ_p(CH₂Cl₂) 147.43, 148.03ppm.

(R,S)-2-methyl-1-phenylpropyl-1-(di-2-cyanoethyl)phosphite δ_p(CH₂Cl₂) 139.61ppm.

(R,S)-1-Phenylpentyl-1-(di-2-cyanoethyl)phosphate (162):

(R,S)-1-phenylpentyl-1-(di-2-cyanoethyl)phosphate (162) (1.71g, 74%) was prepared using a similar method to the preparation of (156).

(EtOAc:CH₂Cl₂ (4:1) R_f=0.30).

δ_p(36MHz:CH₂Cl₂) -3.02ppm.

ν_{max}(thin film) 2970s, 2800w, 2250sharp (CN), 1490w, 1450m, 1275vs (P=O), 1200vs (P-O-alkyl), 760m.

δ_H(300MHz:CDCl₃) 7.40-7.32 (5H, m, C₆H₅), 5.32 (1H, dt, ³J_{P-H} 9.1Hz, J 6.9Hz, PhCH), 4.20-4.08 (1H, m, CH₂-O), 4.06-3.93 (3H, m, CH₂-O), 2.65 (2H, dt, J 10.4, 5.5Hz, CH₂CN), 2.52 (2H, dt, J 8.7, 6.2Hz, CH₂CN), 2.02 (1H, m, CH₂-CH), 1.87 (1H, m, CH₂-CH), 1.33 (4H, m, (CH₂)₂Me), 0.86 (3H, t, J 6.8Hz, CH₃).

δ_C(75MHz:CDCl₃) 139.03(ds, ³J_{P-C} 2.2Hz), 127.70(d), 127.65(d), 125.58(d), 116.21(s,CN), 116.14(s,CN), 81.08(dd, ²J_{P-C} 6.2Hz), 61.35(dt, ²J_{P-C} 5.9Hz), 61.27(dt, ²J_{P-C} 6.1Hz), 36.31(dt, ³J_{P-C} 7.0Hz), 26.38(t), 21.25(t), 18.04(dt, ³J_{P-C} 7.7Hz), 18.35(dt, ³J_{P-C} 7.5Hz), 12.92(q).

m/z(EI): M⁺ 350(5%), (ROPO₂CH₂CH₂CN)⁺ 281(100%), (ROPO₂H)⁺ 216(20%), (C₆H₅CH(Buⁿ))⁺ 147(30%), (C₇H₇O)⁺ 117(90%), (C₆H₅C≡O)⁺ 105(60%), (C₇H₇)⁺ 91(100%), (C₆H₅)⁺ 77(40%).

(R,S)-1-phenylpentyl-1-phosphoramidite δ_p(CH₂Cl₂) 148.61ppm.

(R,S)-1-phenylpentyl-1-(di-2-cyanoethyl)phosphite δ_p(CH₂Cl₂) 138.72ppm.

(R,S)-2,2-Dimethyl-1-phenylpropyl-1-(di-2-cyanoethyl)phosphate (163):

(R,S)-2,2-Dimethyl-1-phenylpropyl-1-(di-2-cyanoethyl)phosphate (163) (680mg, 35%) was prepared using a similar method to the preparation of (156).

(EtOAc:CH₂Cl₂ (4:1) R_f=0.30).

δ_p(36MHz:CH₂Cl₂) -2.82ppm.

ν_{max}(thin film) 2933s, 2790s, 2240m (CN), 1490m, 1390m, 1265s (P=O), 1067s (P-O-CH), 860m.

δ_H(300MHz:CDCl₃) 7.38-7.26 (5H, m, C₆H₅), 5.05 (1H, d, ³J_{P-H} 8.1Hz, PhCH), 4.12-4.05 (2H, m, O-C(H_A)₂), 4.02-3.94 (2H, m, O-C(H_B)₂), 2.64 (2H, m, CH₂CN), 2.42 (2H, m, CH₂CN), 0.97 (9H, s, C(CH₃)₃).

δ_C(75MHz:CDCl₃) 137.56(s), 128.22(d), 127.79(d), 127.75(d), 116.81(s), 116.76(s), 89.03(dd, ²J_{P-C} 7.1Hz), 62.14(dt, ²J_{P-C} 5.2Hz), 62.02(dt, ²J_{P-C} 5.3Hz), 35.93(ds, ³J_{P-C} 7.7Hz), 25.69(q), 19.38(dt, ³J_{P-C} 7.3Hz), 19.28(dt, ³J_{P-C} 7.2Hz).

m/z(EI): M⁺ 350(15%), (ROPO₂CH₂CH₂CN)⁺ 281(90%), (ROPO₂H)⁺ 216(50%), (C₆H₅CH(Bu^t))⁺ 147(35%), (C₇H₇O)⁺ 117(90%), (C₆H₅C≡O)⁺ 105(60%), (C₇H₇)⁺ 91(100%), (C₆H₅)⁺ 77(60%).

(R,S)-2,2-dimethyl-1-phenylpropyl-1-phosphoramidite δ_p(CH₂Cl₂) 147.02, 146.64ppm.

(R,S)-2,2-dimethyl-1-phenylpropyl-1-(di-2-cyanoethyl)phosphite δ_p(CH₂Cl₂) 138.68ppm.

(R,S)-2-Phenylpropyl-1-(di-2-cyanoethyl)phosphate (168):

(R,S)-2-Phenylpropyl-1-(di-2-cyanoethyl)phosphate (163) (1.05g, 45%) was prepared using a similar method to the preparation of (156).

(EtOAc:CH₂Cl₂ (4:1) R_f=0.35).

δ_p(36MHz:CH₂Cl₂) -3.29ppm.

ν_{max}(thin film) 2960s, 2820m, 2760m (O-CH), 2200s (CN), 1490m, 1430m, 1270vs (P=O),

1300s (P-O-CH), 970m, 760m.

δ_{H} (300MHz:CDCl₃) 7.29-7.14 (5H, m, C₆H₅), 4.24-4.16 (4H, m, (CH_AH_B-O)₂), 3.94 (2H, ddd, ³J_{P-H} 5.4Hz, J 15.9, 6.9Hz, CH₂-O), 3.12 (1H, m, PhCH), 2.62 (4H, dt, J 12.0, 6.4Hz, (CH₂CN)₂), 1.28 (3H, d, J 7.0Hz, CHCH₃).

δ_{C} (75MHz:CDCl₃) 145.56(s), 129.34(d), 128.62(d), 127.64(d), 116.62(s), 72.04(dt, ²J_{P-C} 5.3Hz), 62.27(dt, ²J_{P-C} 5.7Hz), 62.16(dt, ²J_{P-C} 5.4Hz), 42.07(dd, ³J_{P-C} 8.7Hz), 19.62(dt, ³J_{P-C} 7.1Hz), 19.54(dt, ³J_{P-C} 7.2Hz), 18.99(q).

m/z(EI): M⁺ 322(10%), (ROPO₃CH₂CH₂CN+H)⁺ 269(40%), (ROPO₃H)⁺ 215(50%), (ROPO₂H)⁺ 199(35%), (PHCH(Me)CH₂)⁺ 119(70%), (C₇H₇)⁺ 91(30%), (C₆H₅)⁺ 77(60%).

(R,S)-2-phenylpropyl-1-phosphoramidite δ_{P} (CH₂Cl₂) 148.23, 147.89ppm.

(R,S)-2-phenylpropyl-1-(di-2-cyanoethyl)phosphite δ_{P} (CH₂Cl₂) 138.90ppm.

(R,S)-2,3-Dimethylbutyl-1-(di-2-cyanoethyl)phosphate (169):

(R,S)-2,3-Dimethylbutyl-1-(di-2-cyanoethyl)phosphate (169) (625mg, 51%) was prepared using a similar method to the preparation of (156).

(EtOAc:CH₂Cl₂ (4:1) R_f=0.24).

δ_{P} (36MHz:CH₂Cl₂) -3.23ppm.

ν_{max} (thin film) 2830s (O-CH), 2200sharp (CN), 1260s (P=O), 1030s (P-O-CH).

δ_{H} (300MHz:CDCl₃) 4.29 (4H, q, J 7.9Hz, (CH_AH_B-O)₂), 4.07 (2H, m, RCH₂-O), 2.79 (4H, t, J 6.1Hz, (CH₂CN)₂), 1.71 (1H, m, CH(Me)₂), 1.46 (1H, m, CHMe), 0.91 (6H, d, J 6.7Hz, CH(CH₃)₂), 0.84 (3H, d, J 6.2Hz, CHCH₃).

δ_{C} (75MHz:CDCl₃) 116.38(s,CN), 72.31(dt, ²J_{P-C} 6.1Hz), 62.16(dt, ²J_{P-C} 4.2Hz), 39.36(dd, ³J_{P-C} 6.6Hz), 28.75(d), 20.33(q), 19.72(dt, ³J_{P-C} 7.3Hz), 18.04(q), 12.49(q).

m/z(EI): M⁺ 278(3%), (ROPO₃H)⁺ 171(45%), (ROPO₂H)⁺ 155(60), (Pr^tCH(Me)CH₂)⁺ 75(80%).

(R,S)-2,3-dimethylbutyl-1-phosphoramidite $\delta_p(\text{CH}_2\text{Cl}_2)$ 148.20, 147.82ppm.

(R,S)-2,3-dimethylbutyl-1-(di-2-cyanoethyl)phosphite $\delta_p(\text{CH}_2\text{Cl}_2)$ 138.90ppm.

(R,S)-3-Methylbutyl-2-(di-2-cyanoethyl)phosphate (164):

(R,S)-3-Methylbutyl-1-(di-2-cyanoethyl)phosphate (**164**) (525mg, 65%) was prepared using a similar method to the preparation of (**156**).

(EtOAc: CH_2Cl_2 (4:1) $R_f=0.23$).

$\delta_p(36\text{MHz}:\text{CH}_2\text{Cl}_2)$ -3.02ppm.

ν_{max} (thin film) 2820s, 2220sharp (CN), 1430m, 1250s (P=O), 1100m, 1070s.

$\delta_{\text{H}}(300\text{MHz}:\text{CDCl}_3)$ 4.62 (1H, dq, $^3J_{\text{P-H}}$ 6.1Hz, J 6.4Hz, MeCH), 4.29 (4H, dq, $^3J_{\text{P-H}}$ 2.0Hz, J 7.4Hz, ($\text{CH}_A\text{H}_B\text{-O}_2$)), 2.86 (4H, t, J 6.0Hz, (CH_2CN)₂), 1.71 (1H, m, CH(Me)₂), 1.36 (3H, d, J 6.3Hz, CH₃), 0.96 (6H, d, J 6.4Hz, CH(CH₃)₂).
 $\delta_{\text{C}}(75\text{MHz}:\text{CDCl}_3)$ 116.42(s), 79.21(dd, $^2J_{\text{P-C}}$ 6.1Hz), 62.35(dt, $^2J_{\text{P-C}}$ 5.6Hz), 30.10(dd, $^3J_{\text{P-C}}$ 5.8Hz), 21.06(dq, $^3J_{\text{P-C}}$ 3.9Hz), 19.64(dt, $^3J_{\text{P-C}}$ 7.2Hz), 17.69(q), 12.61(q).

m/z(EI): M⁺ 274(10%), (ROPO₃CH₂CH₂CN+H)⁺ 220(65%), (ROPO₃H)⁺ 166(40%), (ROPO₂H)⁺ 150(25%), (PrⁱCHMe)⁺ 71(30%).

(FAB, +ve Ion, NOBA): (M+H)⁺ 275(70%), (CNCH₂CH₂OPO₃+2H)⁺ 166(100%), (CNCH₂CH₂OH)⁺ 71(20%).

(R,S)-3-methylbutyl-2-phosphoramidite $\delta_p(\text{CH}_2\text{Cl}_2)$ 148.00, 147.58ppm.

(R,S)-3-methylbutyl-2-(di-2-cyanoethyl)phosphite $\delta_p(\text{CH}_2\text{Cl}_2)$ 139.71ppm.

(R,S)-butyl-2-(di-2-cyanoethyl)phosphate (165):

(R,S)-Butyl-2-(di-2-cyanoethyl)phosphate (**165**) (585mg, 35%) was prepared using a similar method to the preparation of (**156**).

(EtOAc: CH_2Cl_2 (4:1) $R_f=0.20$).

$\delta_p(36\text{MHz}:\text{CH}_2\text{Cl}_2)$ -3.02ppm.

ν_{\max} (thin film) 2250sharp (CN), 1420m, 1370m, 1270s (P=O), 1100m, 1020m.
 δ_H (300MHz:CDCl₃) 4.50 (1H, dq, $^3J_{P-H}$ 6.3Hz, J 6.6Hz, MeCH), 4.26 (4H, dq,
 $^3J_{P-H}$ 1.5Hz, J 7.4Hz, (CH_AH_B-O)₂), 2.84 (4H, t, J 6.0Hz, (CH₂CN)₂), 1.66 (2H, dquin, $^4J_{P-H}$
1.3Hz, J 6.6Hz, CH₂Me), 1.36 (3H, d, J 6.2Hz, CH₃CH), 0.96 (3H, t, J 7.3Hz, CH₃CH₂).
 δ_C (75MHz:CDCl₃) 117.17(s,CN), 79.00(dd, $^2J_{P-C}$ 6.3Hz), 62.35(dt, $^2J_{P-C}$ 5.4Hz), 30.14(dt,
 $^3J_{P-C}$ 5.9Hz), 20.95(dq, $^3J_{P-C}$ 3.2Hz), 19.64(dt, $^3J_{P-C}$ 7.4Hz), 9.42(q).
m/z(EI): M⁺ 260(5%), (ROPO₃CH₂CH₂CN+H)⁺ 191(60%), (ROPO₃H)⁺ 137(25%),
(MeCHEt)⁺ 57(80%).
(FAB, +ve Ion, NOBA): (M+H)⁺ 261(60%), (ROPO₃+2H)⁺ 154(100%), (ROPO₃+H)⁺
137(80%).

(R,S)-butyl-2-phosphoramidite δ_p (CH₂Cl₂) 146.62ppm.
(R,S)-butyl-2-(di-2-cyanoethyl)phosphite δ_p (CH₂Cl₂) 138.62ppm.

The general procedure for the preparation of the monophosphate products (**179-192**) from the corresponding phosphate triesters is illustrated by the synthesis of (R,S)-1-phenylethyl-1-phosphate (**179**).

Analysis of the phosphate products was not always carried out since it has been noted previously that various salts of the alkyl and benzyl phosphates do not analyze well, especially for carbon.^{150,170} The purity of these monophosphate ester substrates was demonstrated by ³¹P, ¹³C and ¹H NMR spectroscopy as well as mass spectroscopy.

(R,S)-1-Phenylethyl-1-phosphate (179):

The (R,S)-1-phenylethyl-1-(di-2-cyanoethyl)phosphate (**156**), (525mg, 1.7mmol) was added to ammonia solution (35%, 10ml) and left to stir at 60°C for two hours. The phosphate product was identified (δ_p (36.2MHz:NH₄OH) 3.02ppm). After allowing the solution to cool to room temperature, it was evaporated down to a white solid. The white

solid was taken up in distilled water (200ml) and isolated by ion exchange chromatography. The buffer eluent used was TEAB of 50mM and 400mM concentrations, run through a DEAE-sephadex column. The eluting solvents were evaporated before the material was successively washed and evaporated with methanol to yield the title compound (**179**) as a putative bis-triethylammonium salt (284mg, 40%).

ν_{max} (thin film) 3364s (NEt₃), 2936s, 2810s (N-CH), 1084s (OPO₃²⁻), 977s.

δ_{H} (300MHz:MeOD) 7.43-7.22 (5H, m, C₆H₅), 5.37 (1H, dq, ³J_{P-H} 15.4Hz, J 6.4Hz, PhCH), 1.55 (3H, d, J 6.4Hz, CHCH₃).

