Mechanistic Studies of Thiophosphoryl Transfer Reactions

by

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at the

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JANUARY 1991
Dedicated to Asha, Anil, Renu and my Parents
STATEMENT

The accompanying thesis submitted for the degree of Doctor of Philosophy entitled "Mechanistic Studies of Thiophosphoryl Transfer Reactions" is based on work conducted by the author in the Department of Chemistry of the University of Leicester between the period October 1986 and December 1989.

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 in this or any other University.

Signed: .................................................. Date: ............................

17/1/91
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PUBLICATIONS

Parts of this work have been published as communications:

A General Configurational Analysis of O-Substituted [^{16}O,^{18}O] Thiophosphates,

Free Monomeric Thiometaphosphate in Protic Solvents: Complete Racemisation at Phosphorus in the Ethanolysis of 4-Nitrophenyl Thiophosphate,

Evidence for Free Monomeric Thiometaphosphate Anion in Aqueous Solution,
MECHANISTIC STUDIES OF THIOPHOSPHORYL TRANSFER REACTIONS

by Rajinder Misra

ABSTRACT

Nucleophilic displacement reactions of monosubstituted thiophosphate esters have been studied. A general synthesis of isotopically chiral O-substituted $^{16}$O,$^{18}$O thiophosphate monoesters has been developed. A new and general method for the configurational analysis of these labelled thiophosphates using high-field $^{31}$P n.m.r. spectroscopy has been developed. The thiophosphate is $S$-alkylated with myrtenyl bromide (10-bromopin-2-ene) followed by $O$-derivatisation either with dimethyl sulphate, diphenyldiazomethane or benzoyl chloride.

Using the above analysis, it was found that the ethanolysis of the monoanion of (Rp)-4-nitrophenyl $^{16}$O,$^{18}$O thiophosphate proceeds with a high degree of racemisation (ca. 80%) and the dianion with complete racemisation, the corresponding solvolysis of the monoanion in aqueous ethanol gives ethyl thiophosphate with ca. 70% racemisation; these data provide the first direct support of a freely-solvated monomeric thiometaphosphate intermediate in the case of the solvolysis of the dianion in ethanol and a relatively long-lived intermediate for the monoanion in ethanol and in aqueous ethanol.

A kinetic study has also been undertaken to investigate the nature of thiophosphoryl transfer reactions in aqueous solution. The rate of hydrolysis of 2,4-dinitrophenyl thiophosphate was observed to be reduced by increased pressure with a volume of activation ($\Delta V^*$) of +11 cm$^3$ mol$^{-1}$. This result indicates that the thiophosphoryl transfer to water is essentially dissociative in nature involving the intermediacy of thiometaphosphate.

Thiophosphoryl transfer reactions involving "front-side" displacement has also been studied. It is found that the formally intramolecular thiophosphoryl transfer reaction of (Rp)-2-(hydroxymethyl)-4-nitrophenyl $^{16}$O,$^{18}$O thiophosphate proceeds with 65% racemisation and 35% excess retention of configuration. This observation shows that a dissociative mechanism can occur with retention of configuration if the nucleophile is constrained to attack the phosphorus centre on the same side as the leaving group.
CONTENTS

CHAPTER 1 - GENERAL INTRODUCTION

Introduction 1
Di- and trisubstituted phosphate esters 4
Penta-coordinate intermediates 4
Monosubstituted phosphate esters 12
Stereochemical tests for the intermediacy of monomeric metaphosphate via isotopic chirality: in protic solvents 22
Stereochemical tests for the intermediacy of monomeric metaphosphate: in aprotic solvents 31
Other tests for monomeric metaphosphate intermediate 36
Monosubstituted thiophosphate esters and monomeric thiometaphosphate 40
Enzyme-catalysed phosphoryl transfer reactions 42
Stereochemical course of phosphotransferase enzymes 45
Interests and aims of the thesis 48

CHAPTER 2 - SYNTHESIS OF ISOTOPICALLY CHIRAL ALKYL, ARYL AND NUCLEOSIDE THIOPHOSPHATE MONOESTERS

Introduction 50
Literature synthesis of isotopically chiral phosphates 50
Literature synthesis of isotopically chiral thiophosphates 54
Synthesis of (Rp)-4-nitrophenyl \[^{16}O,^{18}O\]\ thiophosphate (89a) 68
Synthesis of (Rp)-ethyl \[^{16}O,^{18}O\]\ thiophosphate (90a) 77
Synthesis of nucleoside \[^{18}O\]\ thiophosphates:
a) Synthesis of (Sp)-adenosine-5'-[\beta-^{18}O,\beta-thio]diphosphate [(Sp)-ADP\beta\beta^{18}O] 79
b) Attempted synthesis of adenosine-5'-[\gamma-thio]triphosphate 89
Conclusion 92

CHAPTER 3 - CONFIGURATIONAL ANALYSIS OF ISOTOPICALLY CHIRAL THIOPHOSPHATE MONOESTERS

Introduction 93
Mass spectrometry method 93
\[^{31}P\] n.m.r. spectroscopy method 97
Literature configurational analysis of inorganic \[^{16}O,^{17}O,^{18}O\]\ thiophosphate 99
Literature configurational analysis of \[^{16}O,^{18}O\]\ thiophosphate esters 103
## CONTENTS (Continued) .......

<table>
<thead>
<tr>
<th>Description</th>
<th>Page No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Development of a new and simple configurational analysis of isotopically chiral thiophosphate monoesters</td>
<td>107</td>
</tr>
<tr>
<td>Configurational analysis of 4-nitrophenyl thiophosphate and ethyl thiophosphate</td>
<td>110</td>
</tr>
<tr>
<td>Configurational analysis of (Rp)-4-nitrophenyl [(^{16}\text{O},${}^{18}\text{O})] thiophosphate (89a)</td>
<td>116</td>
</tr>
<tr>
<td>Configurational analysis of (Rp)-ethyl [(^{16}\text{O},${}^{18}\text{O})] thiophosphate (90a)</td>
<td>117</td>
</tr>
<tr>
<td>Conclusion</td>
<td>117</td>
</tr>
</tbody>
</table>

### CHAPTER 4 - STEREOCHEMICAL STUDY: SOLVOLYSIS OF (Rp)-4-NITROPHENYL [\(^{16}\text{O},${}^{18}\text{O}\)]-THIOPHOSPHATE MONOESTER

- **Introduction**                                                                                           | 120      |
- **Literature study of simple thiophosphoryl transfer reactions**                                             | 121      |
- **The stereochemical course of solvolysis of 4-nitrophenyl [\(^{16}\text{O},${}^{18}\text{O}\)]-thiophosphate (89a):** | 123      |
- **Development and analysis of the stereochemical course**                                                  | 123      |
- **The stereochemical course of the ethanolysis of (Rp)-4-nitrophenyl [\(^{16}\text{O},${}^{18}\text{O}\)] thiophosphate (dianion)** | 124      |
- **The stereochemical course of the ethanolysis of (Rp)-4-nitrophenyl [\(^{16}\text{O},${}^{18}\text{O}\)] thiophosphate (monoanion)** | 129      |
- **The stereochemical course of the solvolysis of (Rp)-4-nitrophenyl [\(^{16}\text{O},${}^{18}\text{O}\)] thiophosphate (dianion) in aqueous alcohols** | 133      |
- **Conclusion**                                                                                             | 136      |

### CHAPTER 5 - KINETIC STUDY: EFFECT OF PRESSURE ON THE RATE OF HYDROLYSIS OF 2,4-NITROPHENYL THIOPHOSPHATE DIANION

- **Introduction**                                                                                           | 141      |
- **Volume of activation (\(\Delta V^\pm\)) as a test of reaction mechanism**                                 | 141      |
- **Effect of pressure on the rate of hydrolysis of phosphate monoesters**                                   | 145      |
- **Kinetic study on the hydrolysis of 2,4-dinitrophenyl thiophosphate dianion (141)**                       | 147      |
- **Synthesis of 2,4-dinitrophenyl thiophosphoryl dichloride (142)**                                         | 147      |
- **Rate of hydrolysis of 2,4-dinitrophenyl thiophosphate dianion (141) at atmospheric pressure**             | 148      |
- **Rate of hydrolysis of 2,4-dinitrophenyl thiophosphate dianion (141) at high pressure**                    | 152      |
- **Conclusion**                                                                                             | 156      |
CHAPTER 6 - STEREOCHEMICAL STUDY: INTRAMOLECULAR THIOPHOSPHORYL TRANSFER REACTION OF (Rp)-2-(HYDROXYMETHYL)-4-NITROPHENYL [\(^{16}O,^{18}O\)]THIOPHOSPHATE

Introduction ........................................... 157
Strategy .................................................... 163
Synthesis of 2-hydroxy-5-nitrobenzyl acetate (146a) .......... 166
Synthesis of (Rp)-2-(hydroxymethyl)-4-nitrophenyl [\(^{16}O,^{18}O\)]thiophosphate (145a) .... 167
The thiophosphoryl transfer reaction of (Rp)-2-(hydroxymethyl)-4-nitrophenyl [\(^{16}O,^{18}O\)]thiophosphate (145a) ........................................ 177
Configurational analysis of the products ......................... 182
Conclusion .................................................. 190

EXPERIMENTAL ............................................. 194

APPENDIX ................................................... 244

REFERENCES ............................................. 251
**ABBREVIATIONS AND SYMBOLS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Meaning</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Adenine</td>
</tr>
<tr>
<td>Ac</td>
<td>Acetyl</td>
</tr>
<tr>
<td>Ad</td>
<td>Adenosine</td>
</tr>
<tr>
<td>Ar</td>
<td>Aryl</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine-5'-diphosphate</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine-5'-monophosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine-5'-triphosphate</td>
</tr>
<tr>
<td>β_Lg</td>
<td>Brønsted coefficient for the leaving group</td>
</tr>
<tr>
<td>β_nuc</td>
<td>Brønsted coefficient for the nucleophile</td>
</tr>
<tr>
<td>b.p.</td>
<td>boiling point</td>
</tr>
<tr>
<td>br</td>
<td>broad</td>
</tr>
<tr>
<td>Bu³</td>
<td>tertiary-Butyl</td>
</tr>
<tr>
<td>Bz</td>
<td>Benzyl</td>
</tr>
<tr>
<td>cm</td>
<td>centimetre</td>
</tr>
<tr>
<td>cm⁻¹</td>
<td>wavenumber</td>
</tr>
<tr>
<td>d</td>
<td>doublet</td>
</tr>
<tr>
<td>dd</td>
<td>doublet of doublets</td>
</tr>
<tr>
<td>DEAE</td>
<td>N,N-dimethylformamide</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethyl sulphoxide</td>
</tr>
<tr>
<td>DMSO</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dq</td>
<td>doublet of quartets</td>
</tr>
<tr>
<td>dqn</td>
<td>doublet of quintets</td>
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<tr>
<td>E</td>
<td>Enzyme</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<td>eq</td>
<td>equivalent</td>
</tr>
<tr>
<td>Et</td>
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</tr>
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</tr>
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<td>Ethanol</td>
</tr>
<tr>
<td>g</td>
<td>grammme</td>
</tr>
<tr>
<td>H</td>
<td>Hydrogen</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-2-Hydroxyethylpiperazine-N'-2-ethane sulphonlic acid</td>
</tr>
<tr>
<td>Hz</td>
<td>Hertz</td>
</tr>
<tr>
<td>Hg</td>
<td>Mercury</td>
</tr>
<tr>
<td>^1H n.m.r.</td>
<td>Proton nuclear magnetic resonance spectroscopy</td>
</tr>
<tr>
<td>IR</td>
<td>Infra-Red</td>
</tr>
<tr>
<td>J</td>
<td>coupling constant</td>
</tr>
<tr>
<td>k</td>
<td>rate constant</td>
</tr>
<tr>
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<td>Kilohertz</td>
</tr>
<tr>
<td>m</td>
<td>multiplet</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>M⁺</td>
<td>Molecular ion</td>
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<tr>
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<td>mg</td>
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<td>mM</td>
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<td>mmol</td>
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<tr>
<td>m.p.</td>
<td>melting point</td>
</tr>
<tr>
<td>Myr</td>
<td>myrtenyl</td>
</tr>
<tr>
<td>m/z</td>
<td>mass to charge ration (Mass spectrometry)</td>
</tr>
<tr>
<td>N</td>
<td>Nitrogen</td>
</tr>
<tr>
<td>Na</td>
<td>Sodium</td>
</tr>
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</table>
In the diagrams throughout this thesis the following notations for oxygen isotopes are used:

\[ ^{16}\text{O} \quad \bullet \]
\[ ^{17}\text{O} \quad o \]
\[ ^{18}\text{O} \quad - \]

Because of exchange of protons, fractional bond orders in anionic forms and the involvement of double bonds which shift positions in fully protonated phosphate esters, to avoid confusion the double bonds and formal charges on peripheral oxygen atoms of phosphate esters are omitted in some cases. Also, R and S configurational assignments are made in accordance with the Cahn-Ingold-Prelog rules* on the basis that peripheral oxygen atoms are always singly bonded to phosphorus.

CHAPTER 1
General Introduction
Introduction

In the course of the rapid development of organophosphorus chemistry over the last 30 years, much interest has focused on the mechanism of nucleophilic substitution in tetrahedral phosphorus(V) compounds, such as phosphate esters (1a,b,c).\(^1\)\(^,\)\(^2\) In particular, phosphoryl (PO\(_3\)^−, unsubstituted or substituted) transfer processes involving nucleophilic displacement at the phosphorus atom of phosphate monoesters (below) and diesters have been widely studied as they represent models for enzyme-catalysed processes. Displacement reactions at phosphorus are involved in such diverse processes as photosynthesis, DNA translation and transcription, etc. Such reactions are important in the energy balance of all organisms, and are also involved in cellular control mechanisms at every level.\(^3\)

For nucleophilic substitution reactions of mono-, di- and trisubstituted phosphate esters, there are at least four fundamental mechanisms, Figure 1.1, which operate, along with some borderline ones.

The dissociative process (1a) is a S\(_{N1}(P)\) process, in which the phosphate undergoing substitution expels the leaving group (RO\(^-\)) in the rate determining step, producing a planar monomeric metaphosphate intermediate (2). The nucleophile captures the intermediate in the subsequent fast step. As the metaphosphate is planar and can be attacked by the nucleophile from either face equally, this mechanism will result in loss of configuration...
(1a) Dissociative reaction via a monomeric metaphosphate

\[ \text{RO-P-O}^- \rightarrow \text{RO} + \left[ \begin{array}{c} \text{O}^- \\ \text{P} \\ \text{O} \end{array} \right] \rightarrow \text{Racemic product} \]

(2a) Associative reaction via a penta-coordinate transition state

\[ \text{RO-P-O}^- + \text{Nu} \rightarrow \left[ \begin{array}{c} \text{O}^- \\ \text{P} \\ \text{O} \\ \text{O} \\ \text{O} \end{array} \right] \rightarrow \text{Inversion} \]

(2b) In-line addition-elimination mechanism via a penta-coordinate intermediate

\[ \text{RO-P-O}^- + \text{Nu} \rightarrow \text{RO-P-Nu} \rightarrow \text{Inversion} \]

(2c) Adjacent addition-elimination mechanism involving a pseudorotation

\[ \text{RO-P-O}^- + \text{Nu} \rightarrow \text{RO-P-O}^- \rightarrow \text{RO-P-O}^- \rightarrow \text{Retention} \]

= Pseudorotation

FIGURE 1.1 Fundamental mechanisms for nucleophilic displacement reactions at phosphorus of monosubstituted phosphate esters.
(racemisation) in chiral phosphates. This mechanism is analogous to the $S_N1$ process in carbon chemistry where the metaphosphate can be viewed as analogous to the carbonium ion.

There are three associative mechanisms. The first (2a) is a concerted $S_N2$ process in which the displacing nucleophile attacks from the side opposite the leaving group and displaces it in a single step via a penta-coordinate transition state. This kind of substitution proceeds with inversion of configuration at a chiral phosphorus centre.

The other two associative mechanisms differ from the preceding one in that they involve true intermediates, and they differ from each other in the direction of approach of the nucleophile.

For mechanism (2b) the nucleophile attacks opposite the leaving group to form a penta-coordinate trigonal bipyramidal intermediate. This intermediate has the nucleophile and the leaving group in apical positions and the three remaining substituents in the equatorial plane. The intermediate decomposes to products by the departure of the leaving group from the apical position. The stereochemical outcome of this mechanism is inversion of configuration.

For mechanism (2c) the nucleophile attacks phosphorus adjacent to the leaving group placing the nucleophile in the apical position and the leaving group in the equatorial position of the first trigonal pyramidal intermediate. In the formation and decomposition of such intermediates the attacking nucleophile enters an apical position and the leaving group is expected to depart from an apical position on the grounds of the law of microscopic reversibility (i.e. most favourable pathway from products back to starting materials is the same reaction pathway). The loss of the leaving group must be preceded by a pseudorotatory rearrangement leading to a second intermediate in which the two apical groups are exchanged with two equatorial ligands. The leaving group then departs from the apical position. The overall stereochemical outcome of this mechanism is retention of configuration.
**Di- and trisubstituted phosphate esters**

Higher levels of esterification reduce the negative charge on phosphate esters and this tends to make their nucleophilic displacement reactions more associative in character, presumably because of a reduction of electrostatic repulsion between the nucleophile and the phosphate anions. The evidence supporting an associative mechanism for the hydrolysis of diaryl phosphate diesters and dialkyl aryl triesters can be summarised as follows:

i) Studies on rates of hydrolyses show linear free-energy relationship with the pKa of the leaving group, e.g. the Brønsted coefficient, $\beta_{lg}$ ($lg =$ leaving group) for hydrolysis of diaryl phosphate diesters = -0.97. The rate is also sensitive to the pKa of the nucleophile, e.g. $\beta_{nuc}$ (nuc = nucleophile) = 0.3 - 0.4 for reaction of 2,4-dinitrophenyl, methyl phosphate diester with pyridines.$^4$

ii) A measurable solvent deuterium isotope effect ($k_{H_2O}/k_{D_2O}$) of 1.6 has been found for the hydrolysis of diaryl phosphate diesters.$^5$

iii) Negative entropies of activation ($\Delta S^\ddagger$) of -25 entropy units for these reactions have been reported, which is typical of an associative process.$^5$

**Penta-coordinate intermediates**

The associative addition-elimination mechanism for nucleophilic substitution at tetrahedral phosphorus leads to a penta-coordinate intermediate of trigonal bipyramidal geometry (tbp) (the trigonal bipyramidal geometry is the lowest energy for simple 5-coordinate phosphoranes, although more strained systems are thought to adopt a square pyramidal structure). The concerted $S_N2(P)$ and in-line addition-elimination mechanisms differ only in that for the former the trigonal bipyramid is a transition state whereas for the latter it is an intermediate of finite lifetime. When the trigonal bipyramid species is sufficiently long-lived to be considered an intermediate (3) along the reaction pathway, it may be considered as analogous to the mechanism of reaction at sp$^2$-hybridised carbon (for example nucleophilic attack at carbonyl groups) which are usually assumed to go via a tetrahedral intermediate (4). However, greater mechanistic diversity is observed in the substitution reactions of phosphate esters (mechanisms 2b and 2c, Figure 1.1) relative to
carboxylic acid derivatives, since the dynamics of the penta-coordinate intermediate are far more complex:

i) two types of position exist in the trigonal bipyramid intermediate (5), three equatorial and two apical positions, and therefore ligands are non-equivalent [the apical bonds are longer and weaker than the equatorial bonds because an equatorial bonding electron pair experiences bonding pair \leftrightarrow\ bonding pair repulsion from only two other electron-pairs at 90° (i.e. the apical pairs) whereas the apical bonding-pairs experience repulsion from three electron-pairs at 90° (i.e. the equatorial trio)]; and

ii) ligand reorganization (alternatively termed polytopal or permutational isomerization) may occur, rearranging the position of ligands about phosphorus in the tbp intermediate and as a result several isomeric tbp intermediates may be involved in a single mechanistic pathway.
These points can be clearly illustrated by considering stable penta-coordinate phosphoranes. For instance, phosphorus pentafluoride (PF$_5$) shows two $^{19}$F n.m.r. signals at -85°C in a ratio of 3:2 as expected for the two types of fluorine atoms. However, as the temperature is increased, the $^{19}$F signals coalesce into one signal indicating that all the fluorine atoms are in the same environment. The equivalence of atoms comes about as a result of ligand reorganization which is fast on the n.m.r. timescale leading to a time averaged signal. The observation of phosphorus-fluorine coupling in PF$_5$ indicates that this process must be occurring intramolecularly. Two significant mechanisms have been postulated for this ligand reorganization process, the "turnstile" mechanism$^7$ and the more widely accepted Berry "pseudorotation"$^8$ which is illustrated in Figure 1.2.

\[ \text{FIGURE 1.2 The Berry pseudorotation mechanism.} \]

The pseudorotation process involves the exchange of two equatorial ligands with two apical ligands by a non-dissociative process. The apical atoms become equivalent to two of the equatorial atoms by deforming the trigonal bipyramid to the intermediate square pyramidal structure. Further deformation produces a new trigonal bipyramid in which the new apical groups are derived from two original equatorial ligands. After the pseudorotation, the axis of the trigonal bipyramid is 90$^\circ$ to the original axis; throughout the operations one of the equatorial ligands acts as a pivot and remains equatorial in the isomeric trigonal

\[ \text{FIGURE 1.2 The Berry pseudorotation mechanism.} \]

The pseudorotation process involves the exchange of two equatorial ligands with two apical ligands by a non-dissociative process. The apical atoms become equivalent to two of the equatorial atoms by deforming the trigonal bipyramid to the intermediate square pyramidal structure. Further deformation produces a new trigonal bipyramid in which the new apical groups are derived from two original equatorial ligands. After the pseudorotation, the axis of the trigonal bipyramid is 90$^\circ$ to the original axis; throughout the operations one of the equatorial ligands acts as a pivot and remains equatorial in the isomeric trigonal

- 6 -
Much work can be cited which supports the importance of penta-coordinate phosphoranes and pseudorotation in the displacement reactions of phosphate esters:

1. **Stable penta-coordinate phosphoranes.** Some stable penta-alkoxyphosphoranes have been prepared and characterised, for example pentaethoxyphosphorane (6).

![Image of phosphorane structure]

2. **Rate acceleration and isotope exchange in the hydrolysis of five-membered cyclic phosphates.** Westheimer and workers discovered that the hydrolyses in acid or base of five-membered cyclic esters of phosphoric acid proceed millions of times faster than the hydrolyses of their acyclic counterparts. For example, methyl ethylene phosphate (7) undergoes hydrolysis in acid with 70% ring opening (to 8) and 30% exocyclic cleavage (to 9), both reactions occurring at rates over a million times faster than that for the hydrolysis of dimethyl phosphate:

\[
k / k_{(MeO)3PO} = 2 \times 10^6 \quad (8), 70\%
\]

\[
1 \times 10^6 \quad (9), 30\%
\]
of trimethyl phosphate \([\text{(MeO)}_3\text{PO}]\).

On the other hand, the rates of hydrolysis of six and seven-membered cyclic phosphates (10 and 11) are comparable with those of acyclic analogues.\(^{13}\)

\[
\begin{align*}
\text{(10)} & \\
\text{(11)} & 
\end{align*}
\]

A striking and key observation is that the hydrolysis of the diester (12) in acid or base is not only \(10^8\) times faster than the hydrolysis of the acyclic analogue, dimethyl phosphate \([\text{(MeO)}_2\text{P(O)OH}]\), but that it is accompanied by rapid oxygen isotope exchange into the starting material.\(^{14}\) In fact, oxygen exchange occurs at approximately one-fifth the rate of ring opening hydrolysis, thus this exchange is \(ca.\ 2 \times 10^7\) times faster than the hydrolysis of dimethyl phosphate; this acyclic compound shows no detectable incorporation of \(^{18}\text{O}\).

\[
\frac{k}{k_{\text{(MeO)}_2\text{P(O)OH}}} = 10^8
\]

\[
\begin{align*}
\text{(12)} & \\
\text{hydrolysis 80\%} & \\
\text{exchange 20\%} & 
\end{align*}
\]

Although one might be tempted to argue that relief of ring strain can account for the accelerated opening of the ring in cyclic phosphates, it cannot explain the increased rate of displacement of the exocyclic substituent. The ingenious explanation of these effects, due
largely to Westheimer, invokes a trigonal bipyramidal intermediate that is formed in an accelerated process from the ring-strained cyclic phosphate. The intermediate can then undergo pseudorotation and ligand loss to yield products.

Westheimer rationalized the rate acceleration of cyclic esters by making four assumptions about the penta-coordinate intermediate.15,16

i) The more electronegative ligand prefers to occupy the apical positions (although occupation of the apical position has been shown to depend on more than simple electronegativity).

ii) The five-membered ring will be more stable bridging apical-equatorial positions with an O–P–O bond angle of 90°, rather than bridging equatorial-equatorial positions with an angle of 120°.

iii) Pseudorotation is facile as long as conditions (i) and (ii) are preserved.

iv) Nucleophiles enter an apical position and leaving groups ultimately depart from an apical position, even if this requires several pseudorotations in between ("Extended Principle of Microscopic Reversibility").

On this basis, one can visualize the hydrolysis (endo- and exocyclic cleavage) of (7) as shown in Figure 1.3. From this figure, one can see that the enhanced rate of exocyclic hydrolysis also arises from the relief of strain in the ring on going from the tetra-coordinate (7) to the penta-coordinate configuration (13) with the ring spanning apical-equatorial positions. Pseudorotation then allows the methoxy group to leave from the apical position.

Pseudorotation, as mentioned, was originally proposed to explain the behaviour of stable phosphoranes and has now been extended to trigonal-bipyramidal intermediates. However, in order for pseudorotation to occur, the intermediate must be sufficiently long-lived. In reactions of acyclic compounds this may not be the case, indeed the evidence for trigonal bipyramidal intermediates is sparse. Only when the intermediate is especially stabilized (e.g. by relief of ring strain in five-membered rings) is there clear evidence for stable trigonal bipyramidal intermediates involving pseudorotatory processes.
FIGURE 1.3 The hydrolysis of methyl ethylene phosphate (7) via penta-coordinate intermediates.
Implications from stereochemistry. Some nucleophilic displacement reactions at phosphorus in phosphate esters proceed with retention of configuration.\(^1\) This is most simply explained in terms of an "adjacent" associative mechanism in which the penta-coordinate intermediate pseudorotates once to allow expulsion of the leaving group from an apical position (mechanism 2c, Figure 1.1). Generally, retention of configuration is observed in exocyclic displacement reactions at phosphorus held in a five-membered ring because of the strong preference for the ring to be placed apical-equatorial in the penta-coordinate intermediate.