δ_{C} (75MHz:MeOD) 145.85(ds, ³J_{P-C} 5.3Hz), 129.34(d), 128.37(d), 126.99(d), 74.40(dd, ²J_{P-C} 5.0Hz), 25.35(dq, ³J_{P-C} 4.1Hz).

m/z(FAB, -ve Ion, NOBA): I: 200(100%), (2I+H)⁻ 401(70%), (3I+3H)⁻ 603(20%), (4I+4H)⁻ 804(10%).

(FAB, +ve Ion, NOBA): (M+Et₃N+H)⁺ 304(100%), (M+2Et₃N)⁺ 405(70%).

(R)-1-Phenylethyl-1-phosphate (180):

(R)-1-Phenylethyl-1-phosphate (**180**) (810mg, 36%) was prepared using a similar method to the preparation of (**179**).

δ_{p} (36MHz:NH₄OH) 3.23ppm.

$[\alpha]^{23}_{589} = +23.8$ (c= 6.7 in MeOH).

(S)-1-Phenylethyl-1-phosphate (181):

(S)-1-Phenylethyl-1-phosphate (**181**) (826mg, 56%) was prepared using a similar method to the preparation of (**179**).

δ_{p} (36MHz:NH₄OH) 3.16ppm.

$[\alpha]^{23}_{589} = -25.6$ (c= 7.7 in MeOH).

(R,S)-1-(*para*-Nitrophenyl)ethyl-1-phosphate (189):

(R,S)-1-(*para*-Nitrophenyl)ethyl-1-phosphate (**189**) (370mg, 53%) was prepared using a similar method to the preparation of (**179**).

δ_p (36MHz:NH₄OH) 3.02ppm.

ν_{max} (thin film) 3343s, 2922s, 2773m (N-CH), 1397m (conjugated NO₂), 1162s, 1046s (OPO₃²⁻), 923m.

δ_H (300MHz:MeOD) 8.16 (2H, d, J 8.6Hz, C₆H₄), 7.66 (2H, d, J 8.5Hz, C₆H₄), 5.43 (1H, dq, ³J_{P-H} 15.0Hz, J 6.4Hz, PhCH), 1.57 (3H, d, J 6.4Hz, CHCH₃).

δ_C (75MHz:MeOD) 153.46(ds, ³J_{P-C} 4.8Hz), 148.24(s), 127.89(d), 124.30(d), 73.52(dd, ²J_{P-C} 4.7Hz), 25.04(dq, ³J_{P-C} 4.1Hz).

m/z(FAB, -ve Ion, NOBA): I⁻ 245(60%), (2I+H)⁻ 491(20%).

(FAB, +ve Ion, NOBA): (M+Et₃N+H)⁺ 349(80%), (M+2Et₃N+H)⁺ 450(60%).

(R,S)-1-(*para*-Methoxyphenyl)ethyl-1-phosphate (190):

(R,S)-1-(*para*-Methoxyphenyl)ethyl-1-phosphate (**190**) (307mg, 40%) was prepared using a similar method to the preparation of (**179**).

δ_p (36MHz:NH₄OH) 3.16ppm.

ν_{max} (thin film) 3388s, 2921s (OMe), 1270m (P=O), 1052m (OPO₃²⁻), 930m.

δ_H (300MHz:MeOD) 7.40 (2H, d, J 8.5Hz, C₆H₄), 7.29 (2H, d, J 8.5Hz, C₆H₄), 5.31 (1H, m, PhCH), 3.34 (3H, s, CH₃O-), 1.53 (3H, d, J 6.4Hz, CHCH₃).

δ_C (75MHz:MeOD) 144.48(ds, ³J_{P-C} 5.2Hz), 133.66(s), 129.17(d), 128.56(d), 73.85 (dd, ²J_{P-C} 5.0Hz), 49.04(q), 25.01(dq, ³J_{P-C} 3.5Hz).

m/z(FAB, -ve Ion, NOBA): I⁻ 230(70%), (2I+H)⁻ 461(35%).

(FAB, +ve Ion, NOBA): (M+H)⁺ 234(80%), (M+Et₃N+H)⁺ 335(45%).

(R,S)-1-Phenylpropyl-1-phosphate (182):

(R,S)-1-Phenylpropyl-1-phosphate (**182**) (1.15g, 42%) was prepared using a similar method to the preparation of (**179**).

δ_p (36MHz:NH₄OH) 3.23ppm.

ν_{max} (thin film) 3385s (NEt), 2800m (N-CH), 1397m (P=O), 1163s, 1040s (OPO₃²⁻), 929m, 843m.

δ_H (300MHz:MeOD) 7.40-7.21 (5H, m, C₆H₅), 5.15 (1H, dt, ³J_{P-H} 9.1Hz, J 6.4Hz, PhCH), 2.04 (1H, quin, J 7.0Hz, CH₂Me), 1.87 (1H, quin, J 7.0Hz, CH₂Me), 0.86 (3H, t, J 7.4Hz, CH₃).

δ_C (75MHz:MeOD) 144.10(ds, ³J_{P-C} 4.0Hz), 129.11(d), 128.28(d), 127.64(d), 79.71(dd, ²J_{P-C} 5.1Hz), 32.40(t), 10.19(q).

m/z(FAB, -ve Ion, NOBA): I⁻ 214(100%), (2I+H)⁺ 429(70%), (PhCH₂OPO₃)⁻ 185(10%), (3I+2H)⁺ 644(10%).

(FAB, +ve Ion, NOBA): (M+Et₃N+H)⁺ 318(100%), (M+2Et₃N+H)⁺ 419(50%).

(R,S)-1-Phenylbutyl-1-phosphate (183):

(R,S)-1-Phenylbutyl-1-phosphate (**183**) (1.11g, 47%) was prepared using a similar method to the preparation of (**179**).

δ_p (36MHz:NH₄OH) 3.02ppm.

ν_{max} (thin film) 3380m, 2934m, 2722m (CH-N), 1163s, 1034s (OPO₃²⁻), 930m.

δ_H (300MHz:MeOD) 7.41-7.19 (5H, m, C₆H₅), 5.20 (1H, dt, ³J_{P-H} 9.1Hz, J 6.6Hz, PhCH), 1.91 (1H, m, CH₂-CHPh), 1.71 (1H, m, CH₂-CHPh), 1.32 (2H, m, CH₂Me), 0.91 (3H, t, J 7.2Hz, CH₃).

δ_C (75MHz:MeOD) 144.60(ds, ³J_{P-C} 3.5Hz), 129.10(d), 128.22(d), 127.59(d), 78.34(dd, ²J_{P-C} 5.4Hz), 41.97(dt, ³J_{P-C} 4.7Hz), 19.56(t), 14.69(q).

m/z(FAB, -ve Ion, NOBA): I⁻ 228(100%), (2I+H)⁻ 457(55%), (3I+2H)⁻ 686(10%), (PhCH₂OPO₃)⁻ 185(10%).

(FAB, +ve Ion, NOBA): (M+Et₃N+H)⁺ 332(100%), (M+2Et₃N+H)⁺ 433(60%).

(R,S)-2-Methyl-1-phenylpropyl-1-phosphate (184):

(R,S)-2-Methyl-1-phenylpropyl-1-phosphate (184) (654mg, 48%) was prepared using a similar method to the preparation of (179).

δ_p (36MHz:NH₄OH) 3.18ppm.

ν_{max} (thin film) 3360s, 2975s, 2800m (N-CH), 1086s (OPO₃²⁻), 976m, 918w.

δ_H (300MHz:MeOD) 7.37-7.21 (5H, m, C₆H₅), 4.94 (1H, dd, ³J_{P,H} 9.4Hz, J 13.0Hz, PhCH), 2.04 (1H, dq, J 13.0, 6.7Hz, CH(Me)₂), 0.95 (3H, d, J 6.7Hz, CH(CH₃)₂), 0.85 (3H, d, J 6.7Hz, CH(CH₃)₂).

δ_C (75MHz:MeOD) 143.20(s), 128.70(d), 128.22(d), 127.90(d), 83.37(dd, ²J_{P,C} 5.8Hz), 36.40(dd, ³J_{P,C} 6.0Hz), 19.14(q), 18.85(q).

m/z(FAB, -ve Ion, NOBA): I⁻ 228(90%), (2I+H)⁻ 457(65%), (3I+2H)⁻ 686(10%), (PhCH₂OPO₃)⁻ 185(10%).

(FAB, +ve Ion, NOBA): (M+Et₃N+H)⁺ 332(100%), (M+2Et₃N+H)⁺ 433(60%).

(R,S)-1-Phenylpentyl-1-phosphate (185):

(R,S)-1-Phenylpentyl-1-phosphate (185) (1.11g, 50%) was prepared using a similar method to the preparation of (179).

δ_p (36MHz:NH₄OH) 3.25ppm.

δ_H (300MHz:MeOD) 7.14-7.21 (5H, m, C₆H₅), 5.17 (1H, dt, ³J_{P,H} 9.2Hz, J 6.5Hz, PhCH), 1.93 (1H, m, CH₂-CHPh), 1.81 (1H, m, CH₂-CHPh), 1.28 (4H, m, Me(CH₂)₂), 0.84 (3H, t, J 6.8Hz, CH₃).

δ_C (75MHz:MeOD) 144.65(ds, ³J_{P,C} 3.8Hz), 129.12(d), 128.24(d), 127.63(d), 78.48(dd, ²J_{P,C}

5.4Hz), 39.48(dt, $^3J_{P-C}$ 4.7Hz), 28.55(t), 23.79(t), 14.73(q).

m/z(FAB, -ve Ion, NOBA): I⁻ 242(100%), (2I+H)⁻ 285(70%), (PhCH₂OPO₃)⁻ 185(10%).

(FAB, +ve Ion, NOBA): (M+Et₃N+H)⁺ 346(100%), (M+2Et₃N+H)⁺ 447(50%).

(R,S)-2,2-Dimethyl-1-phenylpropyl-1-phosphate (186):

(R,S)-2,2-Dimethyl-1-phenylpropyl-1-phosphate (**185**) (275mg, 40%) was prepared using a similar method to the preparation of (**179**).

δ_p (36MHz:NH₄OH) 3.16ppm.

δ_H (300MHz:MeOD) 7.36-7.18 (5H, m, C₆H₅), 4.84 (1H, d, $^3J_{P-H}$ 9.5Hz, PhCH), 0.93 (9H, s, C(CH₃)₃).

δ_C (75MHz:MeOD) 142.02(s), 129.23(d), 128.04(d), 127.89(d), 86.06(dd, $^2J_{P-C}$ 6.1Hz), 36.75(ds, $^3J_{P-C}$ 7.0Hz), 26.77(q).

m/z(FAB, -ve Ion, NOBA): I⁻ 242(100%), (2I+H)⁻ 285(80%), (PhCH₂OPO₃)⁻ 185(20%).

(FAB, +ve Ion, NOBA): (M+Et₃N+H)⁺ 346(100%), (M+2Et₃N+H)⁺ 447(70%).

(R,S)-2-Phenylpropyl-1-phosphate (191):

(R,S)-2-Phenylpropyl-1-phosphate (**191**) (895mg, 68%) was prepared using a similar method to the preparation of (**179**).

δ_p (36MHz:NH₄OH) 3.02ppm.

δ_H (300MHz:MeOD) 7.28-7.16 (5H, m, C₆H₅), 3.97 (2H, ddd, $^3J_{P-H}$ 5.6Hz, J 15.9, 6.9Hz, CH-CH₂), 3.06 (1H, m, PhCH), 1.26 (3H, d, J 7.0Hz, CHCH₃).

δ_C (75MHz:MeOD) 145.46(s), 129.44(d), 128.51(d), 127.44(d), 71.29(dt, $^2J_{P-C}$ 5.5Hz), 41.94(dd, $^3J_{P-C}$ 8.9Hz), 18.99(q).

m/z(FAB, -ve Ion, NOBA): I⁻ 214(100%), (2I+H)⁻ 429(85%), (3I+2H)⁻ 644(20%).

(FAB, +ve Ion, NOBA): (M+Et₃N+H)⁺ 318(100%), (M+2Et₃N+H)⁺ 419(80%).

(R,S)-2,3-Dimethylbutyl-1-phosphate (192):

(R,S)-2,3-Dimethylbutyl-1-phosphate (192) (210mg, 28%) was prepared using a similar method to the preparation of (179).

δ_p (36MHz:NH₄OH) 3.06ppm.

δ_H (300MHz:MeOD) 3.85 (2H, m, CH₂-O), 3.05 (1H, m, CHMe), 2.57 (1H, m, CH(Me)₂), 1.19 (3H, d, J 4.3Hz, CHCH₃), 0.94 (6H, d, J 4.4Hz, CH(CH₃)₂).

δ_C (75MHz:MeOD) 73.21(dt, $^2J_{P-C}$ 5.7Hz), 32.86(dd, $^3J_{P-C}$ 5.1Hz), 21.19(d), 17.58(q), 17.40(q), 11.18(q).

m/z(FAB, -ve Ion, NOBA): I⁻ 180(65%), (2I+H)⁻ 361(35%).

(FAB, +ve Ion, NOBA): (M+Et₃N+H)⁺ 284(80%), (M+2Et₃N+H)⁺ 385(20%).

(R,S)-3-Methylbutyl-2-phosphate (187):

(R,S)-3-Methylbutyl-1-phosphate (187) (200mg, 32%) was prepared using a similar method to the preparation of (179).

δ_p (36MHz:NH₄OH) 3.29ppm.

δ_H (300MHz:MeOD) 4.04 (1H, ddq, $^3J_{P-H}$ 8.4Hz, J 9.8, 4.4Hz, MeCH), 2.61 (1H, m, CH(Me)₂), 1.17 (3H, d, J 4.3Hz, CHCH₃), 0.91 (6H, dd, J 6.8, 4.5Hz, CH(CH₃)₂).

δ_C (75MHz:MeOD) 75.62(dd, $^2J_{P-C}$ 5.4Hz), 34.99(dd, $^3J_{P-C}$ 5.2Hz), 19.51(q), 17.69(q), 17.40(q).

m/z(FAB, -ve Ion, NOBA): I⁻ 166(100%), (2I+H)⁻ 333(45%).

(FAB, +ve Ion, NOBA): (M+Et₃N+H)⁺ 270(100%), (M+2Et₃N)⁺ 370(20%).

(R,S)-Butyl-2-phosphate (188):

(R,S)-Butyl-2-phosphate (188) (280mg, 34%) was prepared using a similar method to the preparation of (179).

δ_p (36MHz:NH₄OH) 3.16ppm.

ν_{\max} (thin film) 3320m, 2865m, 2790m, 1376m, 1062s (OPO_3^{2-}), 1000m, 920m.
 δ_{H} (300MHz:MeOD) 5.18 (1H, dq, ${}^3J_{\text{p-H}}$ 11.0Hz, J=6.2Hz, MeCH-O), 1.62 (2H, m, MeCH_2), 1.25 (3H, d, J 6.2Hz, CH_3CH), 0.92 (3H, t, J 7.5Hz, CH_3CH_2).
 δ_{C} (75MHz:MeOD) 75.65(dd, ${}^2J_{\text{p-C}}$ 5.6Hz), 40.97(dt, ${}^3J_{\text{p-C}}$ 4.7Hz), 21.72(q), 19.27(q).
m/z(FAB, -ve Ion, NOBA): I 152(100%), (2I+H)⁺ 305(80%).
(FAB, +ve Ion, NOBA): ($\text{M}+\text{Et}_3\text{N}+\text{H}$)⁺ 256(100%), ($\text{M}+2\text{Et}_3\text{N}+\text{H}$)⁺ 357(70%).

(R)-1-phenylethyl-1-acetate (112):

Into a 50ml round bottom flask was placed the (R)-1-phenylethanol (**105**) (1g, 8.3mmol) and to this was added of pyridine (2ml, 1.95g, 25mmol). This mixture was left stirring at 0°C for five minutes before the acetyl chloride (710mg, 9mmol) was carefully added. After the addition was completed the reaction was allowed to warm to room temperature and stir for a further twenty minutes. Analysis of the reaction by TLC indicated that the reaction had gone to completion. Ether (45ml) was added to the reaction mixture and this was transferred to a separatory funnel. The organic layer was washed successively with saturated copper sulphate solution (2x15ml) and saturated sodium hydrogen carbonate solution (2x15ml). The ether layer was then separated and dried over anhydrous magnesium sulphate. This was filtered and concentrated to a dark oil. The remaining residue was distilled under reduced pressure 110-111°C, 18mmHg (lit.^{167,171} 109°C, 18mmHg) to yield the acetate product (**112**) as a yellow liquid (980mg, 73%)
 $[\alpha]^{23}_{589} = + 110.8$ (neat material) (lit.,¹⁷¹ $[\alpha]^{23}_{\text{D}} = + 127.6$ neat)¹⁷².

ν_{\max} (thin film) 2980m, 2820w (benzene ring), 1720vs (C=O), 1440m, 1360w, 1220vs (C-O), 1020s, 1000s, 920m, 820w.

δ_{H} (300MHz: CDCl_3) 7.32-7.20 (5H, m, C_6H_5), 5.85 (1H, q, J 6.6Hz, CH-O), 1.99 (3H, s, C(O)- CH_3), 1.49 (3H, d, J 6.6Hz, $\text{CH}_3\text{CH-O}$).

δ_{C} (75MHz:CDCl₃) 170.18(s), 141.61(s), 128.42(d), 127.10(d), 126.01(d), 72.26(d), 22.15(q), 21.19(q).

(R)-(para-Nitrophenyl)ethyl acetate (113):

To the acetate (112) (600mg, 3.6mmol) stirring at -20°C was carefully added fuming nitric acid (10ml) over a fifteen minute period. This mixture was left stirring for twenty minutes before being warmed to room temperature and stand for a further ten minutes. The mixture was then added to ice (25g) and stirred carefully before the solution was basified by the slow addition of sodium hydrogen carbonate (15g) to the mixture. The resultant solution was transferred to a separatory funnel and extracted with ether (3x30ml). The organic layer was isolated, dried over anhydrous magnesium sulphate and concentrated to a very dark yellow solution. This solution was then distilled under reduced pressure, 161-162°C, 16mmHg (lit.,^{167,173} 161-163°C, 16mmHg) to yield the nitroacetate derivative (113) as a yellow liquid (465mg, 62%), and subsequently recrystallised.

$[\alpha]^{23}_{589} = + 5.8$.

M.p. = 56-58°C (ethanol) (lit.,^{167,173} 55-56°C).

ν_{max} (thin film) 2980m, 2820w (benzene ring), 1700vs (C=O), 1520m (NO₂-C), 1420m, 1340s (conjugated NO₂ group), 1200vs (C-O), 1040s, 1000s, 900m.

δ_{H} (300MHz:CDCl₃) 8.25 (2H, d, J 8.9Hz, C₆H₄), 7.62 (2H, d, J 8.9Hz, C₆H₄), 6.07 (1H, q, J 6.7Hz, MeCH-O), 2.71 (3H, s, CH₃C(O)-), 1.69 (3H, d, J 6.8Hz, CH₃CH).

δ_{C} (75MHz:CDCl₃) 172.28(s), 147.75(s), 145.89(s), 127.08(d), 124.59(d), 80.33(d), 26.67(q), 19.96(q).

(R)-1-(para-Nitrophenyl)ethanol (114):

To the nitroacetate derivative (113) (400mg, 1.9mmol) was added a solution of 2M hydrochloric acid(15ml) and this mixture was refluxed for two hours. After allowing the

mixture to cool the contents were poured into a beaker of ice (30g). The acidic mixture was carefully neutralised by the addition of sodium hydrogen carbonate (20g) and monitored using pH paper. The resultant mixture was transferred to a separatory funnel and extracted with ether (3x10m). The organic layer was then separated, dried over anhydrous sodium sulphate and concentrated to a dark yellow oil. The remaining yellow residue was carefully distilled under reduced pressure 137-139°C, 2mmHg (lit.,^{167,173} 137-138°C, 2mmHg) to yield the alcohol product as a yellow oil (198mg, 63%).

$$[\alpha]^{23}_{589} = 10.5^{173}.$$

ν_{\max} (thin film) 3400brs (OH), 2820m, 1620vs (benzene ring), 1580s (conjugated benzene ring), 1500s, 1360s, 1300s (conjugated NO₂ group), 1190s, 1110m, 1080vs, 1020m.

δ_{H} (300MHz:CDCl₃) 8.11 (2H, d, J 8.8Hz, C₆H₄), 7.50 (2H, d, J 8.7Hz, C₆H₄), 4.97 (1H, q, J 6.5Hz, MeCH-), 3.22 (1H, brs, MeCH-OH), 1.48 (3H, d, J 6.5Hz, CH₃CH).

δ_{C} (75MHz:CDCl₃) 153.40(s), 146.92(s), 126.13(d), 123.62(d), 69.31(d), 25.37(q).

(R)-2-Phenyl-1-propanol (116):

Lithium aluminium hydride (104mg, 0.6mmol) was added to dry ether (15ml) and left stirring for ten minutes. To this stirred solution was slowly added (S)-2-phenyl-1-propionic acid (**115**) (60mg, 0.4mmol) in dry ether (10ml) over a five minute period. After addition had been completed the reaction mixture was refluxed for sixty minutes. On cooling the mixture was transferred to a beaker and the combined extracts were cooled to 0°C. The excess lithium aluminium hydride was quenched with water (10ml) and ether (25ml). The ether layer from this mixture was carefully decanted off and dried over anhydrous sodium sulphate. After filtering it was concentrated and dried under vacuum. The remaining residue was distilled under reduced pressure 110-111°C, 10mmHg (lit.,¹⁶⁸ 109-111°C, 10mmHg) to yield the title compound (**116**) as a clear liquid (50mg, 92%).

$[\alpha]^{23}_{589} = +15.13$ (benzene) (lit.¹⁶⁸ +16.7).

ν_{max} (thin film) 3340br (OH), 2960s, 2860s, 1490m, 1370m, 1070m, 1000s.

δ_{H} (300MHz:CDCl₃) 7.17 (5H, m, C₆H₅), 3.5 (2H, dd, J 13.5, 6.5Hz, CH₂OH), 3.19 (1H, s, CH₂OH), 2.79 (1H, q, J 7.8Hz, MeCHPh), 1.18 (3H, d, J 9.0Hz, CHCH₃).
 δ_{C} (75MHz:CDCl₃) 143.74(d), 128.05(d), 127.08(d), 126.03(d), 67.93(t), 41.98(d), 17.40(q).

(+)- α -isoPinocamphenol (121)^{95b}:

A 250ml three necked round bottom flask equipped with a septa, magnetic stir bar and a distillation condenser connected to a receiver, was cooled to -78°C. The flask was charged with borane-methyl sulphide complex (25ml, 2.0M, 50mmol) and cooled to -25°C, (+)- α -pinene (**119**) (15.9ml, 100mmol) was added dropwise with stirring over a thirty minute period. The contents were allowed to stir for one hour before being left to warm to 0°C and stand for a further forty five minutes. The dimethylsulphide (DMS) by-product was carefully removed under reduced pressure (0°C, 30mm). The flask was then charged with THF (10ml) and α -pinene (**119**) (2.4ml, 15mmol, 15% excess) and vigorously stirred at 0°C under nitrogen for three hours. The flask was then sealed and stored at -4°C for three days to permit equilibration, and formation of the asymmetric hydroborating agent, (+)- α -diisopinocampheylborane (**120**).

After this time the reaction mixture was methanolised with methanol (5ml) followed by careful addition of 3N sodium hydroxide (20ml) and 20% hydrogen peroxide (20ml). The reaction mixture was heated to 55°C for one hour, cooled and extracted with ether (3x30ml). The extract was subsequently washed with water (10ml) and saturated sodium chloride solution (10ml) before being dried over anhydrous magnesium sulphate. The solution was then evaporated under reduced pressure to yield a thick colourless liquid. The (+)- α -pinene contaminant was removed by distillation under reduced pressure 60-

62°C, 1mmHg, to yield the desired (+)- α -isopinocamphenol product (**121**) (9.23g, 65%).^{95b}

$[\alpha]^{23}_{589} = -31.00$ (neat) 87%ee ($[\alpha]^{23}_D = -35.4$, 99.1%ee)^{95b}.

M.p. 54-55°C (diethyl ether/methanol) (lit.^{95b} 55-57°C).

ν_{max} (thin film) 3200s (OH), 2960m, 2900s, 1450m, 1390m, 1350s, 1320m, 1280m, 1150s, 1050m, 1000w.

δ_H (300MHz:CDCl₃) 4.43 (1H, m, CH-OH), 2.50 (1H, m, CH-OH), 2.32 (1H, m, CHMe), 2.01 (1H, brm, CH-), 1.90 (1H, m, CH-), 1.79 (4H, brm, (CH₂)₂), 1.20 (3H, m, C(CH₃)₂), 1.09 (3H, m, C(CH₃)₂), 0.93 (3H, s, CH-CH₃).

δ_C (75MHz:CDCl₃) 71.42(d), 47.80(d), 46.58(d), 41.68(d), 38.23(t), 33.55(t), 27.67(q), 20.57(q), 14.10(s).

(R)-2,3-Dimethyl-1-butanol (117):

(+)- α -*diiso*Pinocampheyborane (**120**) (87%ee) was used having been prepared as described earlier. To the stirred suspension of Ipc₂BH (50mmol) in THF (25ml) at -20°C was added the 2,3-dimethyl-1-butene (1.6g, 20mmol) and left stirring for three hours. The solid Ipc₂BH slowly disappears, indicating that the trialkylborane intermediate had been produced. The solution was then treated with methanol (4ml), water (5ml), 3N sodium hydroxide (20ml) and 20% hydrogen peroxide (20ml), and this was stirred vigorously at -20°C for twenty minutes before being warmed to room temperature. The reaction was stirred for an additional hour at 55°C, cooled and extracted with ether (3x30ml). The extract was washed successively with water (20ml) and brine (20ml) before being dried. The organic layer was concentrated and the title compound (**117**) and by-product (**118**) was obtained by distillation under reduced pressure 58-59°C, 18mmHg, (lit.^{95a} 58-59°C, 18mmHg), (612mg, 30%). The optical rotation measurement indicated an optical purity of 30% of the (R)-enantiomer (**117**). Both of the enantiomers had been formed in a ratio

of 65:35 (**R**)-(117) to (**S**)-(118).

$[\alpha]^{23}_{589} = -1.76$ (lit.,^{95a} -1.80).

ν_{max} (thin film) 3550br (OH), 2960s, 2880s, 1510m, 1390m, 1360m, 1030s.

(¹H and ¹³C data were the same as the racemic material).

(R)- α -methoxy- α -(trifluoromethyl)phenylacetic acid chloride (MTPA-Cl) (209):⁹³

The (**S**)- α -methoxy- α -(trifluoromethyl)phenylacetic acid (**210**), (398mg, 1.7mmol), sodium chloride (28.5mg) and thionyl chloride (10g, 7ml, 8.4mmol) were refluxed together for seventy hours under a slight positive pressure of nitrogen. On cooling the remaining thionyl chloride was removed by careful distillation under a slightly reduced pressure at room temperature. The oily residue remaining was then transferred to a Kugelrohr apparatus and distilled under reduced pressure 60-61°C, 1mmHg (lit.,⁹³ 56-58°C, 1mmHg) to yield the desired product as a clear liquid (300mg, 65%). The product was then carefully measured out into (50 μ l, 65mg) aliquots and stored in sealed ampoules.

δ_F (282MHz:CDCl₃) -68.56ppm.

δ_H (90MHz:CDCl₃) 7.52-7.37 (5H, m, C₆H₅), 3.47 (3H, s, C-OCH₃).

The general procedure for the preparation of the Mosher's ester derivatives from single enantiomers of the alcohol reactants can be illustrated by the preparation described below, using (**S**)-3-methyl-2-butanol (**110**).

(S)-3-Methyl-2-butyl-[-(R)- α -methoxy- α -(trifluoromethyl)] phenylacetate:

A mixture (**R**)- α -methoxy- α -(trifluoromethyl)phenylacetic acid (**210**), (140mg, 0.6mmol), dicyclohexylcarbodiimide (DCC), (117mg, 0.6mmol), and 4-dimethylamino-pyridine (DMAP), (73mg, 0.6mmol) were added to dichloromethane (4ml) and left stirring for fifteen minutes. To the mixture was slowly added the (**S**)-3-methyl-2-butanol (**110**) (25mg, 0.3mmol) in dichloromethane (2ml) and this was left stirring for twelve hours.

After this time, the flask was washed with water (5ml) and dichloromethane (15ml), and the mixture transferred to a separatory funnel. The organic layer was then isolated, dried over anhydrous magnesium sulphate, concentrated and dried under vacuum. The remaining residue was then submitted for ^{19}F NMR spectroscopy analysis.

δ_{F} (282MHz:CDCl₃) S-(+) = -71.87ppm = 100%

(see Table 2.2 for all other single enantiomer reactions)

The general procedure for the preparation of the Mosher's ester derivatives of racemic alcohols and products of the phosphate-catalysed reactions is illustrated by the following preparation. The racemic alcohol, (R,S)-3-methyl-2-butanol (**96**) is reacted with MTPA-Cl (**209**). The enantiomeric purity of the alcohol reactants can be determined as their diastereomeric esters using ^{19}F NMR spectroscopy analysis.

(R,S)-3-Methyl-2-butyl-[-(R)- α -methoxy- α -(trifluoromethyl)] phenylacetate:

To a dry round bottom flask (10ml) was added the racemic 3-methyl-2-butanol (**96**) (4.3mg, 48.5 μmol) and mixed with dry pyridine (2ml) and carbon tetrachloride (1ml). To this mixture was carefully and quickly added the MTPA-Cl (**209**), (20 μl , 97 μmol) and the resultant solution was left to stand for sixteen hours. Water (1ml) and ether (10ml) were added to the reaction mixture which was transferred to a separatory funnel. The ether layer was washed successively with hydrochloric acid (0.5M, 8ml), water (10ml) and saturated sodium carbonate solution (10ml). The ether solution was dried over anhydrous magnesium sulphate, filtered, evaporated and dried under vacuum. The resultant residue was subsequently submitted for ^{19}F NMR analysis, to determine the enantiomeric purity of the alcohol reactants from the enzyme reactions. The MTPA-Cl (**209**) was reacted with the racemic alcohols to ensure that there was no diastereoselectivity involved in these derivatisation reactions. This method also allowed the relevant Mosher's ester peaks to

be identified in the ^{19}F NMR spectra before the phosphatase alcohol products were reacted with the MTPA-Cl (**209**). The relative percentages of the enantiomers were determined as a function of the areas of the peaks, in their respective spectra.

δ_{F} (282MHz:CDCl₃) S-(-) = -71.87ppm = 49.75%

R-(+) = -71.84ppm = 50.24%

(see **Table 2.1** for racemic reaction results)

(2R,4R,5S)-2-Chloro-3,4-dimethyl-5-phenyl-1,3,2-oxazaphospholidine-2-sulphide

(217):123

A solution of thiophosphoryl chloride (**215**) (4.1ml, 41mmol) in dry benzene (25ml) was slowly added to a suspension of (-)-ephedrine hydrochloride (**216**) (8.2g, 50mmol), triethylamine (35ml, 250mmol) and benzene (100ml). The white suspension was stirred under nitrogen for sixteen hours at room temperature and then poured onto an excess of water (200ml). The organic layer was isolated and the aqueous layer extracted with more benzene (3x15ml). The combined organic extracts were dried over anhydrous sodium sulphate, filtered and concentrated. The resulting light brown oil was purified by flash chromatography (pet ether : CHCl₃ (3:1), R_f=0.42). The product was isolated and the resulting off white solid was recrystallised from diisopropyl ether to give the desired epimer (**217**) as shiny white crystals, m.p. 125-127°C (lit.¹²³ 125-128°C) (5.7g, 53%).