The empirical rules and assumptions, mentioned in point [2], governing the formation and breakdown of penta-coordinate intermediates help us to understand much about the associative displacement reactions at phosphoryl centres. The actual balance between in-line (14) and adjacent (15) approach of the nucleophile very much depends on the nature of:

i) the nucleophile,

ii) the leaving group and

iii) other substituents around the phosphorane.

\[
\begin{align*}
\text{L} & \quad \text{a} \quad \text{b} \quad \text{L} \\
\text{P} & \quad \text{c} \\
\text{Nu} & \quad \text{Nu}
\end{align*}
\]

(14) in-line

\[
\begin{align*}
\text{L} & \quad \text{a} \quad \text{b} \\
\text{P} & \quad \text{c} \\
\text{Nu} &
\end{align*}
\]

(15) adjacent

L = Leaving group

Nu = Nucleophile

The idea of apicophilicity (preference for the apical position in a trigonal bipyramid) helps to rationalise the preference for an observed displacement mechanism by predicting the relative energies of the alternative phosphoranes. A quantitative scale of relative apicophilicities was developed by Trippett\(^2\) (Figure 1.4) and is derived from n.m.r. studies on the dynamic interconversion of stable phosphoranes.
Monosubstituted phosphate esters

While the nucleophilic substitution reactions of phosphate di- and triesters are associative in character, there is much evidence to support the proposal that phosphate monoesters undergo nucleophilic displacement reactions via a dissociative mechanism (mechanism 1a, Figure 1.1), involving a 3-coordinate P(V) monomeric metaphosphate ion intermediate (PO$_3^-$) (2).\textsuperscript{19}

As our interest is in the phosphoryl transfer reactions of monoesters, the evidence for and against the involvement of metaphosphate will be discussed in detail.

It was in fact over 30 years ago (1955) that the monomeric metaphosphate was first postulated as an intermediate in the hydrolysis reactions of phosphate monoesters. Independent reports from the laboratories of Westheimer\textsuperscript{20} and Bunton\textsuperscript{21,22} showed that the rate...
of hydrolysis of phosphate monoesters is maximal near pH 4 and that the pH-rate profile is described by a bell-shaped curve (Figure 1.5). The rate maximum corresponds to the

![Graph showing pH-rate profile](image)

For: $\text{AlkOP(O)(OH)}_2$
$\text{ArOP(O)(OH)}_2$
$\text{MeC} = \text{COP(O)(OH)}_2$
$\text{ROP(S)(OH)}_2$
$\text{NH}_2\text{CO. OP(O)(OH)}_2$
$\text{HSP(O)(OH)}_2$

**FIGURE 1.5** pH-rate profile for the hydrolysis of $\text{ROP(O)(OH)}_2$.

to the maximum concentration of the monoanionic form of the monoester. This observation led both groups to propose the mechanistic pathway shown in Figure 1.6.

A dissociative pathway is also proposed for the hydrolysis of dianions of phosphate monoesters with good leaving groups (pKa <6.5). The evidence supporting a dissociative reaction involving a metaphosphate-like intermediate for the above reactions can be summarised as follows:-

(i) For phosphate monoesters of phenols that have a pKa higher than 6.5 the monoanion is the most reactive species. On the other hand, if the phenol has a pKa lower than 6.5 the monoester is the more reactive in its dianionic form. This is rationalised in terms of a dissociative reaction since it is difficult to see how the introduction of a second negative charge on the phosphate ester could accelerate an associative reaction. Also, monoanions often react faster than neutral species, indicating that rate-limiting nucleophilic attack at phosphorus is unlikely.
a) **Monoanion**

\[
\begin{align*}
\text{RO-P-} & \xrightarrow{\text{O-H}} \text{RO}^+ \text{P-} \\
\text{O-H} & \rightarrow \text{ROH} + \left[ \begin{array}{c}
\text{O-} \\
\text{P} \\
\text{O-} \\
\end{array} \right] \\
\nu^- & \rightarrow \left[ \begin{array}{c}
\text{O} \\
\text{P} \\
\text{O} \\
\end{array} \right]
\end{align*}
\]

b) **Dianion**

\[
\begin{align*}
\text{RO-P-} & \xrightarrow{\text{O-}} \text{RO}^- \\
\text{O-} & \rightarrow \text{RO}^- + \left[ \begin{array}{c}
\text{O-} \\
\text{P} \\
\text{O-} \\
\end{array} \right] \\
\nu^- & \rightarrow \left[ \begin{array}{c}
\text{O} \\
\text{P} \\
\text{O} \\
\end{array} \right]
\end{align*}
\]

**FIGURE 1.6** Pathway for the solvolysis of:

a) monoanion of phosphate monoester with a leaving group of pKa > 6.5

b) dianion of phosphate monoester with a leaving group of pKa < 6.5.
(ii) Monoester hydrolyses have entropy of activation (ΔS⁺) values close to 0 entropy units, which despite the problems of interpreting activation parameters for reactions in structured solvents, is more consistent with a unimolecular reaction.

(iii) The Brønsted coefficient for the leaving group, βlg, for phenolic phosphate monoesters with modest leaving groups (pKa >6.5) is -0.3, consistent with a high degree of bond cleavage in the rate-limiting step and departure of neutral phenol from the zwitterion, derived from the monoanion [Figure 1.6 a)]. In the case of dianions with good leaving groups, the βlg is -1.2, consistent with phenolate as the leaving group and the reaction being sensitive to the pKa of the leaving group [Figure 1.6 b]).

In contrast, the Brønsted coefficient for the attacking nucleophiles (βnuc) in the aminolysis of 4-nitrophenyl phosphate is small (0.13), which indicates that the rate is comparatively insensitive to the basicity of the nucleophile.

(iv) The solvent isotope effect, kH2O/kD2O, for the hydrolysis of acetyl phosphate is negligible. In the hydrolysis of 18O bridge-labelled 2,4-dinitrophenyl phosphate dianion, the kinetic isotope effect (kH1O/kD1O) is found to be 1.02, which indicates substantial P–18O bond cleavage in the transition state.

(v) When solvolyses of monoesters are conducted in aqueous alcohols, the product ratios of alkyl phosphate to inorganic phosphate are close to (although rarely exactly equal to) the mole ratios of alcohol to water. This is thought to be indicative of reaction of a highly reactive and unselective intermediate such as monomeric metaphosphate.

All the above kinetic data relate to experiments conducted in aqueous or mixed aqueous media. However, much more convincing evidence for the metaphosphate intermediate comes from studies carried out in aprotic and less nucleophilic solvents. Besides, if phosphate monoesters do react via the intermediacy of metaphosphate, the change from ground state to transition state involves loss of charge (overleaf), so the ground state is more polar than the transition state; therefore the use of low polarity solvents would help stabilize the
transition state more than the ground state and hence increase the rate of reaction. Use of a less polar medium would also help to remove complications due to reaction with the solvent.

An impressive example of phosphorylation of a nucleophile from potential metaphosphate precursors is provided by the "three-phase test", which was devised by Rebek et al.31-34 as a general test for reactive intermediates.

The principle of the "three-phase test" involves attaching the precursor of the reactive intermediate by covalent bonds to one set of insoluble polystyrene polymer beads (P1). A receptor (containing the nucleophile) for the reactive intermediate is attached to a different set of polymer beads (P2). The two sets of beads are then mixed in a solvent and chemical reaction is allowed to take place. Since a direct (associative) reaction between the functional groups bound to the polymer is impossible, the appearance of the product resulting from reaction of the intermediate with the receptor is proof that an unstable intermediate was formed, which migrates through the solvent from one set of beads to the other.
In the test for metaphosphate, illustrated in Figure 1.7, Rebek et al. attached the metaphosphate precursor, an acyl phosphate, to one set of beads (P1) and glycine residues were attached to the second type of polymer beads (P2). After the beads were mixed in dioxane as solvent, the amino group of the glycine residue was found to be phosphorylated. This provides good evidence that a diffusing electrophilic phosphorylating species, presumed to be monomeric metaphosphate, was formed which passed through the solvent from one set of beads to the other.

\[
\begin{align*}
\text{P1} & \rightarrow \text{P1}^\cdot \\
\text{Dioxan, } 80^\circ C & \\
\text{PO}_3^- & \\
\text{P2} & \rightarrow \text{P2}^\cdot \\
\end{align*}
\]

**FIGURE 1.7** The three-phase test devised by Rebek *et al.*

It has been demonstrated that a species with all the properties expected of monomeric metaphosphate can be generated from the Conant-Swan fragmentation of β-bromophosphonate dianions (16). \(^{19,35,36}\)
It was in fact as long ago as the 1920s that Conant and his group found that the anions of several β-halophosphonates and -phosphinates which they had prepared, decomposed in aqueous solution, see below. Of course, at the time, the idea of monomeric metaphosphate did not arise.

Much later, in 1966, Kenyon et al. examined the stereochemistry of the Conant-Swan fragmentation. The threo and erythro isomers of 1,2-dibromo-1-phenylpropane phosphonic acid (16a) were both prepared and allowed to undergo fragmentation in water or acetonitrile as solvent. The fragmentation was found, as expected, to occur in an anti-periplanar fashion as shown in Figure 1.8.
Interestingly, Westheimer and his co-workers\textsuperscript{43,44} have found that when the Conant-Swan fragmentation is carried out in the presence of acetophenone, it yields the corresponding enol phosphate (17). As carbonyl oxygens are usually not very nucleophilic,

\[
\left[ \text{PO}_3^- \right] \xrightarrow{\text{Ph} \text{C} = \text{O} \text{CH}_3} \text{POCH}_2\text{OH}
\]

(17)

phosphorylation of such sites would presumably require a good electrophile, such as metaphosphate. Indeed, phosphorylation of carbonyl oxygens has been used by Westheimer to be a criterion for the intermediacy of monomeric metaphosphate. He also suggests that one of the biological rôles of phosphorylation may be to activate carbonyl functions.
The most exciting result obtained involving the intermediacy of metaphosphate was the discovery by Clapp, Satterthwait and Westheimer\textsuperscript{45} that when the closely related, methyl metaphosphate (18) [made from pyrolysis of methyl 2-butenyl phosphonate (19)], was bubbled through a solution of neat $N$-methylaniline (20), not only was the expected product $N$-methylphosphoramidate (21) (50\%) obtained, but a substantial amount of ortho- and para-($N$-methylamino)benzenephosphonic acid (22, 23) (total yield 35\%) were also isolated (see Figure 1.9). These latter two products arise from the electrophilic attack of monomeric

\begin{center}
\textbf{FIGURE 1.9} Preparation and electrophilic aromatic substitution reaction of methyl metaphosphate.
\end{center}
methyl metaphosphate on the aromatic ring, indicating that metaphosphate is indeed a potent electrophile.

The evidence thus far presented is entirely consistent with a monomeric metaphosphate intermediate. However, it does not constitute proof of a freely solvated, diffusable intermediate. Indeed, the possibility of direct coordination of solvents (such as dioxan and acetonitrile) with the metaphosphate moiety introduces some ambiguity into some of the early studies.

Monomeric metaphosphate ion is isoelectronic with sulphur trioxide (SO$_3$) and would be expected to add to the oxygen atom of dioxan (24) or to the nitrogen atom of acetonitrile (25) just as SO$_3$ does (26); the adduct of SO$_3$ to dioxan is a well known crystalline zwitterion and mild sulphonating agent.$^{46,47}$

Evidence for such metaphosphate/solvent complexes comes from the previously mentioned study by Clapp et al.$^{48}$ involving the aromatic substitution of N-methylaniline (20) by methyl metaphosphate. The reaction was conducted in a range of solvents and it is found that the amount of aromatic substitution is significantly reduced when dioxan or acetonitrile are used. This is thought to imply that the monomeric metaphosphate coordinates to the heteroatom in these solvents to give a very much less active phosphorylating agent.

In addition, Ramirez and Maracek$^{48}$ found that in the solvolysis of 2,4-dinitrophenyl phosphate in the presence of the base, quinuclidine, they observed (by $^{31}$P n.m.r.) the transient formation of the adduct between monomeric metaphosphate and the base as shown overleaf. All the above results suggest that in the presence of dioxan or acetonitrile, the adduct rather than the free metaphosphate is the phosphorylating agent. These latter
observations imply that even in the case of the "three-phase test" for reactive intermediates conducted in these solvents means that even this criterion is ambiguous.

Much of the data mentioned above seems consistent with the metaphosphate mechanism, especially the earlier kinetic data (i-v) which indicates a large degree of bond cleavage at the transition state of the rate-determining step. However, the characteristics of a transition state do not provide unambiguous proof of the existence of an intermediate. Furthermore, the kinetic data provides no insight into the freeness and lifetime of any metaphosphate intermediate.

In order to probe the longevity of the presumed monomeric metaphosphate intermediate, Knowles\(^4^9\) has suggested that the determination of the stereochemical course of the reactions at phosphorus of phosphate monoesters provides a clocking mechanism for determining the lifetime of a metaphosphate intermediate. Just as stereochemistry has provided valuable information regarding nucleophilic substitution reactions at carbon, it was hoped that such a method would also provide essential insight into the nature of nucleophilic reactions at phosphorus in phosphate monoesters. If a planar trigonal, freely solvated metaphosphate is formed, then an initially chiral phosphorus group would undergo racemisation. Stereochemical investigations of phosphoryl transfer reactions by various groups are reviewed in detail below.

**Stereochemical tests for the intermediacy of monomeric metaphosphate via isotopic chirality: in protic solvents**

The stereochemical course of a displacement reaction at the phosphorus atom of a phosphate monoester and anhydride (27) cannot be determined as long as the three
unesterified oxygens are indistinguishable from one another. However, the degeneracy of the three oxygen atoms in (27) can be broken by isotopic substitution. This is possible because oxygen exists as three stable isotopes, namely $^{16}\text{O}$, $^{17}\text{O}$ and $^{18}\text{O}$. The equivalence of the three peripheral oxygens in (27) can also be destroyed by both isotopic and elemental substitution using sulphur. Both [ $^{16}\text{O},^{17}\text{O},^{18}\text{O}$ ]phosphate monoester (28) and [ $^{16}\text{O},^{18}\text{O}$ ]-thiophosphate monoester (29) are not chiral in the conventional sense but instead are isotopically chiral.