δ_{P} (36.2MHz:CDCl₃) 75.36ppm.

ν_{max} (nujol mull) 1210m, 1180m, 1060s, 960m (P-N), 940s (P-O), 880w, 860w, 750w, 700w.

δ_{H} (300MHz:CDCl₃) 7.32 (5H, s, C₆H₅), 5.81 (1H, brd, J 6.7Hz, O-CH₂Ph), 3.84 (1H, dquin, $^3J_{\text{P-H}}$ 29Hz, J 6.7Hz, CHMe), 2.85 (3H, d, $^3J_{\text{P-H}}$ 15Hz, NCH₃), 0.85 (3H, d, J 7Hz, CH₃).

δ_c (75MHz:CDCl₃) 134.52(ds, ³J_{P-C} 7.6Hz), 128.48(d), 125.58(d), 83.93(dd, ²J_{P-C} 3.3Hz), 60.62(dd, ²J_{P-C} 9.5Hz), 29.32(dq, ²J_{P-C} 6.7Hz), 12.03(q).

The general procedure to prepare the 3,4-dimethyl-5-phenyl-1,3,2-oxazaphospholidine derivatives is illustrated by the reaction of (R,S)-2-butanol (**95**) with (2R,4R,5S)-3,4-dimethyl-5-phenyl-1,3,2-oxazophospholidine-2-sulphide. The enantiomeric purity of the alcohol reactants can be determined as their diastereomeric oxyphospholidine-2-sulphide derivatives using ³¹P NMR spectroscopy.

(1'(R,S),2R,4R,5S)-2-sec-Butyloxy-3,4-dimethyl-5-phenyl-1,3,2-oxazaphospholidine-2-sulphide:⁹⁴

The (R,S)-2-butanol (**95**) (74mg, 60μl, 1.0mmol) was added to dry ether (10ml) and this was left stirring at 0°C for 5 minutes. To the solution was added *n*-butyl lithium¹²⁴ in hexane (1.6M, 400μl, 1.0mmol) and the mixture was left stirring for an additional ten minutes. The phospholidine-2-sulphide (**217**) (290mg, 1.1mmol) was added to the reaction mixture and this was stirred for a further fifteen minutes, before the solution was allowed to reflux for eighteen hours. On cooling the reaction mixture was poured into a beaker containing a solution of ether (20ml) and water (15ml) and stirred vigorously for ten minutes. The organic layer was then separated, dried over anhydrous magnesium sulphate, and concentrated to a thick yellow oil. The diastereomeric alcohol derivatives were analysed by ³¹P NMR spectroscopy without further purification, to determine the enantiomeric purity of the alcohol products. The phospholidine-2-sulphide material was also reacted with mixtures of the racemic alcohol to ensure that there was no diastereoselectivity seen with this derivatisation method. These initial reactions again allowed the relevant oxy-phospholidine peaks to be identified using ³¹P NMR spectroscopy before the enzyme alcohol products were derivatised. The relative percentages of the

enantiomers was determined as a function of the area of the peaks obtained in the ^{31}P NMR spectra.

δ_{p} (36.2MHz:CDCl₃) (2.5K) single enantiomer (R) = 82.38ppm (100%)
racemic mixture (R) = 82.38ppm (52%) low field diastereoisomer
(S) = 82.18ppm (48%) high field diastereoisomer

Difference δ_{p} shifts = 0.20ppm acid by-product = 83.39ppm

(1'R,S),2R,4R,5S)-1-Phenylethoxy-3,4-dimethyl-5-phenyl-1,3,2-oxazaphospholidine-2-sulphide:⁹⁴

The reaction of (217) with (R,S)-1-phenylethanol (80) was exactly the same as that described for the reaction of (R,S)-2-butanol (95) with (217).

δ_{p} (36.2MHz:CDCl₃) (2.5K) single enantiomer (R) = 81.91ppm (100%)
racemic mixture (R) = 81.91ppm (51%) low field diastereoisomer
(S) = 81.71ppm (49%) high field diastereoisomer

Difference δ_{p} shifts = 0.20ppm

(R)-phenyl-tert-butylphosphinothioic acid (223):

This material was kindly provided by Dr. M.J.P. Harger,¹²⁵ Department of Chemistry, Leicester University.

M.p. 96°C (lit.,^{126,174} 96°C). $[\alpha]_D = +28.1$ (c = 2.5 in MeOH).
 δ_{H} (90MHz:CDCl₃) 7.85-7.20 (5H, m, C₆H₅), 6.98 (1H, br, OH), 1.14 (9H, d, $^3\text{J}_{\text{P-H}}$ 18Hz, C(CH₃)₃).

The general procedure to determine the enantiomeric purity of the alcohol products using (R)-phenyl-tert-butylphosphinothioic acid (223)^{125,174} as a chiral shift reagent is illustrated by the reaction of (R,S)-1-phenylethanol (80) with (223).

Addition of (R)-phenyl-*tert*-butylphosphinothioic acid to (R,S)-1-phenylethanol (80):¹²⁵

To the alcohol (80) (20mg, 164mmol) dissolved in CDCl₃ (500μl) in a 5mm NMR tube was carefully added the chiral shift reagent (223) (100mg, 492μmol). The mixture was analysed using the Varian EM-390 spectrometer to record a ¹H NMR spectrum. The region between 0-2ppm (0-80Hz) was then expanded using a low R_f power (0.02) and an amplitude of 3,000. The methyl doublet was split into two sets of doublets arising from the resolution of the enantiomers of 1-phenylethanol (80). The peak areas of the two sets of doublets were then measured and compared to determine the ee of the alcohol products.

δ_H(90MHz:CDCl₃) 7.45-7.19 (10H, m, 2xC₆H₅), 6.98 (1H, br, OH), 5.42 (1H, q, J 6.7Hz, CHMe), 0.91 (9H, s, C(CH₃)₃).

Originally 1.4 (3H, d, J=6.8Hz, CH₃-CH)

Now 1.4-1.3 (3H, 2xd, J=6.4Hz, CH₃-CH) Now see 4 peaks - 2 doublets split by 1.8Hz
2-downfield peaks 1 enantiomer CH₃ group 1 doublet = 51.5%

2-upfield peaks 1 enantiomer CH₃ group 1 doublet = 48.5%

Addition of (R)-phenyl-*tert*-butylphosphinothioic acid to (R,S)-3-methyl-2-butanol (96):

The reaction of (223) with (96) was exactly the same as that described for the reaction of (90) with (223).

δ_H(90MHz:CDCl₃) 7.40-7.20 (5H, m, C₆H₅), 6.98 (1H, br, OH), 4.04 (1H, dq, J 9.8, 4.5Hz, CHMe), 2.61 (1H, m, CH(Me)₂), 1.47 (3H, d, J 4.4Hz, CHCH₃), 0.91 (9H, s, C(CH₃)₃).

Originally 1.25 (6H, dd, J 6.8, 4.5Hz, CH(CH₃)₂)

Now 1.35-1.26 (6H, 2xdd, J 5.4, 5.4Hz, CH(CH₃)₂) Now see 8 peaks - 2 doublet of doublets split by 2.5Hz. 4 central peaks appear to coalesce, however the 4 end peaks are separated and identifiable.

2-downfield peaks 1 enantiomer CH(CH₃)₂ group = 52%

2-upfield peaks 1 enantiomer CH(CH₃)₂ group = 48%

In both of these examples, the percentages of each enantiomer were again determined as a function of the peak areas in the ³¹P NMR spectra.

Diethyl n-butylmalonate (239):

Diethyl malonate (232) (8.0g, 50mmol), bromobutane (238) (7.53g, 50mmol), potassium carbonate (8.3g, 60mmol) and benzyltriethylammonium chloride (1.6g, 7mmol) were placed in a round bottom flask (50ml) and refluxed for two hours. On cooling the mixture was transferred to a separatory funnel and the flask rinsed with cold water (2x15ml). The organic layer was separated and the water wash extracted with ether (2x20ml). The combined organic extracts were dried over anhydrous sodium sulphate and the solvent removed on the rotary evaporator. The remaining residue was distilled to yield the title product (239) as a colourless liquid, b.p. 236-238°C, (lit.,¹³¹ 235-240°C) (4.32g, 40%).

ν_{max} (thin film) 2960m, 2930w, 1750m and 1730s (C=O), 1450br, 1370m, 1170br, 1110w, 1030m.

δ_{H} (300MHz:CDCl₃) 4.14 (4H, q, J 7.0Hz, (CO₂CH₂Me)₂), 1.82 (1H, t, J 7.5Hz, CH(CO₂Et)₂), 1.24 (6H, m, (CH₂)₃Me and 6H, m, (CO₂CH₂CH₃)₂), 0.83 (3H, t, J 7.0Hz, (CH₂)₃CH₃).

δ_{C} (75MHz:CDCl₃) 165.13(s), 60.94(t), 60.71(t), 51.55(d), 29.03(t), 28.00(t), 21.89(t), 13.61(q), 13.33(q).

m/z (FAB, +ve Ion, NOBA): (M+H)⁺ 217(60%), (M-OEt)⁺ 154(45%), (M-CO₂Et)⁺ 136(40%), (CO₂Et)⁺ 80(40%), (OEt)⁺ 62(60%).

Diethyl isopropylidenemalonate (234):

A mixture of dry acetone (10.9g, 187mmol), acetic anhydride (233) (15.93g, 156mmol) and freshly dried zinc chloride (2.83g, 21mmol) were stirred vigorously for twenty minutes. To this mixture was slowly added diethyl malonate (232) (20g, 125mmol) over a fifteen minute period, before the mixture was refluxed for 20 hours. After cooling, the resultant black solution was transferred to a separatory funnel containing dichloromethane (150ml), and the mixture was washed with distilled water (4x100ml). The water washings were extracted with dichloromethane (2x100ml) and combined with the original organic layer. The solution was concentrated by removal of the excess dichloromethane using a rotary evaporator. The remaining concentrate was distilled under reduced pressure, initially the acetic acid and acetic anhydride were collected 40-70°C (18mmHg). The crude diester was isolated 158-160°C (18mmHg). Redistillation of this crude material 110-112°C, 9mmHg (lit.¹²⁹ 111-113°C, 9mmHg) gave the title compound (234) as a pure colourless liquid (12.3g, 50%).

ν_{max} (thin film) 2980m, 2870m, 1720br (C=O), 1680m, 1370s, 1285s, 1210s, 1100.

δ_{H} (300MHz:CDCl₃) 4.21 (4H, q, J 7.0Hz, (CO₂CH₂Me)₂), 2.06 (6H, s, C=C(CH₃)₂), 1.28 (6H, t, J 7.0Hz, (CO₂CH₂CH₃)₂).

δ_{C} (75MHz:CDCl₃) 165.51(s), 154.66(s), 124.95(s), 60.75(t), 22.96(q), 14.15(q).

m/z 200(M⁺) (12), 155(83), 154(72), 127(19), 109(40), 99(100), 82(20), 67(25).

Diethyl-tert-butylmalonate (235):

Prior to use the sample of magnesium (620mg, 25.5mmol) was dried overnight in an oven at 150°C. The magnesium was placed in a three necked flask equipped with a reflux condenser and dropping funnels under a positive pressure of nitrogen. Slowly added to this was dry ether (30ml) and the cloudy mixture produced was cooled to 0°C.

Bromomethane (3.62g, 25mmol) in dry ether (40ml) was carefully added to the mixture over a thirty minute period before being heated for twenty five minutes. The flask was then cooled to 0°C and slowly add to this mixture over a forty minute period was the diethyl *isopropylidenemalonate* (**234**) (3.45g, 17.2mmol) in dry ether (30ml). The mixture was refluxed vigorously for thirty minutes, before cooling to room temperature. The reaction mixture was slowly poured onto ice water (150ml) and the resultant solution acidified by the addition of dilute sulphuric acid. The aqueous layer was extracted with ether (3x20ml) and combined with the original ether layer. The organic layer was dried over anhydrous sodium sulphate before being concentrated to a yellow oil. This was subsequently distilled under reduced pressure 98-102°C, 10mmHg (lit.¹³⁰ 98-101°C, 10mmHg), to yield the product (**235**) as a colourless liquid (2.31g, 62%).

ν_{\max} (thin film) 2980s, 2900s, 2860m, 1680vs (C=O), 1450br, 1370s, 1315br, 1230m, 1140s, 1060br.

δ_H (300MHz:CDCl₃) 4.18 (4H, q, J 7.2Hz, (CO₂CH₂Me)₂), 3.22 (1H, s, CH-C(Me)₃), 1.26 (6H, t, J 7.2Hz, (CO₂CH₂CH₃)₂), 1.12 (9H, s, C(CH₃)₃).

δ_C (75Hz:CDCl₃) 168.13(s), 61.10(d), 60.57(t), 33.38(s), 27.38(q), 13.92(q).

m/z 171(18), 160(92), 155(29), 133(90), 127(18), 109(12).

2-*iso*-Propyl-1,3-propanediol (237**):**

(2.5g, 12.5mmol) (**234**) Was dissolved in dry ether (25ml) and left stirring for ten minutes. To this solution was then carefully added 10% palladium on activated charcoal (176mg), and the flask was fitted with a three way stopcock. Hydrogen from a filled balloon was applied to the reaction mixture for two minutes before the flask was evacuated using a water pump. This procedure was repeated three more times to degas the solvent and the reaction flask. The reaction was then left stirring at room temperature

under a positive pressure of hydrogen for forty five minutes. After this time the reaction was evacuated using the water pump and a new positive pressure of hydrogen was applied. This was repeated every forty minutes until the reaction was terminated after three hours. Upon termination of the reaction, the solution was filtered through a short column of celite to remove the palladium catalyst. The flask was rinsed with ether (2x15ml) and these washings were filtered. The combined organic solution was concentrated and dried under vacuum to yield the crude diethyl *isopropylmalonate* product. Analysis by ^1H NMR spectroscopy and TLC indicated that this hydrogenation had been successful and the material was used without further purification as follows.

Lithium aluminium hydride (4.3g, 2.5mmol) was added to dry ether (40ml) and left stirring for ten minutes. To this stirred mixture was slowly added the diethyl *isopropylmalonate* (2.54g, 12.5mmol) in dry ether (20ml) over a twenty five minute period. After addition had been completed the reaction mixture was refluxed for two hours. On cooling the solution was transferred to a large beaker and the flask rinsed with ether (2x15ml). The combined extracts were cooled to 0°C and the excess lithium aluminium hydride was quenched by careful addition of ice-water (30ml) and ether (50ml). To this stirred creamy solution was added anhydrous sodium sulphate (8.0g) to produce a white crystalline by-product of the lithium aluminium hydride slurry which was filtered from the mixture. The organic layer was decanted off and further dried over anhydrous sodium sulphate, concentrated and dried under vacuum. The clear liquid remaining was distilled under reduced pressure (98-100°C, 2mmHg) to yield the product (237) as a clear liquid (971mg, 66% overall).

ν_{\max} (thin film) 3550br (OH), 2960s, 2880s, 1460m, 1385m, 1365m, 1020br.

δ_{H} (300MHz:CDCl₃) 4.51 (2H, brs, (CH₂OH)₂), 3.76 (2H, dd, J 10.0, 4.0Hz, (CH₂OH)₂),

3.67 (2H, dd, J 10.6, 7.5Hz, $(CH_2OH)_2$), 2.74 (1H, dq, J 13.5, 6.6Hz, $CH-CH_2OH$), 1.5 (1H, m, $CH(Me)_2$), 0.92 (6H, d, J 7.0Hz, $CH(CH_3)_2$).