![Diagram](image)

By the middle of the 1970s $^{17}\text{O}$ and $^{18}\text{O}$ were commercially available as water and dioxygen at enrichment levels that made it possible to prepare both (28) and (29). Currently, $^{17}\text{O}$ is available at about 50 atom % as water whilst $^{18}\text{O}$ is available in excess of 99 atom % as water. However, there were two problems; first, the preparation of (28) and (29) and second, the configurational analysis of the absolute configurations of (28) and (29). Many groups have supplied solutions to both problems and their contributions are reviewed in the subsequent chapters.

In one of the first stereochemical study of its kind, Knowles and his collaborators\textsuperscript{50,51} synthesized (Rp)-[ $^{16}\text{O},^{17}\text{O},^{18}\text{O}$ ] isotopically chiral samples of phenyl phosphate (30) and 2,4-dinitrophenyl phosphate (31) (Figure 1.10) [the synthesis of such compounds is
FIGURE 1.10 Stereochemical study on the solvolysis of $^{16}$O,$^{17}$O,$^{18}$O phosphate monoesters by Knowles and co-workers. 

mentioned in detail in Chapter 2]. They knew from the earlier kinetic work by Kirby and Varvoglis\textsuperscript{23} that at pH 4.7 the monoanion of (30) is considered most likely to undergo solvolysis reactions with the intermediacy of monomeric metaphosphate and that at pH 10.2 the dianion of (31) solvolyses \textit{via} the dissociative mechanism. Therefore, Knowles \textit{et al.} solvolysed (30) and (31) separately in a 1:1 mixture of water and methanol at pH 4.7 and
10.2 respectively. In each case the product, methyl phosphate (32), was purified by ion-exchange chromatography and subjected to the configurational analysis developed for phosphate monoesters (configurational analysis of oxygen chiral phosphate monoesters is described in detail in Chapter 3). In each case the methanolysis was found to proceed with complete (within experimental error) inversion of configuration at phosphorus. If one accepts the vast amount of previous kinetic data, which can be best explained by the generation of metaphosphate as an intermediate in these solvolysis reactions, then this stereochemical result is inconsistent with a fully dissociative mechanism via a freely solvated intermediate but rather with an associative $S_N2$ mechanism. The stereochemical result requires that if a metaphosphate is formed, it is captured before any rotation about the P–O bond can occur. That is, the putative metaphosphate cannot be a liberated intermediate, it is also too reactive to tumble within the cage in which it was generated. So, how can we reconcile the previous kinetic results with this stereochemical data?

It has long been acknowledged that, in addition to the usual clear-cut associative and dissociative reactions, it is necessary to consider various borderline mechanisms. Jencks\textsuperscript{52,53} has considered reaction mechanisms in the borderline region between $S_N1$ and $S_N2$ and has suggested that the apparent conflict between kinetic and stereochemical data be reconciled in terms of a preassociative mechanism. A reaction is forced to go via a preassociative pathway if the intermediate in the reaction is so unstable that the rate of collapse back to the starting material or forward to the product is faster than the rate at which the nucleophile can diffuse away from the encounter complex; this type of mechanism can be more clearly understood by referring to Figure 1.11.
When the complex of $D-O^\cdot PO_3^\cdot A$ collapses back to starting material ($k_{-1}$) more rapidly than the acceptor $A$ can diffuse away ($k_{away}$), i.e. $k_{-1} > k_{away}$, then the forward reaction will proceed by the preassociative route. Furthermore, such preassociative pathways can in principle be concerted or stepwise, see overleaf. A concerted preassociative reaction involves a loose or "exploded" $S_N2$-like transition state in which neither leaving group nor nucleophile is closely associated with the central phosphorus atom and therefore the putative monomeric metaphosphate has no lifetime whatsoever. However, if the postulated metaphosphate has a lifetime between the limits set by the rate of a bond vibration (for a single bond, approximately $10^{13} \text{ s}^{-1}$) and the diffusion rate ($10^{10} \text{ s}^{-1}$ in water), then the reaction becomes preassociative stepwise in character; in the case of the preassociative concerted reaction, the rate constant for collapse of the metaphosphate exceeds that of a bond vibration.
A truly liberated intermediate is generated when the lifetime of the intermediate exceeds the limit set by the diffusion rates. Figure 1.12 shows the difference in free energy profiles for preassociative and dissociative processes.

In both concerted and stepwise preassociative mechanisms, bond breaking dominates the rate-determining step and inversion of configuration is the stereochemical outcome, although strictly speaking, a nucleophile is not necessarily constrained to approach in-line with respect to the leaving group. Therefore, the preassociative mechanism accommodates not only the stereochemical data obtained by Knowles et al. but also the previous kinetic data. However, the stereochemical method is unable to distinguish between the involvement of a concerted or a stepwise preassociative mechanism although it is now generally agreed that phosphoryl transfer reactions in protic media (i.e. water, methanol, ethanol) occur via a preassociative concerted pathway in which the metaphosphate-like species is never free.

Haake and Allen found that, of all the phosphoryl donor species, the product composition in mixed alcohol/water of N-phosphoguanidines (33) most closely matched the solvent ratio. These phosphorylated derivatives appear to undergo solvolysis very rapidly and
Preassociative concerted process

\[ \Delta G \]

Preassociative stepwise (---) and dissociative (-- -) processes

\[ \Delta G \]

FIGURE 1.12 Free energy (\( \Delta G \)) profiles for preassociative (concerted and stepwise) and dissociative (lower profile, dashed line) processes.
according to the authors appear to be among the most reactive precursors of metaphosphates.\textsuperscript{55} Even for such derivatives, Knowles \textit{et al.}\textsuperscript{51} found that the stereochemical course of the phosphoryl transfer from \(N\)-phosphoguanidine to methanol occurred predominately with inversion of configuration, thus implying a preassociative concerted process. This explanation has also been used to accommodate the surprising observation that the phosphoryl group derived from the Conant-Swan fragmentation of 1,2-dibromo-2-phenylethyl-[\((\text{Rp})^{16}\text{O},^{17}\text{O},^{18}\text{O}\)phosphonic acid (34) is transferred to 1-\([(1,1\text{-dimethyl}ethyl)\text{-dimethylsilyloxy}]\)-(S)-butan-3-ol (35), a secondary alcohol, also with inversion of configuration at phosphorus,\textsuperscript{56} (Figure 1.13).

Although the stereochemical method can distinguish between the free intermediate and preassociative mechanisms, it is unable to distinguish between concerted and stepwise preassociation mechanisms. It is difficult to design experiments that will address this problem. However, in an effort to distinguish between these mechanisms two groups, Jencks and Skoog\textsuperscript{57,58} and Bourne and Williams\textsuperscript{59,60} have used linear free-energy relationships (Brønsted plots) as a probe for the existence of intermediates in phosphoryl transfer reactions. They measured the rates of reaction of substituted pyridinium nucleophiles with phosphoryl pyridinium compounds. These substrates are analogues of phosphate monoesters with good leaving groups. Jencks and Skoog reacted \(N\)-phosphorylated 3-methoxypyridine with a series of pyridine nucleophiles (in aqueous solution) whose conjugate acids have pKa values that straddle that of 3-methoxypyridine. Bourne and Williams carried out a similar study with \(N\)-phosphorylated isoquinoline as substrate, also in aqueous solution. If there is a metaphosphate intermediate then, for pyridines more basic than 3-methoxypyridine (or isoquinoline), one would expect the rate-limiting step to be formation of the
bond to the nucleophile. However, for pyridines less basic than 3-methoxypyridine (or isoquinoline), breakage of the bond to the leaving group would be rate-limiting. Both groups found that the logarithms of the observed rate constants show a linear dependence on pKa of the nucleophile, a result which excludes free metaphosphate as a reaction intermediate. A straight line is found with no evidence of a break point at the position where the pKa of the nucleophile equals the pKa of the leaving group. This is inconsistent with a preassociative stepwise mechanism which involves a change in the rate-limiting step. Therefore, the data obtained by these two groups suggest that the transfer of the phosphoryl group from phosphonamidates is best viewed as a preassociative concerted mechanism where the single transition state is a loose exploded S_N2-type transition state in which the leaving group and the nucleophile are weakly bonded to phosphorus, see Figure 1.14.
FIGURE 1.14 Concerted preassociative mechanism for phosphoryl transfer from \( N\)-phosphorylated 3-methoxypyridine (and isoquinoline) to a series of substituted pyridines in aqueous solution.

Stereochemical tests for the intermediacy of monomeric metaphosphate: in aprotic solvents

To complement the stereochemical study of the solvolysis of phosphate monoesters in protic media by Knowles et al., certain groups have looked at the stereochemical course of phosphoryl transfer reactions in aprotic solvents. In such solvents, the putative metaphosphate might have a substantial lifetime.
Cullis and Rous\textsuperscript{61} investigated the stereochemical course of the reaction of isotopically chiral adenosine-5'-\([\beta-(Sp)-^{16}O,^{17}O,^{18}O]\)diphosphate (ADP) (36) with 2-O-benzyl-(S)-propane-1,2-diol (37) nucleophile in acetonitrile, Figure 1.15. Their stereochemical analysis revealed that the phosphoryl transfer reaction proceeded with extensive racemisation (95\%) (and 5\% excess retention of configuration). [Control experiments showed that (a) the starting material (36) had not racemised significantly during the course
of the reaction and (b) the product did not racemise under the reaction conditions.] At first sight, the simplest explanation of the stereochemical result would be that a free, symmetrically solvated, planar metaphosphate is formed during the course of such a reaction which is trapped to give a racemic product. However, this is not the only interpretation of this observation. The solvent acetonitrile is potentially nucleophilic and the possibility exists, as mentioned before, for formation of a specific metaphosphate/solvent adduct. Therefore, racemisation could arise as a result of multiple transfers of the phosphoryl group between solvent molecules before trapping by the acceptor alcohol. Indeed, the formation of such a complex could account for the small extent of retention found in this experiment, which could arise from the occasional trapping of the first-formed acetonitrile adduct. This experiment therefore does not provide unambiguous evidence for the intermediacy of metaphosphate.

However, Cullis and Rous studied the stereochemical course of the phosphoryl transfer from a P,P-disubstituted pyrophosphate derivative (38) to (37) in dichloromethane, Figure 1.16. They reported that the transfer proceeded with a large amount of racemisation (~70%) and a 30% excess inversion of configuration. This study suggests that the majority of the phosphoryl transfer proceeds via a "free" metaphosphate. This result using dichloromethane as the solvent is less ambiguous (than the study which used acetonitrile) since this would presumably require coordination of the metaphosphate to the lone pairs on the chlorine substituents which is certainly less likely than the acetonitrile complex.

One way to increase the lifetime of the postulated metaphosphate intermediate could be to lower the concentration of the nucleophile in the medium or lower the potency of the nucleophile by electronic or steric means. Using this approach, Ramirez and Marecek investigated the phosphorylation of sterically hindered nucleophiles such as tert-butanol (BuOH) in a number of solvents. Their kinetic investigation found that in acetonitrile, the rates of reaction of aryl phosphate monoester dianions with tert-butanol and with water are almost the same, despite the fact that tert-butanol is a much more sterically hindered nucleophile. Also, when the phosphoryl transfer was conducted in the presence of
competing alcohol nucleophiles, it was observed that tert-butanol competes effectively with methanol. These results, however, contrast sharply with the findings from the solvolysis of aryl phosphate monoester dianions in aqueous solution. Here, although methanol and ethanol compete very well with water for the phosphoryl group to form methyl and ethyl phosphate respectively, in aqueous isopropanol, isopropyl phosphate can barely be detected. The results of these experiments led Ramirez and Marecek to propose that the formation of tert-butyl phosphate is proof that aryl phosphate monoester dianions react by a dissociative mechanism since the rate of an associative reaction is assumed to be more sensitive to the steric bulk in the nucleophile. Furthermore, no tert-butyl phosphate is produced from a phosphate ester not thought to react via a dissociative mechanism, e.g. 2,4-dinitrophenyl phosphate monocation.

Indeed, it has been suggested that the formation of tert-butyl phosphate could be used as a criterion for the intermediacy of monomeric metaphosphate.
In view of the above proposal and to test the soundness of the mechanistic criterion, Knowles and co-workers investigated the stereochemical course of phosphoryl transfer reactions to tert-butanol in non-aqueous solution. Initially, the phosphoryl transfer from the dianion of (Rp)-phenyl [\(^{16}O,^{17}O,^{18}O\)]phosphate (30) to the tertiary alcohol in acetonitrile\(^{69,70}\) was studied, Figure 1.17. The product, tert-butyl [\(^{16}O,^{17}O,^{18}O\)]phosphate (39),

\[
\begin{array}{c}
\text{O-P-O} \\
\text{CH_3CN, 70°C} \\
\rightarrow \\
\text{Bu'OH} \\
\end{array}
\]

\begin{equation}
(30) \rightarrow (39)
\end{equation}

**Result**: 94% racemisation
6% excess retention

**FIGURE 1.17** Stereochemical study of phosphoryl transfer reactions of aryl phosphate monoesters to tert-butanol by Knowles et al.

was purified by ion-exchange chromatography and subjected to their configurational analysis. The product was found to be largely racemic. However, in view of the problems associated with the use of acetonitrile as a solvent, Knowles and his collaborators decided to study the phosphorylation in neat tert-butanol.\(^{70,71}\) (Rp)-4-nitrophenyl [\(^{16}O,^{17}O,^{18}O\)]-phosphate dianion (40) was solvolyzed in the tertiary alcohol at 30°C. The product (39) was

\[
\begin{array}{c}
\text{O-N-} \\
\text{O-P-O} \\
\text{Bu'OH} \\
\rightarrow \\
\text{Bu'OH} \\
\end{array}
\]

\begin{equation}
(40) \rightarrow (39)
\end{equation}

**Result**: 100% racemisation
found in this case to be completely racemic. This stereochemical study, therefore, provided the first direct evidence for a freely solvated monomeric metaphosphate intermediate in the alcoholysis of a monosubstituted phosphate ester. The result also confirmed the proposal of Ramirez and Marecek that the formation of tert-butyl phosphate is, in such reactions, diagnostic of the metaphosphate intermediate.