δ_c (75MHz:CDCl₃) 63.24(t), 48.02(d), 26.51(d), 20.82(q).

m/z 18(5), 100(3), 85(17), 69(20), 69(9), 68(16), 57(12), 55(25).

Cyclohexylidene cyanoacetic acid (246):

Into a 250ml round bottom flask was placed cyanoacetic acid (245) (12g, 141mmol), cyclohexanone (244) (15.2g, 155mmol), ammonium acetate (1g, 13mmol) and benzene (75ml). The flask was attached to a Dean and Stark continuous water separator and a vigorous reflux of the mixture was maintained for two hours. The collected water by-product was removed from the separator at regular intervals. Once all the water had been collected, the mixture was refluxed for a further hour. After allowing the flask to cool more benzene (30ml) was added to the solution and quickly reheated. While still hot, the solution was poured into a separatory funnel and the reaction flask rinsed with ether (2x25ml). The combined mixture was allowed to cool to room temperature before being washed with cold water (2x30ml). The organic layer was concentrated to half its original volume by evaporation under reduced pressure, and then allowed to slowly cool. The title compound (246) crystallised as a colourless solid which was filtered and washed with portions of cold benzene (2x15ml) before being dried under vacuum.

M.p. 110-111°C (lit.¹³² 110-111°C) (15g, 68%).

ν_{max} (nujol mull) 2220m (CN), 1700s (conjugated C=O), 1590s (conjugated C=C), 1340m, 1320m, 1255m, 1220w.

δ_h (300MHz:CDCl₃) 9.98 (1H, brs, CO₂H), 3.00 (2H, t, J 6.0Hz, $(CH_2)_2-C=C$), 2.71 (2H, t, J 6.0Hz, $(CH_2)_2-$), 1.71 (6H, brm, C₆H₆ of C₆H₁₀).

δ_c (75MHz:CDCl₃) 121.21(s), 117.25(s), 115.23(s), 105.14(s), 37.45(t), 32.00(t), 28.78(t),

25.53(t).

m/z 164(M⁺+H) (28), 147(13), 137(21), 121(26), 119(23), 111(10), 104(12).

Cyclohexylcyanoacetic acid (247):

Cyclohexylidenecyanacetic acid (**246**), (2.6g, 16mmol) was dissolved in dry ether (35ml) and left stirring for ten minutes. To this solution was carefully added 10% palladium on activated charcoal (200mg). Reduction of the alkene functionality to the saturated alkane was performed by a hydrogenation experiment as described previously in the preparation of 2-*iso*-propyl-1,3-propanediol (**237**). This yielded the product (**247**) as a white crystalline solid, m.p. 61-65°C (pet ether) (2.61g, 82%).

ν_{max} (thin film) 3500br and 3200br (OH), 2920s, 2850s, 2250m (CN), 1710br (conjugated C=O), 1450m, 1290s, 1255m, 1200m.

δ_{H} (300MHz:CDCl₃) 10.7 (1H, brs, CO₂H), 3.43 (1H, d, J 5.4Hz, CH-CN), 2.00 (1H, brm, CH-CH(CN)), 1.66 (5H, br, C₆H₁₀), 1.27 (5H, brm, C₆H₁₀).

δ_{C} (75MHz:CDCl₃) 175.14(s), 121.21(s), 44.67(d), 38.57(d), 31.02(t), 29.15(t), 27.75(t), 25.62(t), 25.53(t).

m/z (E-Spray) M 167(70%), (M-CO₂) 125(100%), (cyclohexyl) 83(20%), (CO₂) 44(85%).

C ₉ H ₁₃ NO ₂	requires:	C 65.06%	H 7.83%	N 8.43%	O 18.68%
(pet ether)	found:	C 65.15%	H 8.00%	N 8.11%	O 18.74%

Cyclohexylmalonic acid (248):

To the cyclohexylcyanoacetic acid (**247**), (540mg, 3.3mmol) was carefully added concentrated sulphuric acid (5ml) and this vigorously stirred mixture was heated to 65°C for thirty minutes. After cooling, water (15ml) was added to the mixture *via* the reflux condenser and this mixture was refluxed for an additional hour. On cooling, the mixture was extracted with ether (3x20ml), before being washed with sodium hydroxide (0.25M,

40ml) and dried over anhydrous sodium sulphate. The organic layer was concentrated to a yellowish solid and dried under vacuum. Recrystallisation from diethyl ether in the presence of activated charcoal gave the cyclohexylmalonic acid (248) as a white crystalline material, m.p. 122-124°C (diethyl ether) (570mg, 85%).

ν_{max} (nujol mull) 3400br (OH), 1690br and 1650br (C=O), 1240w, 1200m, 1130m, 920m, 720m.

δ_{H} (300MHz:CDCl₃/DMSO) 8.64 (2H, brs, CH(CO₂H)₂), 3.01 (1H, d, J 9.2Hz, CH-CO₂H), 1.96 (1H, m, CH-CH(CO₂H)₂), 1.71 (5H, br, C₆H₁₀), 1.17 (5H, brm, C₆H₁₀).

δ_{C} (75MHz:CDCl₃/DMSO) 170.63(s), 58.41(d), 37.52(d), 30.55(t), 25.92(t), 25.86(t).

m/z (M⁺) 186(15), 169(15), 133(30), 132(90), 119(100), 117(10), 105(25), 101(18), 82(90).

C₉H₁₄O₄ requires: C 58.07% H 7.53% O 34.40%

(diethyl ether) found: C 58.08% H 7.56% O 34.36%

The general procedure for the preparation of the 2-alkyl-1,3-propanediols is by reducing the parent diethyl malonate derivatives. This is illustrated by the preparation of 2-phenyl-1,3-propanediol (228) from diethyl phenylmalonate (224).

2-Phenyl-1,3-propanediol (228):

Using a round bottom flask (100ml), condenser and a dropping funnel which were charged with a positive pressure of nitrogen, the following reaction was carried out. The lithium aluminium hydride (2.9g, 17mmol), was added dry ether (35ml) and left stirring for ten minutes. To this stirred mixture was slowly added the diethyl phenylmalonate (224) (2g, 85mmol) in dry ether (25ml) over a twenty five minute period. After addition had been completed the reaction mixture was refluxed for ninety minutes. On cooling, the solution was transferred to a large beaker and the flask rinsed with ether (2x15ml). The combined extracts were cooled to 0°C and the excess hydride was quenched by the careful

addition of ice (20ml) and ether (50ml). To the solution was added anhydrous sodium sulphate (12g) to produce a white solid by-product of the lithium slurry and subsequently filtered from the mixture. The organic layer from the filtrate was carefully decanted from the mixture, dried over anhydrous sodium sulphate, concentrated and dried under vacuum. The clear liquid remaining was distilled under reduced pressure 135-137°C, 2mmHg (lit.,¹²⁷ 136-138°C, 2mmHg) to yield the title compound (**228**) as a white crystalline solid.

M.p. 48-49°C (lit.,¹²⁷ 48-49°C) (900mg, 70%).

ν_{max} (nujol mull) 3300br (OH), 1200m, 1160w, 1080w, 1040br, 1010br, 970w.

δ_{H} (300MHz:CDCl₃) 7.21 (5H, m, C₆H₅), 3.86 (2H, dd, J 10.0, 7.0Hz, C(H_A)₂OH), 3.79 (2H, dd, J 10.8, 5.5Hz, C(H_B)₂OH), 3.53 (2H, brs, (CH₂OH)₂), 2.97 (1H, quin, J 4.0Hz, CH-CH₂OH).

δ_{C} (75MHz:CDCl₃) 139.52(s), 128.60(d), 127.97(d), 126.99(d), 65.39(t), 49.62(d).

m/z (M⁺)152(5), 121(10), 105(12), 104(100), 102(40), 91(50), 77(50).

2-tert-Butyl-1,3-propanediol (236):

2-tert-Butyl-1,3-propanediol (**236**) (670mg, 64%) was prepared using a similar method to the preparation of (**228**).

B.p.120-125°C, 9mmHg, m.p.56-57°C (lit.,¹³⁰ 123-125°C, 9mmHg, 56-58°C).

ν_{max} (nujol mull) 3300br (OH), 1230w, 1175m, 1100m, 1050m, 1010s, 950m.

δ_{H} (300MHz:CDCl₃) 3.95 (2H, dd, J 12.0, 3.0Hz, C(H_A)₂OH), 3.75 (2H, dd, J 12.3, 6.0Hz, C(H_B)₂OH), 3.30 (2H, brs, (CH₂OH)₂), 1.61 (1H, quin, J 4.5Hz, CH-CH₂OH), 0.92 (9H, s, C(CH₃)₃).

δ_{C} (75MHz:CDCl₃) 65.54(t), 51.05(d), 31.37(s), 28.15(q).

m/z 84(40), 69(70), 56(42), 57(100), 43(100), 41(30).

2-Butyl-1,3-propanediol (240):

2-Butyl-1,3-propanediol (**240**) (1.47g, 82%) was prepared using a similar method to the preparation of (**228**).

B.p. 98-100°C, 2mmHg (lit.¹²⁷ 99-100°C, 2mmHg).

ν_{\max} (thin film) 3200br (OH), 2950s, 2870s, 1460m, 1375w, 1190w, 1160s.

δ_{H} (300MHz:CDCl₃) 4.18 (2H, brs, (CH₂OH)₂), 3.70 (2H, dd, J 10.8, 4.2Hz, C(H_A)₂OH), 3.57 (2H, dd, J 10.7, 7.4Hz, C(H_B)₂OH), 1.68 (1H, m, CH-CH₂OH), 1.26 (6H, m, (CH₂)₃Me), 0.89 (3H, t, J 7.0Hz, CH₂CH₃).

δ_{C} (75MHz:CDCl₃) 64.77(t), 42.19(d), 29.43(t), 27.58(t), 23.01(t), 14.02(q).

m/z 84(90), 71(40), 69(45), 57(75), 56,55(100), 45(80), 43(90).

2-Propyl-1,3-propanediol (231**):**

2-Propyl-1,3-propanediol (**231**) (620mg, 72%) was prepared using a similar method to the preparation of (**228**).

B.p. 96-98°C, 3mmHg (lit.¹²⁷ 97-98°C, 3mmHg).

ν_{\max} (thin film) 3320br (OH), 2960s, 1460m, 1375m, 1200w, 1065s, 1040s, 1020s, 960m.

δ_{H} (300MHz:CDCl₃) 4.57 (2H, brs, (CH₂OH)₂), 3.69 (2H, dd, J 10.0, 4.0Hz, C(H_A)₂OH), 3.59 (2H, dd, J 10.0, 7.5Hz, C(H_B)₂OH), 1.69 (1H, septet, J 4.0Hz, CH-CH₂OH), 1.33 (2H, m, CH₂Et), 1.22 (2H, m, CH₂Me), 0.91 (3H, t, J 7.0Hz, CH₂CH₃).

δ_{C} (75MHz:CDCl₃) 64.42(t), 42.04(d), 30.09(t), 20.23(t), 14.35(q).

m/z 100(5), 82(25), 71(50), 70(100), 69(45), 57(80), 55(100), 43(100).

2-Ethyl-1,3-propanediol (230**):**

2-Ethyl-1,3-propanediol (**230**) (576mg, 69%) was prepared using a similar method to the preparation of (**228**).

B.p. 86-88°C, 2mmHg (lit.¹²⁷ 83-86°C, 2mmHg).

ν_{\max} (thin film) 3320br (OH), 2930s, 1460m, 1360w, 1200w, 1100m, 1040s.

δ_{H} (300MHz:CDCl₃) 4.56 (2H, brs, (CH₂OH)₂), 3.66 (2H, dd, J 11.0, 4.2Hz, C(H_A)₂OH), 3.57 (2H, dd, J 11.0, 7.2Hz, C(H_B)₂OH), 1.61 (1H, m, CH-CH₂OH), 1.30 (2H, qun, J 7.2Hz, CH₂Me), 0.93 (3H, t, J 7.8Hz, CH₂CH₃).
 δ_{C} (75MHz:CDCl₃) 63.96(t), 43.96(t), 20.63(t), 11.57(q).
m/z 86(15), 71(40), 57(80), 56(100), 55(75), 43(50), 41(100), 39(50).

2-Methyl-1,3-propanediol (229):

2-Methyl-1,3-propanediol (229) (603mg, 78%) was prepared using a similar method to the preparation of (228).

B.p. 83-85°C, 3mmHg (lit.¹²⁸ 83-84°C, 3mmHg).
 ν_{max} (thin film) 3320br (OH), 2960w, 1460br, 1360w, 1200m, 1100m, 1040s.
 δ_{H} (300MHz:CDCl₃) 4.61 (2H, bvs, (CH₂OH)₂), 3.54 (4H, d, J 4.5Hz, CH(CH₂OH)₂), 1.84 (1H, sextet, J 4.5Hz, CH-CH₂OH), 0.87 (3H, d, J 7.0Hz, CH-CH₃).
 δ_{C} (75MHz:CDCl₃) 65.78(t), 37.45(d), 13.36(q).
m/z 91(M⁺H⁺) (20), 71(40), 56(90), 54(80), 46(75), 45(100).

2-Cyclohexyl-1,3-propanediol (243):

2-Cyclohexyl-1,3-propanediol (243) (280mg, 74%) was prepared using a similar method to the preparation of (228).

M.p. 87-90°C (diethyl ether).

ν_{max} (nujol mull) 3400br (OH), 1100m, 1070s, 1050m, 1020s, 990s, 950m.
 δ_{H} (300MHz:CDCl₃) 3.84 (2H, dd, J 10.5, 3.8Hz, C(H_A)₂OH), 3.77 (2H, dd, J 13.0, 7.6Hz, C(H_B)₂OH), 2.91 (2H, brs, (CH₂OH)₂), 1.71 (5H, br, C₆H₁₀), 1.56 (1H, m, CH-CH₂OH), 1.39 (1H, m, CH-Cy), 1.17 (5H, brm, C₆H₁₀).
 δ_{C} (75MHz:CDCl₃) 65.01(t), 47.07(d), 36.45(d), 30.60(t), 26.67(t), 26.47(t).
m/z 140(10), 138(15), 125(20), 122(42), 111(10), 110(65), 109(45).

$C_9H_{18}O_2$ requires: C 68.35% H 11.39% O 20.26%

(diethyl ether) found: C 67.86% H 11.51% O 20.63%

2-Propylpropyl-1,3-bisphosphate (255):

2-Propyl-1,3-propanediol (**231**), (118mg, 1mmol) was dissolved in dichloromethane (5ml) and to this was added the *N,N*-di*isopropylethylamine* (516mg, 4mmol) and the mixture was stirred at 0°C for ten minutes. 2-Cyanoethyl-*N,N*-di*isopropylchlorophosphoramidite* (**132**) (806mg, 3mmol) was then carefully added to this mixture and left stirring for an hour at 25°C to yield the four 1,3-bisphosphoramidite diastereoisomers identified by the two peaks in the ^{31}P NMR as δ_p (36.2MHz: CH_2Cl_2) = 148.00, 147.74ppm. After this time 2-cyanoethanol (284mg, 4mmol) and tetrazole (280mg, 6mmol) were added and the mixture was again left stirring at 25°C for an additional hour. This yielded the 2-propylpropyl-1,3-bis(di-2-cyanoethyl)phosphite identified in the ^{31}P NMR by a peak at δ_p (36.2MHz: CH_2Cl_2) = 139.12ppm. The phosphite triester moiety was oxidized in the same reaction flask by cooling the reaction to -78°C and slowly adding *tert*-butylhydroperoxide (360mg, 5mmol). The mixture was stirred for thirty minutes before being warmed to room temperature and stirred for an additional twenty minutes, to yield the phosphate triester derivative, identified by its chemical shift of δ_p (36.2MHz: CH_2Cl_2) = -2.82ppm. The reaction mixture was taken up in dichloromethane (2x20ml) and washed with distilled water (2x10ml) to remove all the chloride salt by products. The organic layer was then separated and dried over anhydrous sodium sulphate before being concentrated to a dark yellow oil. The mixture was then run through a short column of silica gel to remove any remaining amine salts before undergoing the ammonolysis reaction.