An important conclusion that can be drawn from all the studies reviewed so far is that the reaction medium can cause the selection of a mechanism, these results can be summarized as shown in Figure 1.18.

\[
\begin{array}{ccc}
\text{Associative} & \text{Preassociative} & \text{Dissociative} \\
\text{concerted }/ \text{ stepwise} & & (\text{CH}_3\text{CN})? \\
\text{H}_2\text{O} & \text{CH}_2\text{Cl}_2 & \text{CH}_3\text{OH} \\
\text{CH}_3\text{OH} & & \text{Bu}^+\text{OH}
\end{array}
\]

**FIGURE 1.18** Range of mechanisms of phosphoryl transfer reactions of phosphate monoesters in a variety of solvents.

**Other tests for monomeric metaphosphate intermediate:**

*Positional isotope exchange (P.I.X.) as a test for metaphosphate.*

Positional isotope exchange (given the acronym P.I.X.) studies arguably represent the most sensitive probe of events occurring within the solvent cage during phosphoryl transfer reactions. It can provide information into the existence, however fleeting, of metaphosphate. The principle of the P.I.X. analysis for pyrophosphate (41) is shown in Figure 1.19. Provided that the lifetime of the dissociation complex (42) within the solvent cage is long enough to allow P–O bond rotation and that the back reaction can compete effectively with the forward reaction, then the reaction of bridge $^{18}\text{O}$-labelled (41) proceeding via a metaphosphate intermediate will lead to scrambling of the label in the reisolated starting material; (bridge and non-bridge labelled $^{18}\text{O}$ species can be distinguished by high-field $^{31}\text{P}$ n.m.r. spectroscopy, such analysis is discussed later). One can see why P.I.X. is such a
more sensitive probe than stereochemical analysis in detecting the fleeting existence of an intermediate. In the latter case, metaphosphate will only signal its presence if it can tumble within the solvent cage or it is long-lived enough to escape the solvent cage completely to allow racemisation.

\[
\begin{align*}
\text{(41)} & \quad \text{(42)} \\
\end{align*}
\]

**FIGURE 1.19** Principle of positional isotope exchange (P.I.X.) analysis.

Lowe and Tuck\textsuperscript{72} undertook P.I.X. experiments with adenosine-5'-[\(\beta^{18}\text{O}_4\)]diphosphate (ADP\(\beta^{18}\text{O}_4\)) (43) in both a protic and an aprotic solvent. They initially incubated (43) in tris-HCl aqueous buffer in the presence of (a) magnesium chloride and (b) ethylenediaminetetraacetate (EDTA) at various pHs for 3 weeks at 20°C. After this time, they found that 20% of (43) had been hydrolyzed to adenosine-5'-[\(^{18}\text{O}\)]monophosphate (AMP) (44) and inorganic \(^{18}\text{O}_3\) phosphate (P\(_1\)) (45), Figure 1.20. The high-field \(^{31}\text{P}\) n.m.r. spectrum of the recovered starting material (43) showed no evidence of positional \(^{18}\text{O}\) exchange between the P\(_\alpha\)-O-P\(_\beta\) bridge and the non-bridging positions at P\(_\alpha\) in either experiment.

However, when the tris-(tetra-n-butylammonium) salt of (43) was incubated on its own in acetonitrile at 70°C for 2 days, they isolated four compounds. They were identified as AMP (43), adenosine-2',5'-biphosphate (pAp) and adenosine-2'-phospho-5'-diphosphate...
(ppAp). The high resolution $^{31}$P n.m.r. spectrum of reisolated (43) showed that extensive $^{18}$O exchange from the Pα-O-Pβ bridge to the non-bridging site at Pα had taken place.

\[
\text{AdO—P ———> aqueous buffer} \quad (43) + \quad \text{AdO—P}
\]

\[
\text{No P.I.X.} \quad (43) + \quad \text{AdO—P}
\]

\[
\text{No P.I.X.} \quad (43) + \quad \text{AdO—P} + \text{O—P}
\]

\[
(43) \xrightarrow{\text{CH}_3\text{CN, 70°c}} \quad \text{AdO—P ———> AMP} \quad (44)
\]

\[
+ \quad \text{pAp} + \text{ppAp}
\]

**FIGURE 1.20** Positional isotope exchange experiments of ADPβ$^{18}$O$_4$ in protic and aprotic solvents.

The observation of no P.I.X. in ADPβ$^{18}$O$_4$ in aqueous solution is very much consistent with a preassociative concerted mechanism, as confirmed before, and the phosphoryl transfer would be expected to proceed with inversion of configuration at phosphorus.

However, the P.I.X. result with ADPβ$^{18}$O$_4$ in acetonitrile is very much consistent with a presumed preassociative stepwise mechanism. Racemisation would also be observed if the lifetime of the metaphosphate intermediate is long enough or if multiple transfers in acetonitrile are involved.
In another similar study, Cullis and Nicholls investigated the P.I.X. in the reaction of adenosine-5′-[αβ-18O]diphosphate trianion (46) in neat acetonitrile, in acetonitrile/tert-butanol and in neat tert-butanol. In each case, after ca. 70% extent of reaction, they found significant levels of scrambling of the bridged oxygen in the reisolated ADP. These results are consistent with the transient formation of monomeric metaphosphate, although in the case of acetonitrile, the possible phosphorylation of the solvent leads to ambiguity in interpretation.

Volume of activation as a test for metaphosphate.

Le Noble et al. have suggested that the sign and magnitude of the volume of activation (ΔV*) for a reaction may be an unambiguous indicator of an associative or dissociative reaction pathway (Chapter 5 is devoted to our work on ΔV*). They measured the ΔV* for the hydrolysis of 2,4-dinitrophenyl phosphate dianion and found that the rate was accelerated by pressure (ΔV* = -4.8 cm³ mol⁻¹). Normally for a dissociative process increase in pressure slows down the rate of reaction as the rate-limiting step is expansive; in this case the sign of ΔV* is positive. On the other hand, an associative process is accelerated by the application of pressure since the rate-limiting step involves shrinkage; in this case ΔV* is negative in sign. Le Noble’s result is not, therefore, compatible with a free metaphosphate but rather it is concluded that the hydrolysis proceeds via nucleophilic attack by water at phosphorus.

Generation of metaphosphate in non-liquid phases.

Arguably one of the most direct and convincing evidence for the existence of monomeric metaphosphate comes from studies conducted in the gas phase. Originally, calculations
suggested that such an intermediate can exist in the gas media; later, experiments conducted in the gas phase suggested that such an intermediate can exist in the gas media; later, experiments conducted in the gas phase identified metaphosphate monoanion by mass spectrometry and metaphosphate derivatives were produced in the gas phase by pyrolysis. Surprisingly, metaphosphate is thermodynamically stable and is not particularly reactive as either a nucleophile or electrophile in the gas phase. Therefore, the high reactivity of metaphosphate in solution is attributed not to its intrinsic instability, but to its environment and to the high stability in solution of the 4-coordinate products resulting from nucleophilic attack at phosphorus.

Despite the fact that metaphosphate (and similar 3-coordinate P(V) species) has resisted isolation, other 3-coordinate P(V) compounds have been isolated and characterised. The nitrogen analogue of monomeric metaphosphate, (47), has been prepared by Niecke and Flick and Scherer and Kuhn. Niecke et al. later characterised this compound by X-ray crystallography and found, as expected, that (47) is indeed trigonal planar in shape. Naturally, extra stability can be conferred in 3-coordinate P(V) species by the attachment of bulky groups on the ligand bonded to phosphorus; however, as oxygen is divalent, this is not possible in the case of monomeric metaphosphate.

Monosubstituted thiophosphate esters and monomeric thiometaphosphate

Although there is convincing evidence that a relatively "free" metaphosphate intermediate does not exist in substitution reactions in aqueous solution, this does not preclude the possibility of reactions of related species going via analogous intermediates. For instance, the sulphur analogue, monomeric thiometaphosphate (48), has also been implicated in displacement reactions of O-substituted thiophosphate monoesters (49), as shown overleaf.
Thiophosphate monoesters show many of the characteristics of a dissociative pathway in the nucleophilic displacement reactions that they undergo. Rate is insensitive to the nature of the nucleophile; their hydrolysis reaction shows a typical bell-shaped pH-rate profile (similar to Figure 1.5); they hydrolyse faster than their oxy counterparts both as the monoanion and the dianion, in contrast to the diminished reactivity of thiophosphate di- and triesters in associative nucleophilic displacement reactions with respect to the corresponding phosphates. Moreover, monomeric metaphosphate has only really been detected in the gas phase whereas Roesky et al. have recently isolated trithiometaphosphate (50) as its tetraphenylarsonium salt, which suggests that successive substitution of oxygen by sulphur produces thiometaphosphates which may possess greater kinetic stability than metaphosphate itself. Indeed, one can probably predict this on the basis of the polarisability of sulphur compared to oxygen, which in the former case should lead to reduced electrophilicity at phosphorus.

There have been very few studies of simple chemical thiophosphoryl transfer reaction.
Despite the extensive use made of thiophosphate substrate analogues as stereochemical probes for enzyme-catalysed phosphoryl transfer reactions.

**Enzyme-catalysed phosphoryl transfer reactions**

Many of the multitude of studies on the reactivity of phosphorus species towards nucleophilic substitution have been aimed at gaining a clearer understanding of the chemistry of biomolecules containing phosphorus. The enzyme-catalysed phosphoryl-group transfer reactions of phosphate esters and anhydrides play a main rôle in the structure, operation and replication of living systems.

Phosphorylated biomolecules, especially nucleoside triphosphates (also referred to as nucleotides), e.g. adenosine-5'-triphosphate (ATP) (51), are important energy sources, releasing energy on their hydrolysis.

![Phosphoryl group](image)

Figure 1.21 summarises the classes of enzyme that catalyse nucleophilic substitution reactions at phosphorus. Enzymes that catalyse phosphoryl transfer reaction of phosphate monoesters and anhydrides, known as phosphotransferases, fall into three categories:

a) **Phosphokinases** catalyse phosphoryl transfer of the terminal (γ) phosphoryl group of a nucleoside triphosphate, e.g. ATP, to an acceptor nucleophile which can be a hydroxyl group, e.g. protein kinase, hexokinase; a nitrogen group, e.g. creatine kinase, arginine kinase; a carboxyl group, e.g. acetate kinase; or a phosphate group, e.g. adenylate kinase, nucleoside diphosphate kinase.

b) **Phosphatases** are enzymes that catalyse phosphoryl transfer to water which acts as the acceptor nucleophile (hydrolysis), e.g. ATPases catalyse transfer of the terminal phosphoryl group of ATP to H₂O.
**Reaction**

\[
\text{RO} - \text{P} - \text{O}^- + \text{XH} \rightarrow \text{ROH} + \text{O} - \text{P} - \text{X} \quad \text{Phosphokinases}
\]

\[
\text{RO} - \text{P} - \text{O}^- + \text{H}_2\text{O} \rightarrow \text{ROH} + \text{O} - \text{P} - \text{OH} \quad \text{Phosphatases}
\]

\[
\text{O} - \text{P} - \text{O}^- \quad \text{X} \text{YH} \rightarrow \text{O} - \text{P} - \text{O}^- \quad \text{Phosphomutases}
\]

\[
\text{RO} - \text{P} - \text{OR}' + \text{XH} \rightarrow \text{R'OH} + \text{RO} - \text{P} - \text{X} \quad \text{Nucleotidyl transferases}
\]

\[
\text{RO} - \text{P} - \text{OR}' + \text{H}_2\text{O} \rightarrow \text{R'OH} + \text{RO} - \text{P} - \text{OH} \quad \text{Nucleases}
\]

**Type of enzymes**

Nucleotidyl cyclases
Pyrophosphokinases
Phosphodiesterases

**FIGURE 1.21** Classes of enzymes that catalyse reactions at phosphoryl centres.
c) **Phosphomutases** catalyse phosphoryl transfer to an acceptor which is another functional group on the donor molecule, i.e. an intramolecular reaction, e.g. phosphoglycerate mutase, phosphoglucomutase, etc.

The enzymes that handle phosphate diesters are either hydrolytic (e.g. the nucleases) or nucleotidyl transfer catalysts.

The most basic questions that have been asked about enzyme-catalysed phosphoryl transfer are:

1) Are there covalent intermediates (e.g. phosphoenzyme) along the reaction pathway?
2) When do substrates combine and products leave the catalytic cycle?
3) What is the nature of the displacement step? Is it dissociative or associative?

The first two questions have classically been addressed through kinetics. It appears that, in general, kinases exhibit kinetics consistent with a sequential mechanism (ternary complex of enzyme and the two substrates, donor and acceptor) rather than a ping-pong mechanism (binary complex). However, it is unclear as to whether the ternary complex is formed in an obligatory order (ordered sequential) or whether the free enzyme does in fact combine with either of the substrates (random sequential).