Ammonium solution (35%, 8ml) was added to the reaction mixture and heated to

60°C for two hours. The 1,3-bisphosphate product was identified by ^{31}P NMR (δ_{p} (36.2MHz:NH₄OH) 2.60ppm). After being cooled the solution was evaporated to a white solid and taken up in distilled water (250ml). The product was isolated by ion exchange chromatography. The buffer eluent used was TEAB 100mM and 700mM and run through a DEAE-sephadex column. The eluting solvents were evaporated before the material was successively washed and evaporated with methanol to yield the 2-propylpropyl-1,3-bisphosphate product as a putative triethylammonium salt (435mg, 62%).

δ_{p} (36MHz:buffer) 4.17ppm.

ν_{max} (thin film) 3354s, 2960s, 2880m (N-CH), 1404m, 1085vs (OPO₃²⁻), 970s.

δ_{H} (300MHz:D₂O) 3.87 (4H, ddd, $^3\text{J}_{\text{P-H}}$ 12.0Hz, J 10.5, 5.2Hz, (CH₂-OP)₂), 2.45 (1H, dt, J 13.0, 5.1Hz, CH-CH₂OP), 1.72 (2H, m, CH₂Et), 1.37 (2H, m, CH₂Me), 0.94 (3H, t, J 6.7Hz, CH₂CH₃).

δ_{C} (75MHz:D₂O) 66.07(dt, $^2\text{J}_{\text{P-C}}$ 5.6Hz), 42.88(ds, $^3\text{J}_{\text{P-C}}$ 7.2Hz), 31.19(t), 21.21(t), 14.95(q).
m/z(FAB, -ve Ion, NOBA): (M+H)⁺ 279(10%), (M+Et₃N)⁺ 380(25%), (M+2Et₃N)⁺ 481(40%), (2M+H)⁺ 557(30%).

(FAB, +ve Ion, NOBA): (M+H)⁺ 279(25%), (M+Et₃N+H)⁺ 380(20%), (M+2Et₃N)⁺ 480(80%).

2-Phenylpropyl-1,3-bisphosphate (252):

2-Phenylpropyl-1,3-bisphosphate (252) (300mg, 30%) was prepared using a similar method to the preparation of (255).

δ_{p} (36MHz:NH₄OH) 3.22ppm. δ_{p} (36MHz:buffer) 4.17ppm.

ν_{max} (thin film) 3360s, 2980s, 1652s, 1455w, 1085s (OPO₃²⁻), 978s, 912w.

δ_{H} (300MHz:MeOD) 7.38-7.21 (5H, m, C₆H₅), 4.17 (4H, m, CH-(CH₂)₂), 3.28 (1H, brm, CHPh).

δ_c (75MHz:MeOD) 141.48(s), 129.59(d), 129.42(d), 127.87(d), 66.99(dt, $^2J_{P-C}$ 4.8Hz), 4.80(d).

m/z(FAB, -ve Ion, NOBA): (M-H)⁻ 311(30%), (2M-H)⁻ 623(50%), (2M+H)⁻ 625(20%).
(FAB, + Ion, NOBA): (M+H)⁺ 313(60%), (M+Et₃N+H)⁺ 414(40%), (M+2Et₃N+H)⁺ 515(15%).

2-phenylpropyl-1,3-bisphosphoramidite δ_p (CH₂Cl₂) 147.71, 147.46ppm.

2-phenylpropyl-1,3-bis(di-2-cyanoethyl)phosphite δ_p (CH₂Cl₂) 138.76ppm.

2-phenylpropyl-1,3-bis(di-2-cyanoethyl)phosphate δ_p (CH₂Cl₂) -3.02ppm.

2-tert-Butylpropyl-1,3-bisphosphate (258):

2-*tert*-Butylpropyl-1,3-bisphosphate (258) (95mg, 32%) was prepared using a similar method to the preparation of (255).

δ_p (36MHz:NH₄OH) 3.83ppm. δ_p (36MHz:buffer) 4.10ppm.

ν_{max} (thin film) 3330s, 2957s (N-CH), 1406m, 1085s (OPO₃²⁻), 985s, 966s.

δ_h (300MHz:MeOD) 4.07 (2H, ddd, $^3J_{P-H}$ 9.2Hz, J 14.4, 6.1Hz, C(H_A)₂-OP), 4.05 (2H, ddd,

$^3J_{P-H}$ 9.2Hz, J 14.2, 6.1Hz, C(H_B)₂-OP), 1.59 (1H, m, CH-CMe₃), 0.95 (9H, s, C(CH₃)₃).

δ_c (75MHz:MeOD) 65.89(dt, $^2J_{P-C}$ 5.2Hz), 44.88(d), 33.85(s), 30.50(q).

m/z(FAB, +ve Ion, NOBA): (M+Et₃N)⁺ 393(35%), (M+2Et₃N)⁺ 495(20%), (M+2Et₃N+H)⁺ 496(15%).

2-*tert*-butylpropyl-1,3-bisphosphoramidite δ_p (CH₂Cl₂) 146.98, 146.77ppm.

2-*tert*-butylpropyl-1,3-bis(di-2-cyanoethyl)phosphite δ_p (CH₂Cl₂) 138.64ppm.

2-*tert*-butylpropyl-1,3-bis(di-2-cyanoethyl)phosphate δ_p (CH₂Cl₂) -2.82ppm.

2-Butylpropyl-1,3-bisphosphate (257):

2-Butylpropyl-1,3-bisphosphate (257) (35mg, 10%) was prepared using a similar method

to the preparation of (**255**).

δ_p (36MHz:NH₄OH) 2.62ppm.

m/z(FAB, -ve Ion, NOBA): (M-H)⁻ 291(40%), (M+Et₃N)⁻ 392(30%), (2M-H)⁻ 583(50%).

(FAB, +ve Ion, NOBA): (M+Et₃N+H)⁺ 394(20%), (M+2Et₃N+H)⁺ 495(40%).

2-butylpropyl-1,3-bisphosphoramidite δ_p (CH₂Cl₂) 147.83ppm.

2-butylpropyl-1,3-bis(di-2-cyanoethyl)phosphite δ_p (CH₂Cl₂) 138.75ppm.

2-butylpropyl-1,3-bis(di-2-cyanoethyl)phosphate δ_p (CH₂Cl₂) -3.02ppm.

2-iso-Propylpropyl-1,3-bisphosphate (256):

2-*iso*-Propylpropyl-1,3-bisphosphate (**258**) (229mg, 30%) was prepared using a similar method to the preparation of (**255**).

δ_p (36MHz:NH₄OH) 3.03ppm. δ_p (36MHz:buffer) 4.30ppm.

ν_{max} (thin film) 3352s, 2962s (N-CH), 1653m, 1200m (P=O), 1089s (OPO₃²⁻).

δ_H (300MHz:MeOD) 4.01 (2H, ddd, ³J_{P,H} 9.1Hz, J 14.4, 6.1Hz, C(H_A)₂-OP), 3.99 (2H, ddd, ³J_{P,H} 9.2Hz, J 14.1, 6.0Hz, C(H_B)₂-OP), 3.01 (1H, m, CH-CH₂), 1.78 (1H, m, CH(Me)₂), 1.02 (6H, d, J 7.4Hz, (CH₃)₂).

δ_C (75MHz:MeOD) 64.28(dt, ²J_{P,C} 5.7Hz), 43.21 (d), 32.64(d), 26.81(q).

m/z(FAB, -ve Ion, NOBA): (M+H)⁻ 279(20%), (M+Et₃N)⁻ 380(35%), (M+2Et₃N)⁻ 481(15%), (2M+H)⁻ 557(40%).

(FAB, +ve Ion, NOBA): (M+H)⁺ 279(30%), (M+Et₃N+H)⁺ 380(40%), (M+2Et₃N)⁺ 480(60%).

2-*iso*-propylpropyl-1,3-bisphosphoramidite δ_p (CH₂Cl₂) 147.80ppm.

2-*iso*-propylpropyl-1,3-bis(di-2-cyanoethyl)phosphite δ_p (CH₂Cl₂) 139.31ppm.

2-*iso*-propylpropyl-1,3-bis(di-2-cyanoethyl)phosphate δ_p (CH₂Cl₂) -2.62ppm.

2-Ethylpropyl-1,3-bisphosphate (254):

2-Ethylpropyl-1,3-bisphosphate (**254**) (108mg, 26%) was prepared using a similar method to the preparation of (**255**).

δ_p (36MHz:NH₄OH) 2.83ppm.

δ_H (300MHz:D₂O) 3.99 (4H, brm, (CH₂)₂), 1.86 (1H, m, CH-CH₂), 1.30 (2H, quin, J 7.1Hz, CH₂Me), 1.02 (3H, t, J 7.4Hz, CH₂CH₃).

δ_C (75MHz:D₂O) 66.41(dt, ²J_{P-C} 4.3Hz), 46.12(d), 22.76(t), 16.81(q).

m/z(FAB, +ve Ion, NOBA): (M+Et₃N)⁺ 367(60%), (M+2Et₃N)⁺ 468(20%).

2-ethylpropyl-1,3-bisphosphoramidite δ_p (CH₂Cl₂) 148.00ppm.

2-ethylpropyl-1,3-bis(di-2-cyanoethyl)phosphite δ_p (CH₂Cl₂) 139.10ppm

2-ethylpropyl-1,3-bis(di-2-cyanoethyl)phosphate δ_p (CH₂Cl₂) -2.42ppm.

2-Methylpropyl-1,3-bisphosphate (253):

2-Methylpropyl-1,3-bisphosphate (**253**) (112mg, 27%) was prepared using a similar method to the preparation of (**255**).

δ_p (36MHz:NH₄OH) 2.22ppm.

ν_{max} (thin film) 3350s, 2964s, 1198m (P=O), 1085vs (OPO₃²⁻), 975w.

δ_H (300MHz:D₂O) 4.02 (4H, brm, (CH₂)₂-OP), 1.87 (1H, m, CH-CH₂), 0.91 (3H, d, J 6.4Hz, CH-CH₃).

δ_C (75MHz:D₂O) 66.27(dt, ²J_{P-C} 4.8Hz), 38.40(d), 20.11(q).

m/z(FAB, +ve Ion, NOBA): (M+Et₃N+H)⁺ 348(60%), (M+2Et₃N+H)⁺ 449(40%).

2-methylpropyl-1,3-bisphosphoramidite δ_p (CH₂Cl₂) 148.20ppm.

2-methylpropyl-1,3-bis(di-2-cyanoethyl)phosphite δ_p (CH₂Cl₂) 139.10ppm.

2-methylpropyl-1,3-bis(di-2-cyanoethyl)phosphate δ_p (CH₂Cl₂) -3.02ppm.

2-Cyclohexylpropyl-1,3-bisphosphate (259):

2-Cyclohexylpropyl-1,3-bisphosphate (**259**) (98mg, 21%) was prepared using a similar

method to the preparation of (**255**).

δ_p (36MHz:NH₄OH) 3.63ppm. δ_p (36MHz:D₂O) 1.07ppm.

2-cyclohexylpropyl-1,3-bisphosphoramidite δ_p (CH₂Cl₂) 147.36ppm.

2-cyclohexylpropyl-1,3-bis(di-2-cyanoethyl)phosphite δ_p (CH₂Cl₂) 138.73ppm.

2-cyclohexylpropyl-1,3-bis(di-2-cyanoethyl)phosphate δ_p (CH₂Cl₂) -3.09ppm.

The general procedure for the preparation of the monobenzoyl protected 2-phenyl- (**228**)- and 2-propyl-(**231**)-1,3-propanediols is illustrated below by the synthesis of 3-benzoyl-2-phenyl-1-propanol (**260**).

3-Benzoyl-2-phenyl-1-propanol (260):

Into a 50ml round bottom flask was placed the 2-phenyl-1,3-propanediol (**228**), (1g, 3.3mmol) and to this was added pyridine (2.0ml, 1.95g, 25mmol). This mixture was left stirring at room temperature for five minutes before the benzoyl chloride (920mg, 3.3mmol) was carefully added. After the addition had been completed the reaction was left stirring for thirty minutes. Analysis of the reaction by TLC clearly indicated that the reaction had gone to completion. Ether (30ml) was added to the reaction mixture, and transferred to a separatory funnel. The organic layer was washed successively with saturated copper sulphate solution (2x10ml) and saturated sodium hydrogen carbonate solution (2x10ml). The ether layer was further washed with water (15ml) before being separated and dried over anhydrous sodium sulphate. The organic phase was then filtered and concentrated to a dark yellow liquid. The liquid was then purified by flash chromatography (Et₂O:CH₂Cl₂ (1:1) R_f=0.6) to yield the desired product (**260**) (650mg, 40%).

ν_{max} (thin film) 3500brs (OH), 2980s, 1720s (C=O aryl ester), 1460m, 1320s, 1260s, 1200m, 1120s, 1100s, 1030s, 700s.

δ_{H} (300MHz:CDCl₃) 7.99-7.29 (10H, cm, C₆H₅, C₆H₅CO), 4.68 (2H, d, J 6.7Hz, CH₂OCOPh), 3.98 (2H, d, J 6.7Hz, CH₂OH), 3.63 (1H, dt, J 12.6, 5.1Hz, CH-CH₂OH), 3.08 (1H, brs, CH₂OH).
 δ_{C} (75MHz:CDCl₃) 166.23(s), 138.35(s), 132.96(d), 129.63(s), 129.51(d), 128.73(d), 128.30(d), 127.95(d), 127.45(d), 65.55(t), 53.56(t), 44.11(d).

3-Benzoyl-2-propyl-1-propanol (261):

This was prepared using a similar procedure to (260) to give (261) in (570mg, 35%) yield.
(Et₂O:CH₂Cl₂ (1:1) R_f=0.62).

ν_{max} (thin film) 3500brs (OH), 3000s, 1720s (C=O aryl ester), 1450w, 1320m, 1280s, 1260s, 1100s, 1070m, 1030w, 700s.

δ_{H} (300MHz:CDCl₃) 8.05-7.27 (5H, cm, C₆H₅), 4.42 (2H, d, J 6.7Hz, CH₂OCOPh), 3.64 (2H, d, J 6.4Hz, CH₂OH), 3.41 (1H, brs, CH₂OH), 2.31 (1H, dt, J 13.0, 5.1Hz, CH-CH₂OH), 1.51 (2H, m, CH₂Et), 1.42 (2H, m, CH₂Me), 1.18 (3H, t, J 7.0Hz, CH₂CH₃).
 δ_{C} (75MHz:CDCl₃) 166.39(s), 132.94(d), 130.5(s), 129.55(d), 128.36(d), 65.00(t), 53.51(t), 37.41(d), 30.65(t), 20.09(t), 14.26(q).

3-Hydroxy-2-phenylpropyl-1-phosphate (262):

The general procedure for the preparation of the monophosphate 2-phenyl-(262)- and 2-propyl-(263)-3-propanols was the same as that described previously in the synthesis of the 2-alkylpropyl-1,3-bisphosphates in which the monophosphate is the only product finally isolated by ion exchange chromatography (241mg, 55%).

δ_{H} (300MHz:MeOD) 7.33-7.20 (5H, m, C₆H₅), 4.19 (2H, ddd, ³J_{P-H} 12.8Hz, J 12.4, 6.4Hz, CH₂-OP), 3.82 (2H, dd, J 12.5, 6.7Hz, CH₂OH), 3.12 (1H, m, CH-CH₂OH).
 δ_{C} (75MHz:MeOD) 142.02(s), 129.47(d), 129.37(d), 127.71(d), 67.07(dt, ²J_{P-C} 5.3Hz), 64.43(t), 50.29(ds, ³J_{P-C} 7.3Hz).