However, kinetic methods cannot answer such questions as whether the conversion

\[
\text{enzyme/phosphoryl donor/acceptor} \rightarrow \text{enzyme/donor/phosphoryl acceptor}
\]

proceeds by direct transfer of the phosphoryl group between the two bound substrates or by a double displacement involving a covalent phosphoenzyme intermediate. In some studies it has been possible to show the existence of a phosphorylated enzyme intermediate directly by isolation (e.g. nucleoside diphosphate kinase, acetate kinase, hexokinase) or indirectly by trapping experiments. Nevertheless, kinetics cannot always reveal the participation of a transient phosphoenzyme intermediate. Stereochemical investigation can, however, provide a more sensitive probe of events occurring within the active site of the enzyme.
Stereochemical course of phosphotransferase enzymes

The first stereochemical study of a displacement reaction at phosphorus used thiophosphate analogues of the natural substrates since experimentally this was straightforward. However, the development of the all-oxygen approach to stereochemical studies at phosphorus was brought about because of problems encountered in the thiophosphate approach. It is observed that thiophosphates in general are poor substrates for phosphotransferases and are processed very slowly in comparison to their all-oxygen counterparts; in some extreme cases, thiophosphates are not substrates at all, e.g. phosphoglycerate mutase. These shortcomings meant there were fears about the reliability of results obtained with thiophosphates. Subsequent comparisons between isotopically chiral phosphates and thiophosphates as stereochemical probes have revealed no contradiction, and thiophosphates are accepted as reliable alternative substrates. The advantage of using oxygen isotopes ($^{17}$O and $^{18}$O) is that it does not disturb the chemistry or the enzymology of the substrate.

The results of experiments that have used the $^{16}$O, $^{17}$O, $^{18}$O methodology to investigate the stereochemical course of phosphoryl transfer reactions catalysed by phosphokinases, phosphatases and phosphomutases are listed in Table 1.1. Also shown in this table are results from experiments in which the stereochemical course of the corresponding thiophosphoryl transfers have been assessed.

The majority of the enzymes catalyse transfer of the phosphoryl group with inversion of configuration at phosphorus except for nucleoside diphosphate kinase, some of the phosphatases and all of the phosphomutases which catalyse the transfer with retention of configuration.

The simplest interpretation of inversion of configuration is that the phosphoryl transfer proceeds by a single associative in-line phosphoryl transfer between the two substrates in the ternary complex. However, overall inversion of configuration would also be consistent with a pathway involving an odd number of phosphoryl transfer or even a double displacement entailing an in-line displacement followed by an adjacent displacement (see
### TABLE 1.1 Stereochemical course of enzymic phosphoryl and thiophosphoryl transfer reactions

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Method</th>
<th>Stereochemical course</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PHOSPHOKINASES</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetate kinase</td>
<td>2</td>
<td>Inversion</td>
<td>100</td>
</tr>
<tr>
<td>Adenosine kinase</td>
<td>1</td>
<td>&quot;</td>
<td>101</td>
</tr>
<tr>
<td>Adenylate kinase</td>
<td>1</td>
<td>&quot;</td>
<td>102</td>
</tr>
<tr>
<td>Creatine kinase</td>
<td>2</td>
<td>&quot;</td>
<td>103</td>
</tr>
<tr>
<td>Glucokinase</td>
<td>2</td>
<td>&quot;</td>
<td>104</td>
</tr>
<tr>
<td>Glycerol kinase</td>
<td>1, 2</td>
<td>&quot;</td>
<td>100,105,106</td>
</tr>
<tr>
<td>Hexokinase</td>
<td>1, 2</td>
<td>&quot;</td>
<td>100,105,107</td>
</tr>
<tr>
<td>Nucleoside diphosphate kinase</td>
<td>1</td>
<td>Retention</td>
<td>108</td>
</tr>
<tr>
<td>Nucleoside phosphotransferase</td>
<td>1</td>
<td>&quot;</td>
<td>109</td>
</tr>
<tr>
<td>Phosphofructokinase</td>
<td>2</td>
<td>Inversion</td>
<td>110</td>
</tr>
<tr>
<td>Polynucleotide kinase</td>
<td>1, 2</td>
<td>&quot;</td>
<td>106,111,112</td>
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<tr>
<td>Pyruvate kinase</td>
<td>1, 2</td>
<td>&quot;</td>
<td>100,105,113</td>
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<tr>
<td>Ribulose phosphate kinase</td>
<td>1</td>
<td>&quot;</td>
<td>114</td>
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<td><strong>PHOSPHATASES</strong></td>
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<tr>
<td>Acid phosphatase</td>
<td>2</td>
<td>Retention</td>
<td>115</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>2</td>
<td>&quot;</td>
<td>116</td>
</tr>
<tr>
<td>Mitochondrial ATPase</td>
<td>1</td>
<td>Inversion</td>
<td>117</td>
</tr>
<tr>
<td>Myosin ATPase</td>
<td>1</td>
<td>&quot;</td>
<td>118</td>
</tr>
<tr>
<td>Sarcoplasmic reticulum ATPase</td>
<td>1</td>
<td>Retention</td>
<td>119</td>
</tr>
<tr>
<td>Elongation factor G GTPase</td>
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<td>Inversion</td>
<td>120</td>
</tr>
<tr>
<td>Elongation factor T GTPase</td>
<td>1</td>
<td>&quot;</td>
<td>121</td>
</tr>
<tr>
<td>Thermophilic bacterium PSE ATPase</td>
<td>1</td>
<td>&quot;</td>
<td>122</td>
</tr>
<tr>
<td>Pyrophosphatase</td>
<td>1</td>
<td>&quot;</td>
<td>123</td>
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<tr>
<td>Glucose-6-phosphatase</td>
<td>2</td>
<td>Retention</td>
<td>124</td>
</tr>
<tr>
<td>Snake venom-5'-nucleotidase</td>
<td>1</td>
<td>Inversion</td>
<td>125</td>
</tr>
<tr>
<td>Enzyme</td>
<td>Method</td>
<td>Stereochemical course</td>
<td>References</td>
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<tr>
<td>---------------------------------------------</td>
<td>--------</td>
<td>-----------------------</td>
<td>------------</td>
</tr>
<tr>
<td>MUTASES</td>
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<tr>
<td>Phosphoglucomutase</td>
<td>2</td>
<td>Retention</td>
<td>126</td>
</tr>
<tr>
<td>Phosphoglycerate mutase (muscle)</td>
<td>2</td>
<td>&quot;</td>
<td>127</td>
</tr>
<tr>
<td>Phosphoglycerate mutase (wheat germ)</td>
<td>2</td>
<td>&quot;</td>
<td>127</td>
</tr>
</tbody>
</table>

Method 1: $[^{16}O,^{18}O]$thiophosphate
Method 2: $[^{16}O,^{17}O,^{18}O]$phosphate
mechanism 2c, Figure 1.1). However, there is no firm evidence which supports multiple (>2) phosphoryl transfer reactions or other composite pathways for simple phosphokinases. Besides, it is argued that an adjacent displacement which involves pseudorotation is unlikely on two grounds: (i) a pseudorotation pathway will need a multi-step mechanism and (ii) the molecular movement involved in pseudorotation may demand a conformational change of the active site of the enzyme to accommodate this motion.

Moreover, in most of the cases where overall retention of configuration is observed, there is either presumptive or compelling evidence for the intermediacy of a phospho-enzyme which would accord with a mechanism involving two in-line displacements, as shown below. The symbol $\overset{\hat{P}}{E}$ specifies the phosphoryl group being transferred from the donor substrate $\overset{\hat{P}}{D}$ to the acceptor substrate $\overset{\hat{P}}{A}$ through the phosphorylated enzyme intermediate, $\overset{\hat{P}}{E}$, where $X$ is a nucleophilic group on the enzyme.

Although individual steps in enzyme-catalysed phosphoryl transfer reactions would accord with an associative in-line displacement mechanism, the question of whether it is truly associative or dissociative in character is not addressed since a metaphosphate intermediate may be captured before it is allowed to tumble within the active site of the enzyme, leading to inversion of configuration.

**Interests and aims of the thesis**

We have shown that the study of the chemical phosphoryl transfer reactions of phosphate monoesters are important in relation to reactions that occur in living systems. The stereochemical, kinetic, thermodynamic and P.L.X. evidence presented earlier suggests that metaphosphate is too unstable to exist as a fully liberated species in water and most other hydroxylic solvents, f-butanol being a notable exception. In contrast, stereochemical studies in aprotic solvents on analogous phosphoryl transfer reactions have shown that they
go with extensive racemisation which leaves open the possibility of a metaphosphate intermediate with an appreciable lifetime. In relation to these studies, our interest is in the properties and lifetime of the closely related thiometaphosphate, the closest relative to metaphosphate itself. Despite the extensive use made of thiophosphate substrate analogues to delineate enzymatic reaction mechanism, simple chemical thiophosphoryl transfer reactions have not been widely studied. These studies would be of much interest since (a) the stereochemical course of enzymic thiophosphoryl transfer reactions have often been assumed to be the same as for the natural phosphoryl transfer reactions and it is of importance to determine whether these reactions are indeed stereochemically equivalent and (b) in the literature there is a claim that thiophosphate monoesters react more rapidly via a dissociative pathway than analogous phosphate monoesters in aqueous alcoholic media, indicating that thiometaphosphate intermediate might have an appreciable lifetime in aqueous solution.

In order to study such reactions from a stereochemical viewpoint, our initial aim was to:

1) synthesize isotopically chiral \( [^{16}O,^{18}O \text{ or } ^{17}O] \) thiophosphate monoesters of either the Rp or Sp absolute configuration and,

2) devise a simple independent configurational analysis of such labelled compounds.

As well as using a stereochemical method to probe the lifetime of the thiometaphosphate intermediate in aqueous alcoholic media, we also aimed to use a kinetic method, volume of activation (\( \Delta V^+ \)), to assess its stability in aqueous solution.
CHAPTER 2
Synthesis of Isotopically Chiral Alkyl, Aryl and Nucleoside Thiophosphate Monoesters
Introduction

Analysing reactions by a stereochemical method represents one of the best ways of elucidating reaction mechanisms. Therefore, in order to investigate the stereochemistry of phosphoryl and thiophosphoryl transfer reactions (chemical or enzymic) a great deal of effort has gone into the synthesis and configurational analysis of isotopically chiral phosphate (28) and thiophosphate (29) monoesters [R = alkyl, aryl, nucleoside/nucleotide].

The following sections review the literature synthesis of isotopically chiral phosphates and thiophosphates. (Methods for their configurational analysis is reviewed later, in Chapter 3).

Literature synthesis of isotopically chiral phosphates

In 1978, two independent studies described the syntheses of the first oxygen chiral \[^{16}O,^{17}O,^{18}O\] phosphate esters. The first was reported by Knowles\(^{49,128}\) and his collaborators at Harvard followed by Lowe\(^{129,130}\) and his collaborators at Oxford. Both syntheses rely on the stereocontrolled exocyclic displacement reactions at phosphorus held in five-membered rings.\(^{10}\)

Knowles's synthesis of labelled phosphate monoesters is shown in Figure 2.1 and is based on the work of Inch \textit{et al.}\(^{131}\) \(^{17}\)O-enriched phosphoryl chloride (52) (prepared by reacting \text{PCl}_5\) with \text{H}_2^{17}\text{O}\) was reacted with \((\cdot)-\text{ephedrine} (53) in the presence of triethylamine to give an epimeric mixture of five-membered ring phosphoramidic chlorides (54) (only the major \text{cis} epimer is shown in Figure 2.1). This mixture was reacted directly (even though the epimers are separable by chromatography) with 2-benzyl-(S)-propane-1,2-diol (55) to yield an epimeric mixture of cyclic phosphoramidic esters (56a,b) which were separated by chromatography. There is good precedent for assuming that this exocyclic displacement reaction of oxazaphospholidinone (and thione) occurs stereospecifically with
FIGURE 2.1 Synthesis of the Rp diastereoisomer of [\textsuperscript{16}O, \textsuperscript{17}O, \textsuperscript{18}O]phospho-(S)-1,2-propanediol devised by Knowles et al.
retention of configuration at phosphorus because of the strong preference for the ring to be placed axial-equatorial in the penta-coordinate intermediate. The major phosphoramidic ester, known to have the ester functionality cis to the phenyl group of the ephedrine moiety, was subjected to acid-catalysed hydrolysis in H\textsubscript{2}\textsuperscript{18}O, which introduced another isotope of oxygen. The stereochemical course of this ring opening reaction is known to proceed with inversion of configuration through the work of Inch et al.\textsuperscript{131}

Hydrogenolysis of the acyclic [\textsuperscript{17}O, \textsuperscript{18}O] diester (57) removes the ephedrine moiety and the benzyl protecting group to give the isotopically chiral phosphate monoester (58).

The absolute configuration follows from the knowledge of the stereochemistry of the key steps in the synthesis; on this basis phosphate ester (58) was predicted to have the Rp configuration. This was later confirmed by an independent analysis method (full details in Chapter 3). The opposite configuration can be obtained, if desired, by simply reversing the order of introduction of \textsuperscript{17}O and \textsuperscript{18}O in the synthetic pathway.

Further experiments by Knowles et al. have shown that this synthetic method is general and has been successively applied to the synthesis of nucleoside phosphates such as [\textsuperscript{\gamma-16}O,\textsuperscript{17}O,\textsuperscript{18}O]ATP\textsuperscript{132} (discussed in detail later), as well as a number of alkyl and aryl monoesters.\textsuperscript{116}

Lowe’s general method for the synthesis of labelled phosphate monoesters was first applied to methyl phosphate (32),\textsuperscript{129} and is illustrated in Figure 2.2. This differs from Knowles’s method in that it does not involve the introduction of oxygen isotopes in any displacement reaction at phosphorus. The key step is the stereospecific synthesis of meso-hydrobenzoin (63) which is chiral by virtue of oxygen isotopes. This precursor was prepared in a number of steps starting with the synthesis of (S)-benzoin (60) by reacting (S)-mandelic acid (59) with phenyl lithium. Acid-catalysed hydrolysis of the corresponding ethylene ketal (61) in H\textsubscript{2}\textsuperscript{18}O introduced the isotope in the carbonyl oxygen. Reduction of the labelled (S)-benzoin (62) with lithium aluminium hydride gave exclusively meso-[\textsuperscript{18}O] hydrobenzoin (63). Reaction of this key intermediate with \textsuperscript{17}O-enriched phosphorus oxychloride introduced another isotope of oxygen in the resulting single five membered ring
FIGURE 2.2 Synthesis of the Sp enantiomer of methyl \([^{16}O, ^{17}O, ^{18}O]\)phosphate monoester (32) reported by Lowe et al.
chloride. The chloride was treated with methanol to give the corresponding methyl triester (64). Then finally, catalytic hydrogenolysis gave the $^{16}O,^{17}O,^{18}O$ methyl ester of Sp configuration. The Rp isotopomer can be isolated by reversing the introduction of the isotopes or preparing the compound from (R)-mandelic acid.