δ_p (36MHz:NH₄OH) 3.02ppm.

m/z(EI): M⁺ 256(25%), (C₆H₅CO₂H)⁺ 122(30%), (C₆H₅C≡O)⁺ 105(80%), (C₇H₇)⁺ 91(100%), (C₆H₅)⁺ 77(100%).

3-benzoyl-2-phenylpropyl-1-phosphoramidite δ_p 147.46, 147.75ppm.

3-benzoyl-2-phenylpropyl-1-(di-2-cyanoethyl)phosphite δ_p 138.74ppm.

3-benzoyl-2-phenylpropyl-1-(di-2-cyanoethyl)phosphate δ_p -3.29ppm.

3-Hydroxy-2-propylpropyl-1-phosphate (263):

δ_H (300MHz:MeOD) 3.86 (2H, ddd, ³J_{P-H} 12.0Hz, J=10.5, 5.2Hz, CH₂-OP), 3.57 (2H, d, J 5.6Hz, CH₂OH), 2.41 (1H, dt, J 13.0, 5.1Hz, CH-CH₂OH), 1.72 (2H, m, CH₂Et), 1.36 (2H, m, CH₂Me), 0.92 (3H, t, J 6.7Hz, CH₂CH₃).

δ_C (75MHz:MeOD) 66.07(dt, ²J_{P-C} 5.5Hz), 62.90(t), 42.87(ds, ³J_{P-C} 7.2Hz), 31.09(t), 21.17(t), 14.75(q),

δ_p (36MHz:NH₄OH) 3.36ppm.

m/z(EI): M⁺ 222(30%), (C₆H₅CO₂H)⁺ 122(40%), (C₆H₅C≡O)⁺ 105(60%), (C₇H₇)⁺ 91(25%), (C₆H₅)⁺ 77(90%).

3-benzoyl-2-propylpropyl-1-phosphoramidite δ_p 147.67, 147.96ppm.

3-benzoyl-2-propylpropyl-1-(di-2-cyanoethyl)phosphite δ_p 138.68ppm.

3-benzoyl-2-propylpropyl-1-(di-2-cyanoethyl)phosphate δ_p -3.16ppm.

ENZYME EXPERIMENTAL:

The enzyme assays, kinetic studies and enantioselective reactions were all studied using alkaline phosphatases purchased from Sigma.

Bovine-	Bovine intestinal mucosa alkaline phosphatase
Bacterial-	<i>Escherichia Coli</i> alkaline phosphatase
Rabbit-	Rabbit intestine alkaline phosphatase
Pigeon-	Pigeon intestinal mucosa alkaline phosphatase
Trout-	Trout intestine alkaline phosphatase

Racemic monophosphate (R,S)-1-phenylethyl-1-phosphate (179):

Preparation of Buffer:^{61a}

This was made up as standard stock solution using potassium carbonate (30.02g, 300mmol), potassium hydrogen carbonate (41.46g, 300mmol), magnesium acetate (106mg, 750 μ mol) and zinc acetate (1.5mg, 8.0 μ mol) all of which were dissolved up in distilled water and made up to a volume of 1 litre. The pH of the solution was then adjusted to pH 8.0 using acetic acid.

Enzyme Assay:

The alkaline phosphatase reactions with the various racemic monophosphates were carried out in 5mm NMR tubes and these were kept in a thermostated water bath at 37°C. The progress of the enzyme reaction was monitored by ^{31}P NMR spectroscopy in which the monophosphate ester substrate peaks typically around $\delta_{\text{p}} = 3.00\text{ppm}$ were seen to slowly decrease, while the peak at $\delta_{\text{p}} = 2.69\text{ppm}$ corresponding to the inorganic phosphate product was seen to increase. The determination of the extent of the reaction of a particular substrate, at a given time point, involved a simple procedure. First, the phosphorus spectra accumulated were printed at a large expansion

and the areas (height x width at half height) of the substrate (monophosphate) and product (inorganic phosphate) peaks were measured. From these values the percentage of the enzyme reaction was determined. The P(OH)₄^{143a} material in the 10mm tube was used as an internal standard in these calculations.

³¹P NMR Spectroscopy Specifications:

The ³¹P NMR were obtained on a JEOL FX90Q (36.2MHz) spectrometer. The temperature of the probe was maintained at 35°C throughout the enzyme experiments. The reaction solution in the 5mm tube was placed inside a 10mm tube with an external lock of D₂O to which a small amount of P(OH)₄ had been added.^{143a} A sweep width of 10KHz, pulse width of 30°, pulse delay of 1 second and an exponential window of zero were used. All ³¹P spectra obtained were completely decoupled.

Typically for concentrated solutions of the phosphate substrates of 200mM and 100mM, 200 scans were required to give clear spectra of the progress of the enzyme catalysed reaction. More dilute solutions, 25mM and 10mM required more scans of between 300 to 500 to allow the substrate and product peaks to be clearly identified.

Preparation of samples for enantiomeric excess determination by G.C.:

As the enzyme reaction progressed, the enantiomeric excess (ee) was determined by removing from the tube 100μl aliquots of the reaction mixture at a known percentage of completion and carrying out the following procedure, in order to prepare the sample for analysis by G.C.

The aliquot was added to a separatory funnel containing Et₂O (20ml) and water (2ml). The mixture was shaken vigorously to extract the 1-phenylethanol product into the organic layer. The Et₂O layer was separated, dried over a small amount of anhydrous Na₂SO₄, filtered and dried under vacuum for 10 minutes. The product was

then taken up in 200-500 μ l of dry Et₂O, and to this was added the internal standard of 2-cyanoethanol (25 μ l). The solution was then ready for analysis by gas chromatography. This preparation enabled determination of the ratio of (R):(S) enantiomers of 1-phenylethanol products, thus determining the %ee at a known percentage of completion of the enzyme reaction. At 50% completion, the reaction was terminated by pouring the remaining mixture into a separatory funnel and extracting it with Et₂O (40ml) and water (8ml). The reaction tube was rinsed with Et₂O (2x3ml) and water (2x2ml). These washings were combined, extracted and prepared as described previously.

This method was repeated and the enzyme reactions terminated at increasing percentages of completion. The course of the alkaline phosphatase's reactions were monitored by ³¹P NMR spectroscopy as described earlier in Chapter 3. From these studies it was possible to correlate how the %ee of the (R,S)-1-phenylethanol product varied with the progress of the phosphatase reactions with the (R,S)-phenylethyl-1-phosphate substrate (179).

Once the enzyme solution had been taken up in Et₂O there was no possibility of further turnover of the phosphate derivative by the alkaline phosphatase. It is known that the addition of Et₂O to the reaction mixture inhibits the activity of the phosphatases.

Gas Chromatography analysis:

The gas chromatography work was carried out on a Pye Unicam PU4500 capillary chromatograph, using a SP4270 integrator from Spectra Physics and a R.W. Jennings chart recorder (300mm· h⁻¹). The chiral column used to separate the enantiomeric alcohols was a Cyclodex-B column supplied by SGE Chromatography.

Sample sizes of $1\text{-}2\mu\text{l}$ were regularly used as injection volumes on the column.

The conditions used for baseline separation of the (R)- and (S)-1-phenylethanols was possible using a hydrogen flow rate of 11 lb/in^2 , and an air flow rate of 8 lb/in^2 . Helium was used as a carrier gas and had a flow rate of 16 lb/in^2 . The temperature of the column was 137°C , and the temperatures of the injector and detector were 250°C and 280°C respectively. Peak assignment of the enantiomers was made possible by analysis of authentic samples of the (R)- and (S)-1-phenylethanol materials.

<u>compound</u>	<u>retention time</u>	<u>%material</u>
(R)-(+)	7 min 44 sec	49.80
(S)-(-)	7 min 57 sec	50.20
2-cyanoethanol (internal standard)	5 min 24 sec	-----

Determining %ee:¹⁷⁵

At 50% completion a sample was extracted and subsequently analysed by G.C.
(R,S)-1-phenylethanol product.

<u>compound</u>	<u>area</u>
2-cyanoethanol	19797
(R)-(+)	11550
(S)-(-)	5435

$$0.274 + 0.583 = 0.857$$

$$(R)-(+)=0.583/0.857=68\% \quad ee = (R) - (S) / (R) + (S) \times 100\%$$

$$(S)-(-)=0.274/0.857=32\%$$

$$\%ee^{175}=(R)-(+)-(S)-(-) \quad 68 - 32 = 36\% \quad ee = 36\%$$

Sample experiments from all the alkaline phosphatase reactions were allowed to go to 100% completion, and the alcohol products from these reactions were isolated and analysed by G.C. The purpose of this was firstly to confirm complete turnover of both the (R)- and (S)- phosphate enantiomers. Secondly, to ensure that the extraction method employed is efficient. This was further confirmed as the (R)- and (S)-1-phenylethanol products were detected in equal amounts, as a racemate.

Bovine intestinal mucosa alkaline phosphatases:

The alkaline phosphatase was purchased as a sample of 2,000units which was taken up in 4ml of buffer solution with a few drops of glycerol. The samples were stored as 200 μ l fractions in plastic vials and kept under dry conditions at -4°C until used. 50 μ l (25units) volumes were used in the enzyme assays of the phosphate substrates.

The (R,S)-1-phenylethyl-1-phosphate (179) product was dissolved into the buffer solution to make a standard stock solution of 500mM. From this aliquots of the appropriate amount were taken to make 1ml solutions of the phosphate substrate of required concentrations (10mM, 25mM, 50mM, 100mM and 200mM).

<u>concentration</u>	<u>stock solution</u>	<u>buffer</u>	<u>enzyme</u>
25mM	50 μ l	900 μ l	50 μ l
50mM	100 μ l	850 μ l	50 μ l
100mM	200 μ l	750 μ l	50 μ l
200mM	400 μ l	550 μ l	50 μ l

Kinetic studies:

Measuring the alkaline phosphatase activity of this altered substrate, involved the use of (R)-1-phenylethyl-1-phosphate (180) and (S)-1-phenylethyl-1-phosphate (181) enantiomers which were synthesised from their corresponding (R)-(105) and (S)-(106)-1-phenylethanol products. The pure enantiomeric phosphates were made up into

500mM stock solutions as described previously, and 1ml solutions of 5mM, 25mM, 50mM, 100mM, 200mM and 300mM concentrations were made up by appropriate dilutions of the standard. The bovine alkaline phosphatase (50 μ l, 25units) was added to the 1ml aliquots of these various concentrations and these bovine alkaline phosphatase reactions were monitored as described below.

For each concentration the amount of free phosphate product ($\delta_p = 2.69\text{ppm}$) was measured at regular intervals of 10 minutes until the reaction reached 40% completion. The amount of inorganic phosphate produced at these intervals was calculated from the relative areas of the substrate and product peaks in the ^{31}P NMR spectrum. From these results, a plot of concentration of product versus time, was produced for each substrate concentration of the (R)-(180) and (S)-(181)-1-phenylethyl-1-phosphates. The slope of this plot was measured to obtain a reaction rate for a particular substrate concentration. These experiments were repeated for each concentration of the (R)-(180) and (S)-(181)-phosphate enantiomers to produce a series of reaction rates for the various concentrations under investigation. These were plotted to produce the rate profiles of the (R)-(180) and (S)-(181)-1-phenylethyl-1-phosphate enantiomers with bovine alkaline phosphatase. These experiments were repeated with *E.coli* alkaline phosphatase (200 μ l, 10units) using the various concentrations of (R)-(180) and (S)-(181) phosphate substrates.

pH changes:

The standard buffer solutions were made up with pH values of 11.0, 10.0, 9.0, and 7.0. The pH values below 11.0 were adjusted using acetic acid. The (R,S)-phenylethyl-1-phosphate (179) was dissolved up in these various pH solutions to make a series of 75mM stock solutions. To 950 μ l aliquots of these stock solutions was

added bovine alkaline phosphatase ($50\mu\text{l}$, 25units). The progress of these reactions were monitored using ^{31}P NMR spectroscopy as described earlier.

Changes in Mg(OAc)_2 and Zn(OAc)_2 concentrations:

The buffer solutions were made up with different amounts of zinc acetate and magnesium acetate to produce two new solutions with 50 fold increases in the concentration of zinc and magnesium ions. The (R,S)-phenylethyl-1-phosphate (179) was dissolved in these two concentrations to produce stock solutions of 75mM. The bovine alkaline phosphatase ($50\mu\text{l}$, 25units) was added to $950\mu\text{l}$ aliquots of the solutions and these reactions were monitored as described previously.

	<u>standard</u>	<u>x50</u>
Mg(OAc)_2	106mg, $750\mu\text{mol}$	5.3g, 37.5mmol
Zn(OAc)_2	1.5mg, $8.0\mu\text{mol}$	75mg, $408\mu\text{mol}$

Various NaCl concentrations:

To standard buffer solutions (pH 8.0), were added weighed amounts of sodium chloride to produce a series of solutions containing increased concentrations of NaCl (10mM, 100mM, 300mM, 500mM). Samples of the (R,S)-phenylethyl-1-phosphate (179) were dissolved in these NaCl buffer solutions to produce a series of 75mM solutions. To $950\mu\text{l}$ aliquots of these NaCl mixtures was added bovine alkaline phosphatase ($50\mu\text{l}$, 25units) and the reactions were monitored as described earlier, using ^{31}P NMR spectroscopy. NaCl/1mole/58.5g

<u>[NaCl]</u>	<u>weight</u>	<u>volume</u>
500mM	7.31g	250ml
300mM	4.38g	250ml
100mM	1.46g	250ml
10mM	146mg	250ml

Organic solvents:

150 μ l aliquots of the 500mM standard solution of (R,S)-phenylethyl-1-phosphate (179) were taken and added to the buffer solution (700 μ l) making up solutions of 850 μ l. To these volumes was added a 100 μ l sample of organic solvent (ethanol, methanol, acetonitrile or ethyl acetate), and bovine alkaline phosphatase (50 μ l, 25units). The reactions were monitored using ^{31}P NMR spectroscopy as described earlier. concentration - 75mM

<u>10% solvent</u>	<u>stock solution</u>	<u>buffer</u>	<u>enzyme</u>
100 μ l EtOH	150 μ l	700 μ l	50 μ l
100 μ l MeOH	150 μ l	700 μ l	50 μ l
100 μ l MeCN	150 μ l	700 μ l	50 μ l
100 μ l EtOAc	150 μ l	700 μ l	50 μ l

Escherichia coli alkaline phosphatases:

This was purchased as a sample of 100units, which was taken up in 2ml of buffer solution and a few drops of glycerol. The samples were stored as 200 μ l (10units) fractions in plastic vials and kept under dry conditions at -4°C until used. The 200 μ l (10units) volumes were used in studies with the racemic monophosphate substrates (179, 182-192).

<u>concentration</u>	<u>stock solution</u>	<u>buffer</u>	<u>enzyme</u>
25mM	50 μ l	750 μ l	200 μ l
50mM	100 μ l	700 μ l	200 μ l
100mM	200 μ l	600 μ l	200 μ l
200mM	400 μ l	400 μ l	200 μ l

Rabbit intestine alkaline phosphatases:

This was purchased as a sample of 100mg containing 46units. The sample was stored as 11mg (5units) fractions in small sample vials and kept under dry conditions

at -4°C until used. The 11mg (5units) samples were used in studies with the 1ml solutions of the racemic monophosphate esters (179, 182-192).

<u>concentration</u>	<u>stock solution</u>	<u>buffer</u>	<u>enzyme</u>
25mM	50 μ l	950 μ l	11mg
50mM	100 μ l	900 μ l	11mg
100mM	200 μ l	800 μ l	11mg
200mM	400 μ l	600 μ l	11mg

Pigeon intestinal mucosa alkaline phosphatases:

This alkaline phosphatase was purchased as a sample of 250mg containing 80units. The sample was stored as 50mg (20units) fractions in small sample vials and kept under dry conditions at -4°C until used. The 50mg (20units) samples were used in studies with 2ml solutions of the phosphate substrates (179, 182-192).

<u>concentration</u>	<u>stock solutions</u>	<u>buffer</u>	<u>enzyme</u>
25mM	100 μ l	1900 μ l	50mg
50mM	200 μ l	1800 μ l	50mg
100mM	300 μ l	1700 μ l	50mg
200mM	400 μ l	1600 μ l	50mg

Trout intestine alkaline phosphatases:

This alkaline phosphatase was purchased as a sample of 100mg containing 12units. The sample was stored as 50mg (6units) fractions in small sample vials and stored under dry conditions at -4°C until used. The 50mg (6units) samples were used in studies with 2ml solutions of the racemic monophosphate esters (179, 182-192).

<u>concentration</u>	<u>stock solution</u>	<u>buffer</u>	<u>enzyme</u>
25mM	100 μ l	1900 μ l	50mg
50mM	200 μ l	1800 μ l	50mg
100mM	300 μ l	1700 μ l	50mg

Other racemic monophosphate substrates:

The preparation of the 100mM stock solutions of the other monophosphate ester derivatives was the same as that described previously for the (R,S)-phenylethyl-1-phosphate (179) material, from which aliquots were taken to make 1 or 2ml solutions of the appropriate concentrations.

<u>concentration</u>	<u>stock solution</u>	<u>buffer</u>	<u>enzyme</u>
25mM	50 μ l	900 μ l	50 μ l
50mM	100 μ l	850 μ l	50 μ l
100mM	200 μ l	750 μ l	50 μ l
200mM	400 μ l	550 μ l	50 μ l

The reactions of the monophosphate esters with the various alkaline phosphatases were the same as those described previously, in which their progress was monitored by ^{31}P NMR spectroscopy. With these substrates (182-192), their progress was allowed to continue until the reactions reached 50% completion. The reaction mixture was poured into a separatory funnel containing Et₂O (20ml) and water (5ml). The 5mm tube was then rinsed with Et₂O (2x2ml) and water (2x2ml). The solutions were combined and shaken vigorously before the organic layer was separated. It was then dried over anhydrous Na₂SO₄, filtered and concentrated to a dark yellow solution. Analysis of this material by TLC and G.C. revealed that the desired alcohol product from the enzyme reaction had been isolated. Confirmation of this was made by comparing these materials with authentic samples of the racemic alcohols. This material was further purified to remove the triethylamine and other extracts present in the yellow solution by running it on a small preparative silica plate (15cm x 5cm) alcohol product; EtOAc:CH₂Cl₂ (4:1) Rf=0.59-0.63. The band of product was

identified and the silica removed from the plate with a spatula. The alcohol was subsequently extracted from the silica by vigorous stirring with Et₂O (80ml) in a round bottom flask for 25 minutes. The silica was removed by filtration through a glass frit Buchner funnel. The filtrate was evaporated down to yield the purified alcohol product from the enzyme reaction.

The other alcohols involved in this study, including (R,S)-1-(*para*-nitrophenyl)ethanol (98), (R,S)-2-phenyl-1-propanol (103) and (R,S)-2-butanol (95), all proved difficult to separate as their respective (R) and (S) enantiomers on the chiral G.C. column. In several cases separation proved very difficult, various temperature changes and alterations to carrier gas flow were made, but baseline separation of the enantiomers into two distinguishable peaks proved impossible. The ee of the (R) and (S) alcohols from the enzyme reactions involving the monophosphate substrates (182-192) were determined using various other methods already described in the synthesis section of Chapter 7. Two of these methods involved reacting the alcohol products with chiral acid chlorides to form diastereoisomers. These included the formation of Mosher's esters by reacting the alcohols with MTPA-Cl (209), which were analysed by ¹⁹F NMR spectroscopy and the de's determined, from which the ee's were obtained; or by reacting with the phospholidine-2-sulphide derivative (217) to produce the oxy-3,4-dimethyl-5-phenyl-1,3,2-oxazaphospholidine-2-sulphides as diastereoisomers which were analysed by ³¹P NMR spectroscopy from which their ee's were determined. The experimental error in determining the ee's by measuring the peak areas of the diastereoisomers is negligible. The composition of the ester products are exactly the same. Consequently, their spin-spin relaxation times and levels of saturation will be the same. The only difference is in the configuration of the atoms about the benzylic

chiral centre. Had the observed enantioselectivity of the alkaline phosphatases been significantly high, the accuracy of determining the ee's by measuring the peak areas would have been evaluated further. The other method involved, adding the chiral shift reagent, (R)-phenyl-*tert*-butylphosphinothioic acid (223) to the alcohol products. This split the ^1H NMR signals of the alkyl groups allowing the ee's to be determined. With all these derivatives, the enzyme reactions were allowed to go to 100% completion and the alcohol products from these reactions were isolated and analysed by G.C., TLC and optical rotation. This was to confirm complete turnover of the (R)- and (S)- enantiomers, and extraction of the desired alcohol products.

2-Alkylpropyl-1,3-bisphosphates (252-259):

The preparation of the standard stock solutions for the various 1,3-bisphosphates were the same as those described previously for the racemic monophosphates using the alkaline buffer. From these, aliquots were taken to make 1ml solutions of the required concentrations.

The reactions of these substrates with the various alkaline phosphatases were the same as those described for the monophosphate esters. The progress of the enzyme reaction was monitored by ^{31}P NMR spectroscopy. The reactions were allowed to continue to 50% completion, before they were terminated. The reaction was poured into a separatory funnel containing Et_2O (20ml) and water (20ml). The NMR tube was rinsed with Et_2O (2x2ml) and water (2x2ml). The solutions were combined and shaken vigorously and the organic layer was separated, dried over anhydrous Na_2SO_4 and concentrated to a dark yellow liquid. Analysis of this material by TLC against an authentic sample of the 2-alkyl-1,3-propanediol was carried out to confirm that the undesired 2-alkyl-1,3-propanediol by-products had been obtained from the reactions

with the bovine phosphatase. These products were subsequently analysed by ^1H and ^{13}C NMR spectroscopy to validate these observations. The aqueous layer was taken up in distilled water (200ml) and isolated by ion exchange chromatography. The buffer eluent used was TEAB of 50mM and 700mM concentrations, run through a DEAE-sephadex column. The inorganic phosphate product of the enzyme reaction and the unreacted bisphosphate were identified and isolated. The eluting solvents were evaporated, before the materials were successively washed with MeOH to yield the monophosphate product as a bis(triethylammonium) salt and the bisphosphate material as a putative triethylammonium salt. If any of the monophosphate product had been isolated, it would have been converted to a phosphate triester derivative. The free hydroxyl group would have subsequently been derivatised with a chiral acid chloride such as MTPA-Cl (209), to determine the chirality at the C-2 carbon of the propyl chain. Thereby, determining the chirality of the monophosphate intermediates from the reactions of the alkaline phospahptases with the 2-alkylpropyl-1,3-bisphosphates.

Preparation of the Tris-HCl buffer:^{57,58}

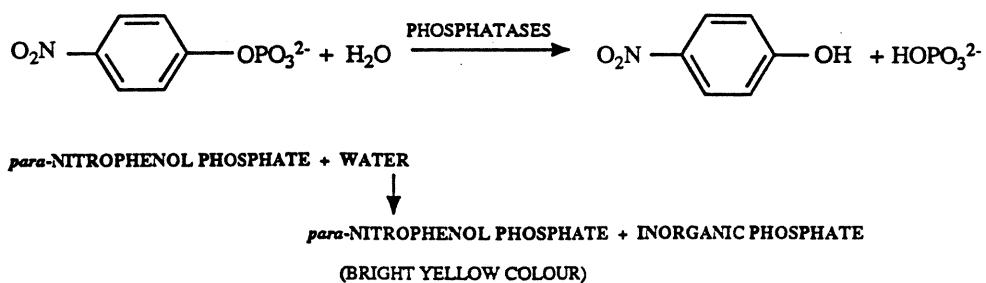
This was made up as a standard stock solution using tris(hydroxymethyl)aminomethane (2.42g, 20mmol) and potassium chloride (3.75g, 50mmol) which were dissolved in distilled water and made to a volume of 1 litre. The pH of the buffer was then adjusted to pH 8.0 using hydrochloric acid.

Preparation of para-nitrophenyl phosphate:

This was made as a fresh solution each day. A 500 μM standard solution was made by dissolving 22mg (50 μmol) of *para*-nitrophenyl phosphate bis(cyclohexylammonium) salt monohydrate in 100ml of distilled water.

Spectrophotometric assays:

Initial rates were measured on a Shimadzu UV-Visible spectrophotometer UV 240. The technique used for quantitating the rate of transfer of the phosphate group to the aqueous solution is shown below.



The enzyme reacts with the *para*-nitrophenyl phosphate to yield *para*-nitrophenol and inorganic phosphate. The absorbance of the *para*-nitrophenol is measured spectrophotometrically at 405nm ($E^{mm}_{405} = 18.5$) as the reaction proceeds.

Unit definition: One unit will hydrolyse 1.0 micromole of *para*-nitrophenyl phosphate to *para*-nitrophenol and inorganic phosphate at pH 8.0 and 25°C.

Alkaline phosphatases standard assay:

The standard 1ml cuvette assay contained tris-HCl buffer, 50mM KCl and various concentrations of the *para*-nitrophenyl phosphate substrate. The reaction was initiated by the addition of alkaline phosphatase and monitored by the increase in absorbance at 405nm at 25°C. The standard assay mixture was used over a wide range of concentrations from 500μM to 1μM and these reactions were performed in duplicate.

e.g.	<u>concentration</u>	<u>stock solution</u>	<u>buffer</u>	<u>enzyme</u>
	100 μ M	200 μ l	780 μ l	20 μ l
	50 μ M	100 μ l	880 μ l	20 μ l
	10 μ M	20 μ l	940 μ l	20 μ l
	5 μ M	10 μ l	970 μ l	20 μ l

E. coli - 20 μ l - 0.05units
 Bovine - 20 μ l - 0.5units

The absorbance· minutes⁻¹ values were calculated from linear intercepts of the spectrophotometric data. Using these values along with the extinction coefficient, in the Beer-Lambert equation, the rates for the various concentrations were calculated. From these results, plots of rate (V) versus concentration ([S]) and reciprocal plots of 1/rate versus 1/[S] were produced. Using the Lineweaver-Burk plots 1/V_{max} was derived as the intercept on the vertical axis and -1/K_m, the intercept on the horizontal axis. The K_m/V_{max} value was obtained from the gradient of the plot. These were subsequently used to determine the K_m and V_{max} values, from which the k_{cat} was calculated as shown below.

Beer-Lambert equation A = ε c l l = path length
 c = rate concentration/min
 A = absorbance
 ε = extinction coefficient

$$V_{\text{max}} = 0.028 \mu\text{mol} \cdot \text{min}^{-1} \quad K_m = 13 \mu\text{M}$$

$$k_{\text{cat}} [E_0] = V_{\text{max}} \quad [E_0] = \text{mass/RMM} = 2.105 \times 10^{-7} / 92,000$$

$$= 2.288 \times 10^{-12} \text{ moles} \times 10^6$$

$$= 2.288 \times 10^6 \mu\text{moles}$$

$$k_{\text{cat}} = V_{\text{max}} / [E_0]$$

$$= 0.028 / 2.288 \times 10^6 = 12237.76 \text{ min}^{-1} / 60 = 208 \text{ sec}^{-1}$$

$$\underline{k_{\text{cat}} = 208 \text{ sec}^{-1}}$$

Inhibition studies of *E.coli* and bovine alkaline phosphatases with the 2-alkylpropyl-1,3- bisphosphate derivatives (253-259):

The 1ml cuvette assays for these inhibition studies contained the tris-HCl buffer, 50mM KCl, 2mM (100 μ l) or 1mM (50 μ l) or 500 μ M (25 μ l) of the various 2-alkylpropyl-1,3-bisphosphates and the various concentrations of the *para*-nitrophenyl phosphate substrate. The reaction was initiated by the addition of the alkaline phosphatases and monitored by the increase in absorbance at 405nm at 25°C. All these reactions were performed in duplicate for each concentration. 2-alkylpropyl-1,3-bisphosphates- methyl (253), ethyl (254), propyl (255), *iso*-propyl (256), butyl (257), *tert*-butyl (258), and cyclohexyl (259) derivatives e.g. 2-methylpropyl-1,3-bisphosphate (253) added to all the solutions 1mM (50 μ l) to make up 1ml solutions.

<u>substrate</u>	<u>stock solution</u>	<u>buffer</u>	<u>enzyme</u>
250 μ M	500 μ l	430 μ l	20 μ l
100 μ M	200 μ l	730 μ l	20 μ l
50 μ M	100 μ l	830 μ l	20 μ l
10 μ M	20 μ l	910 μ l	20 μ l

E. coli - 20 μ l - 0.05units

Bovine - 20 μ l - 0.5units

The rates for each concentration of the *para*-nitrophenyl phosphate substrate in the presence of the 1,3-bisphosphate derivatives (2mM, 1mM, 500 μ M) were calculated as described in Chapter 6. From these results reciprocal plots of 1/rate versus 1/[S] were produced for each of the respective 1,3-bisphosphate concentrations, and drawn alongside standard plots of the *para*-nitrophenyl phosphate concentrations. These 1,3-bisphosphate inhibitor plots were then compared to the standard Lineweaver-Burk plots,^{83,148} and from these comparisons it was possible to determine

the type of inhibition (competitive, non-competitive or uncompetitive) of the bisphosphates. The 1,3-bisphosphates were identified as competitive inhibitors of the alkaline phosphatases. Using the Lineweaver-Burk plots, $1/V_{max}$ was obtained as the intercept on the vertical axis and $1/K_m$ ($1 + i/K_i$) the intercept on the horizontal axis. These were used to determine the various K_i (inhibitor binding constants) values for the 1,3-bisphosphates as shown by the following example.

e.g. $K_i = K_m \times i/K_p - K_m$

K_i = inhibitor constant
i = inhibitor concentration
K_m = $13.6\mu M$ (bovine phosphatase)
K_p = inhibitor intercept on horizontal axis

<u>bisphosphate concentration</u>	<u>K_p</u>	<u>K_i</u>
$500\mu M$	53	$174\mu M$
$1000\mu M$	91	$176\mu M$
$2000\mu M$	167	$178\mu M$ <u>$Av = 176\mu M$</u>

This was repeated for the various 2-alkylpropyl-1,3-bisphosphates (253-259), at each concentration, and then repeated using *E.coli* alkaline phosphatase.

Inhibition studies of bovine alkaline phosphatase with organic solvents:

The 1ml cuvette assays for these organic solvent studies contained the tris-HCl buffer, 50mM KCl, $500\mu M$ ($500\mu l$) *para*-nitrophenyl phosphate substrate and various volumes of the organic solvents. The reaction was initiated and monitored as described previously. Organic solvents - hexane, toluene, chloroform, diethyl ether, pyridine, ethyl acetate, tetrahydrofuran, dichloromethane, acetone, ethanol, acetonitrile, methanol, dimethylformamide and dimethyl sulphoxide.

para-nitrophenyl phosphate - $500\mu M$ - $500\mu l$ standard used in all the 1ml solutions.

e.g. ethanol

<u>percentage</u>	<u>solvent</u>	<u>buffer</u>	<u>enzyme</u>
0%	0.0 μ l	500 μ l	20 μ l
10%	100 μ l	400 μ l	20 μ l
20%	200 μ l	300 μ l	20 μ l
30%	300 μ l	200 μ l	20 μ l
40%	400 μ l	100 μ l	20 μ l
50%	500 μ l	0.0 μ l	20 μ l

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