Lowe and his co-workers have since used this methodology to synthesize a range of chiral phosphate monoesters, including $[^\gamma^{16}O,^{17}O,^{18}O]ATP$.107

These two synthetic methods have both used five membered ring phosphoryl compounds and their known stereospecific reactions to introduce chirality at phosphorus.

**Literature synthesis of isotopically chiral thiophosphates:**

**A) Nucleoside thiophosphates**

Much of the early work on the synthesis of isotopically chiral thiophosphates (29)

![Diagram](29)

concentrated on adenine nucleotides because of their great potential as stereochemical probes for enzyme catalysed phosphoryl transfer reactions in nature. What makes thiophosphate analogues unique to a certain extent is that chirality is generated at the phosphorus centre when only two stable isotopes of oxygen ($^{16}O$ and $^{18}O$) are linked to the thiophosphoryl group. This opens the way for the use of these compounds to investigate stereochemical aspects of enzymatic reactions, most notably phosphatases for which thiophosphates offer the only available methodology.

Eckstein in 1968, reported the synthesis of the first thiophosphate, uridine-2',3'-cyclic diester (65), which was used to determine the stereochemical course of the reaction catalysed by ribonuclease A.134 Since that initial experiment, numerous studies notably from the laboratories of Eckstein, Knowles, Frey, Benkovic and Stec have resulted in the syntheses of thiophosphate analogues of the common adenine nucleotides. These studies, as well as others, have reported the use of these compounds, often with $^{18}O$ labels, in the
Stereochemical studies using isotopically chiral phosphate esters are usually more
demanding than those utilizing chiral thiophosphates. This is because in the preparation of
isotopically chiral thiophosphates, it is often possible to separate diastereoisomers which
are epimeric at the chiral phosphorus atom by normal ion-exchange chromatography. Also,
some enzymes have the ability to distinguish the prochiral oxygen atoms of a
thiophosphoryl group; the analogous distinction of prochiral phosphoryl oxygen atoms is
not possible.

Both of these features have usually led to efficient syntheses of the thiophosphate
analogues of almost all the possible diastereoisomers of adenine nucleotides.

i) Terminal thiophosphoryl group

In order to study enzyme-catalysed thiophosphoryl transfer reactions, it is necessary to
use adenine nucleotides with terminal thiophosphoryl groups, e.g. adenosine-5′-[γ-\(^{18}\)O,
thio ]triphosphate (ATPγS\(^{18}\)O). For instance, Richard and Frey\(^{135}\) have used imaginative
chemistry to synthesize [\(^{16}\)O,\(^{18}\)O ]-chiral samples of AMPS, ADPβS and ATPγS as shown
in Figures 2.3, 2.4 and 2.5.

The Rp and Sp diastereoisomers of [\(^{16}\)O,\(^{18}\)O ]AMPS were prepared by a chemical and
enzymic method as shown in Figure 2.3. Activation of AMPS\(^{18}\)O\(_2\) (66) (prepared by the
reaction of adenosine with P(S)Cl\(_3\), followed by work up with H\(_2\)\(^{18}\)O) with diphenyl
phosphorochloridate and coupling with AMP produced (67) and (68) as an epimeric
mixture. After separation by chromatography, separate hydrolyses of these diastereomeric
FIGURE 2.3 The synthesis of the diastereoisomers of [\(^{16}\text{O},^{18}\text{O}\)]AMPS.
FIGURE 2.4 The synthesis of the diastereoisomers of [β-16O, 18O]ADPβS.
FIGURE 2.5  The synthesis of the Rp diastereoisomer of [γ-^{16}O, {^{18}}O]ATPYS.
mixed anhydrides using nucleotide pyrophosphatase gave diastereomerically pure samples of \([^{16}O,^{18}O]AMPS\) (69) and (70) along with some AMP. This enzymic reaction does not alter the configuration of the thiophosphoryl group when the AMP group is removed. Although the enzymic step is efficient, the overall yield of (69) and (70) was 39%.

The Rp and Sp diastereoisomers of \([\beta^{16}O,^{18}O]ADP\beta S\) were synthesised by the methodology illustrated in Figure 2.4. Again, the important steps in this synthesis are the separation of the diastereomeric mixed anhydrides (72) and (73) (formed by reaction of 2',-3'-methoxymethylidene (71) with AMPS via activation with diphenyl phosphorochloridate) and the use of the adenosine moiety associated with the AMPS, as a blocking group which was removed by periodate cleavage to yield (Rp) and (Sp)-ADP\beta S^{18}O, (74) and (75).

A similar synthetic strategy was employed in the synthesis of the Rp diastereoisomer of \([\gamma^{16}O,^{18}O,\text{thio}]ATP\) (76), see Figure 2.5.

**ii) Internal thiophosphoryl group**

Nucleotidyl transfer reactions involve nucleophilic substitution at an internal phosphorus atom of nucleotides, therefore in order to study such reactions stereochemically, methods have been devised to synthesize adenine nucleotides with internal sulphur and oxygen-18 atoms. Diastereomerically pure samples of ADP and ATP analogues with internal thio-

\[
\begin{align*}
\text{AdO} & \text{P} \text{O} \text{O} \text{P} \text{O} \text{O} \text{O} \\
\text{O} & \text{O} \text{O} \text{O}
\end{align*}
\]

\[\text{Nucleotidyl transfer}\]

phosphoryl groups can be made from nucleotide analogues with terminal thiophosphoryl groups by making use of the stereospecificity of a number of kinase reactions. A large number of oxygen labelled thiophosphate derivatives of ADP and ATP have been synthesized this way.

The enzymatic reactions that can be used to prepare the various ADP and ATP analogues are summarized in Figure 2.6. Eckstein and Goody have reported enzymatic reactions
that can convert ADPβS into either the Rp or Sp isomer of ATPβS. Coupled action of adenylate (also known as myokinase) and pyruvate kinase on AMPS can give (Sp)-ATPαS as shown by Sheu and Frey. \(^{137}\) Modifications of these reactions have been reported by various workers and these are summarized in Figure 2.6.

**B) Inorganic thiophosphate**

Phosphoryl transfers to water either chemically or enzymically (as in phosphatases and synthetases) involve the direct or indirect production of inorganic phosphate (45). However, such reactions cannot be investigated by an all oxygen-isotope approach as there are only three stable isotopes of oxygen and consequently the inorganic phosphate cannot be made isotopically chiral. Therefore, to distinguish between the four identical substituents in the product inorganic phosphate, \([^{16}O,^{17}O,^{18}O]\) chiral inorganic thiophosphate (77) has been employed. The synthesis and configurational analysis of such species has been addressed by a number of groups. Webb and Trentham\(^ {138}\) reported the first synthesis of the enantiomers of inorganic \([^{16}O,^{17}O,^{18}O]\)thiophosphate by employing both chemical and enzymatic reactions and also showed that their configurations could be assigned unambiguously by a \(^{31}P\) n.m.r. method (discussed in Chapter 3). Later, Tsa\(^ {139}\) reported the enzymatic preparation of isotopically chiral inorganic thiophosphate along with a similar \(^{31}P\) n.m.r. configurational analysis method.

The synthesis of isotopically chiral inorganic thiophosphate of either Rp or Sp
FIGURE 2.6 Methods for the syntheses of the diastereoisomers of ADPαS, ATPαS and ATPβS.
configuration devised by Trentham and Webb is shown in Figure 2.7. [18O2]AMPS (78) was prepared chemically and reacted with diphenyl phosphorochloridate to randomly activate each of the prochiral oxygen atoms of the thiophosphoryl group for displacement by [17O4]phosphate. This gave a 1:1 mixture of (Sp)-ADPαS (79) and (Rp)-ADPαS (80) diastereoisomers. The Sp diastereoisomer was enzymatically converted to ATPαS with use of pyruvate kinase and phosphoenolpyruvate. This allowed ATPαS and ADPαS to be separated by chromatography; the ATPαS sample was then converted to (Sp)-ADPαS with hexokinase. Subsequently each of the ADPαS diastereoisomers was oxidized with periodate and treated with base to give two samples of thiopyrophosphate. Hydrolysis of these thiopyrophosphates at the phosphoryl group with pyrophosphatase gave the separate enantiomers of inorganic [16O,17O,18O]thiophosphate. The incomplete stereospecificity of pyruvate kinase means this strategy has some limitations.

A much more recent study by Lowe and Arnold reported the synthesis of isotopically chiral inorganic thiophosphate by a chemical method and closely parallels Lowe's synthesis of [16O,17O,18O]phosphate monoesters, mentioned earlier. This latest method is illustrated in Figure 2.8.

C) Alkyl and aryl thiophosphates

The methods for the synthesis of isotopically chiral nucleoside thiophosphates just mentioned are not in general applicable to the synthesis of simple thiophosphate monoesters (29; R = alkyl or aryl). However, synthesis of these thiophosphates based on the meso-

\[
\text{RO-}\overset{\text{S}}{\text{P}}\overset{\text{O}}{\text{O}}
\]

(29)

hydrobenzoin route to isotopically chiral phosphate monoesters has been reported by Jarvest and Lowe. Again, the key step is the synthesis of the [18O]-labelled meso-hydrobenzoin (63) (Figure 2.9) which was reacted with thiophosphoryl bromide with varying amounts of pyridine. When two equivalents of pyridine are used, the diastereoisomer (81) (kinetic
FIGURE 2.7 The synthesis of (Rp) and (Sp)-inorganic $^{16}$O, $^{17}$O, $^{18}$O thiophosphates by Trentham and Webb.
FIGURE 2.8 Route to (Rp) and (Sp)-inorganic $[^{16}\text{O},^{17}\text{O},^{18}\text{O}]$thiophosphate by Lowe and Arnold.
FIGURE 2.9 Synthesis of $[^{16}\text{O},^{18}\text{O}]$ thiophosphate monoesters by Jarvest and Lowe.
product) is the predominant product; however in the presence of an excess of pyridine, reversible ring opening of the cyclic phosphobromidate is possible, allowing the kinetically favoured product to be transformed into the thermodynamically more stable one (82). Reaction of these bromidates with, for example, methanol gave the corresponding triesters (83) and (84). Reduction of the hydrobenzoin framework with sodium in liquid ammonia gave the enantiomeric methyl [\(^{16}\text{O},^{18}\text{O}\)]thiophosphates (85) and (86) which were purified by ion-exchange chromatography. The yield of the product was quite low due to competing aminolysis of the five-membered ring and difficulty in removing the hydrobenzoin framework. This and the fact that the \(^{18}\text{O}\) meso-hydrobenzoin is difficult to prepare in large amounts means this route is not widely used.

An alternative method has been reported in preliminary work by Cullis et al.\(^{142,143}\) who used the simple thiophosphate monoesters to study thiophosphoryl transfer reactions. This method is analogous to the previously published route to [\(^{16}\text{O},^{17}\text{O},^{18}\text{O}\)]phosphate monoesters by Knowles et al.,\(^{49,128}\) mentioned at the beginning of this Chapter. Like Knowles’s syntheses, Cullis’s method exploits the stereocontrolled displacement reactions of 2-substituted 1,3,2-oxazaphospholidine-2-ones and thiones (87), which have established precedent in the work of Inch et al.\(^{131}\)

\[
\begin{align*}
\text{Ph} & \text{O} \\
\text{Me} & \text{N} \\
X & \text{Me} \\
\text{Cl} & \text{P}
\end{align*}
\]

\(X = \text{O, S}\)

\(\text{(87)}\)

The two general routes to isotopically chiral [\(^{16}\text{O},^{18}\text{O}\) (or \(^{17}\text{O}\))]thiophosphate monoesters of either the Rp or Sp absolute configuration devised by Cullis et al. are shown in Figure 2.10. This synthetic route is potentially general; 4-nitrophenyl [\(^{16}\text{O},^{18}\text{O}\)]thiophosphate and ethyl [\(^{16}\text{O},^{18}\text{O}\)]thiophosphate monoesters are examples that have been prepared this way. However, the extension of the published route to isotopically chiral phosphate monoesters to the synthesis of thiophosphate monoesters is non-trivial: the thiophosphoro-chloridate (88a) is less reactive than its oxy counterpart as would be expected from the
FIGURE 2.10  Two general syntheses of isotopically chiral $[^{16}\text{O},{}^{18}\text{O}]$thiophosphate monoesters.
known difference in reactivity of trisubstituted thiophosphates as compared to phosphates in associative nucleophilic substitution reactions;\textsuperscript{144} furthermore, removal of the ephedrine framework is difficult, a situation similar to the problems encountered by Jarvest and Lowe\textsuperscript{141} in their study.

The work in our study has used the preliminary work by Cullis \textit{et al.} to improve the synthesis of isotopically chiral thiophosphate monoesters with respect to increasing the yields and purity of the intermediate and final compounds in the synthetic pathway. In particular, as the stereochemical course of the ethanolysis of 4-nitrophenyl thiophosphate (89a) (below) was to be extensively studied, \textit{O-4-nitrophenyl [\textsuperscript{16}O,\textsuperscript{18}O]thiophosphate} (89a)

\begin{equation}
\begin{array}{c}
\text{O}_2\text{N} \quad \text{O} \quad \text{P} \quad \text{S} \\
\downarrow \quad \text{EtOH} \quad \longrightarrow \\
\text{EtO} \quad \text{P} \quad \text{S} \\
\text{O} \quad \text{O} \\
\end{array}
\end{equation}

(89a)

(89a) (90a)

(starting material) and \textit{O-ethyl [\textsuperscript{16}O,\textsuperscript{18}O]thiophosphate} (90a) (product) were synthesised. Studying the solvolysis of 4-nitrophenyl thiophosphate is ideal because as the leaving group has a pKa \~7.2, it is reactive in both its mono- and dianionic state.

Although the following sections describe the syntheses of labelled monoesters, reaction conditions were first perfected with unlabelled materials.

**Synthesis of (Rp)-4-nitrophenyl [\textsuperscript{16}O,\textsuperscript{18}O]thiophosphate (89a)**

The early part of this synthesis is based upon the original ideas of Inch \textit{et al.}\textsuperscript{131} The initial step in our study involved the preparation of \textit{cis} 2-chloro-3,4-dimethyl-5-phenyl-1,3,2-oxazaphospholidine-2-thione (88a) (spatial relationship between the phenyl group and chloro group designates the \textit{cis} or \textit{trans} configuration). This was prepared as the major epimer (cis:trans \textit{ca.} 8:1) by the reaction of (-)-ephedrine (53) with thiophosphoryl chloride (91) in the presence of triethylamine at room temperature.

This major chloro epimer (kinetic product) was isolated in pure form by chromatography and then recrystallisation. A small amount of the trans material (88b) (thermodynamic product) was isolated by concentration of the mother liquor.
Assignments of configuration in 1,3,2-oxazaphospholidines can be made on the basis of observed chemical shifts. Protons in a 1,3 cis relationship to a P=X group (X = O or S) are deshielded, therefore H-4 and H-5 resonate at lower field in compound (88a) than in (88b). The deshielding effect is greater for H-5 than for H-4, a result which is expected in a puckered ring in which P=S is closer to H-5 than H-4.

Although not reported in the work of Inch, the epimers (88a) and (88b) have $^{31}$P n.m.r. resonances which differ by ~5 p.p.m. (cis +75.2 p.p.m.; trans +80.3 p.p.m.).

Reaction of the cis-chloro adduct (88a) with 4-nitrophenoxide gave essentially a single diastereoisomer as judged by $^1$H and $^{31}$P n.m.r. spectroscopy, the latter showed a singlet (+77.2 p.p.m.) downfield from the cis-chloro compound (88a). As has frequently been observed, exocyclic displacement reactions at phosphorus held in a five-membered ring of this kind proceed with retention of configuration. This was confirmed by the X-ray
crystal structure of the product (92), which has been isolated for the first time as pure colourless crystals; previously both Inch and Cullis had reported it as an oil. The X-ray structure (Figure 2.11) clearly shows the cis relationship between the nitrophenoxy and the phenyl group. The five-membered ring adopts an envelope structure in which the carbon atom bearing the methyl group is out-of-plane with the rest of the ring atoms. This presumably lessens the steric interaction between the methyl and the phenyl group. Knowledge of the structure of (92) is important as the isotopic chirality of the resulting thiophosphate monoester at the end of the synthetic scheme follows from the absolute configuration of this nitrophenyldiazocyclodioxan.

The next stage in the synthesis involved endocyclic cleavage of the P–N bond in (92) leading to the incorporation of an $^{18}$O atom by the use of labelled water ($^{2}H_{2}^{18}O$) and trifluoroacetic acid. This acid-catalysed ring opening occurs essentially stereospecifically with inversion of configuration at phosphorus.$^{131}$ Other related studies have demonstrated that acid-catalysed cleavage of P–N bonds in acyclic phosphinates$^{145}$ and acyclic alkyl-methyl phosphonamidothioates$^{146}$ occurs with inversion of configuration.

The absence of any P–NMe coupling in the $^{1}$H n.m.r. spectrum of the diester (93) and the presence of just one peak in the $^{31}$P n.m.r. spectrum (δP +55.2 p.p.m.) shows exclusive P–N bond cleavage with no P–O bond cleavage, evidently showing that the P–N bond in the triester (92) is very labile in the presence of acid, (it is also labile in the presence of base$^{131}$).

Indeed, the initial step involves protonation of the nitrogen followed by direct $S_N^2$ displacement of the now good leaving group (-NH$_2$Me) by H$_2^{18}$O.

The next step involved removal of the ephedrine framework via C–O bond cleavage. However, some of the methods available for this task are inappropriate, see Figure 2.12.
FIGURE 2.11 X-ray crystal structure of cis (4S, 5R)-2-(4-nitrophenoxy)-3,4-dimethyl-5-phenyl-1,3,2-oxazaphospholidine-2-thione (92).
Inch et al. removed the ephedrine moiety from a variety of thiophosphorus acid derivatives (94) by treatment with strong base which led to elimination of the thiophosphoryl component (95) with concomitant aziridine (96) formation (see below).

However, when this method is used on arylthiophosphates (93a) with good leaving groups, it only leads to the formation of 2-oxy-oxazaphospholidine (97), shown below.

The presence of sulphur in (93) prevents the use of hydrogenolysis to effect C–O bond cleavage as divalent sulphur deactivates metal catalysts.

Removal of the ephedrine moiety through the use of sodium metal in liquid ammonia is
The reaction is thought to proceed through a mechanism involving nucleophilic attack of the phospharyl oxygen on silicon followed by dealkylation of a phosphonium intermediate. The silyl groups attached to oxygen can be removed by hydrolysis under mild conditions to give the free phosphorus acid.

We chose to use trimethylsilyl iodide (TMSiI) as it reacts much faster than any of the other trialkylsilyl halides.
However, removal of the ephedrine framework in (93) is extremely slow because the initial silylation occurs preferentially on oxygen (oxygen forms much stronger bonds to silicon than sulphur does) as shown in Figure 2.13 (pathway X), giving the P=S compound (98), which reacts very slowly with TMSiI. This low philicity of P=S compounds with trimethylsilyl halides is well documented in the literature. For instance, the removal of the O-ethyl groups from tetraethyl dithiopyrophosphate (99) requires extreme conditions as shown below.

\[
\begin{array}{c}
\text{EtO} \quad \text{P} \quad \text{O} \quad \text{P} \quad \text{OEt} \\
\text{OEt} \quad \text{OEt} \\
\end{array}
\xrightarrow{TMSiI, 110^\circ C, 12 \text{ h}}
\begin{array}{c}
\text{TMSiO} \quad \text{P} \quad \text{O} \quad \text{P} \quad \text{O} \quad \text{TMSi} \\
\text{TMSi} \quad TMSi \quad TMSi \quad TMSi \\
\end{array}
\]

(99)

To overcome this problem with (93), the sulphur, a soft nucleophilic centre, was selectively methylated with a soft electrophile, methyl iodide (20 equivalents), prior to the addition of TMSiI (4 equivalents). This leads to a P=O compound (100) (Figure 2.13, pathway Y) which is considerably more reactive towards TMSiI. S-Alkylation of (93) causes the \(^{31}\text{P}\) n.m.r. resonance to shift upfield (to +27.8 p.p.m.) as the P–S bond order changes. The \(^{31}\text{P}\) n.m.r. chemical shifts of thiophosphates are highly bond order dependent, for example, moieties with P=S bonds resonate around 75 p.p.m. whereas P=S bonds resonate around 25 p.p.m. (see Table 2.1). Ring structures and substituents attached to phosphorus exert their own influence and these effects are approximately additive, (extrinsic factors, such as pH, temperature, etc., obviously will also effect chemical shift values). This bond order dependence has been used throughout this thesis as a guide to assigning the structure of compounds.

Following the reaction with TMSiI, iodide attack at the benzylic centre to remove the ephedrine moiety rather than de-methylation gives the thermodynamically more stable P=O intermediate (101) (δp + 13.5 p.p.m.) which undergoes further reaction with TMSiI to give the bis-(trimethylsilyl) 4-nitrophenyl thiophosphate (102) in good yield. This C–O bond cleavage with TMSiI does not effect the stereochemistry at phosphorus.

Importantly, each of the intermediate steps in the reaction sequence just described.
FIGURE 2.13 Favoured pathway for the cleavage of the C—O bond in (93) using MeI / TMSiI.
TABLE 2.1 Trends in $^{31}$P n.m.r. chemical shifts of thiophosphates

<table>
<thead>
<tr>
<th>Compound</th>
<th>P–S Bond Order</th>
<th>$^{31}$P n.m.r. Chemical Shift / p.p.m.</th>
</tr>
</thead>
<tbody>
<tr>
<td>MeO $\text{P}\equiv\text{S}$ MeO</td>
<td>2.00</td>
<td>73</td>
</tr>
<tr>
<td>$\left[\text{EtO}_2\text{P}^{\text{Na}^+}\right]$</td>
<td>1.50</td>
<td>57</td>
</tr>
<tr>
<td>$\left[\text{EtO}_2\text{PO}^{2\text{Na}^+}\right]$</td>
<td>1.33</td>
<td>44</td>
</tr>
<tr>
<td>$\left[\text{OP}_3\right]^{3\text{Na}^+}$</td>
<td>1.25</td>
<td>37</td>
</tr>
<tr>
<td>$\left[\text{EtO}_2\text{PSMe}\right]$</td>
<td>1.00</td>
<td>29</td>
</tr>
</tbody>
</table>

proceed essentially quantitatively, as judged by $^{31}$P n.m.r. spectroscopy.

The bis-(trimethylsilyl) ester (102) spontaneously hydrolysed in aqueous sodium bicarbonate and mercaptoethanol to give 4-nitrophenyl thiophosphate (89a) which was purified by ion-exchange chromatography on DEAE-Sephadex resin. Isotopically-chiral 4-nitrophenyl thiophosphate (89a) was isolated in 65% yield.

As the absolute configuration of (89a) follows from the synthesis, the enantiomeric purity (>95% Rp) was independently established by our new configurational analysis method, which is described in the next chapter. The route to (Rp)-4-nitrophenyl [ $^{16}$O,$^{18}$O ] thiophosphate just described is summarised in Figure 2.14.

The Sp enantiomer of (89a) can be synthesized by following the alternative pathway depicted in Figure 2.10 which employs similar chemistry as above.

**Synthesis of (Rp)-ethyl [ $^{16}$O,$^{18}$O ]thiophosphate (90a)**

![Chemical structure of (90a)](image)

(Rp)-ethyl [ $^{16}$O,$^{18}$O ]thiophosphate was prepared by the same route as the aryl thiophosphate (89a) with some obvious modifications.

The ethoxy functionality was introduced by reacting the chloro adduct (88a) with sodium ethoxide in ethanol which gave essentially one epimer, as judged by $^1$H and $^{31}$P n.m.r. spectroscopy. Naturally, this product was assumed to be (103) from our knowledge of exocyclic displacement reactions at phosphorus held in five-membered rings which occur with retention of configuration.$^{10,131}$
FIGURE 2.14 Summary of the synthetic route to (Rp)-4-nitrophenyl $^{[16}O, ^{18}O]$-thiophosphate (89a).

The P–N bond was cleaved as before with labelled $[^{18}O]$-water and trifluoroacetic acid to give only the diester (104) as its zwitterion.

Trimethylsilyl iodide (TMSiI) was not used to facilitate removal of the ephedrine group in (104) as in the case of its aryl counterpart because iodide ion would also cleave off the
O-ethyl group leading to unwanted inorganic thiophosphate (77a). However, the use of sodium in liquid ammonia to effect reductive cleavage of the benzylic group in (104) proved to be satisfactory, indicated by $^{31}$P n.m.r. spectroscopy ($\delta p +59.5 \rightarrow +42.8$ p.p.m.), giving isotopically chiral ethyl thiophosphate (90a) in a modest yield (52%) after purification by ion-exchange chromatography. $^1$H-coupled $^{31}$P n.m.r. of (90a) shows the expected triplet splitting pattern for phosphorus coupling to methylene protons ($J_{PH} \sim 10$ Hz).

**Synthesis of nucleoside [^{18}O]thiophosphates:**

a) **Synthesis of (Sp)-adenosine-5'-$^{18}$O, $^{18}$O-thio]diphosphate [(Sp)-ADP$^{18}$O]** (75)

We have shown how the stereocontrolled displacement reactions of 2-substituted 1,3,2-oxazaphospholidine-2-thione, like (88a), have been exploited in making enantiomerically-pure labelled alkyl and aryl thiophosphate monoesters. As an important
extension of this, we were interested in seeing whether thiopyrophosphate monoesters could be synthesized in isotopically chiral form.

The pyrophosphates of general interest are biophosphate derivatives of adenine nucleotides, adenosine-5′-diphosphate (ADP) (105) and adenosine-5′-triphosphate (ATP) (51); the latter is the universal currency of free energy in living systems and as such is involved in a great many enzyme-catalysed reactions.
Thio analogues of adenine nucleotides can introduce isotopic chirality without the need of $^{17}$O and have been used extensively to study the stereochemical course of enzyme-catalysed thiophosphoryl transfer reactions since they are substrates common to many of the phosphoryl transferase enzymes in nature.

We have already seen how Knowles et al. have synthesized isotopically-chiral phosphate monoesters using the oxazaphospholidin-2-one (54). This application was extended to the synthesis of adenosine [(S)-$^{\gamma-16}$O,$^{17}$O,$^{18}$O]triphosphate (ATP) (106) which is outlined in Figure 2.16. The yield of (106) was 40% [from (54)], such low yields are quite common in the preparation of some nucleotides. By analogy with this synthesis, it should be possible to prepare the (S) diastereomeric forms of [(S)-$^{\beta-16}$O,$^{18}$O]ADP$\beta$S (75) and [(S)-$^{\gamma-16}$O,$^{18}$O]ATP$\gamma$S (107) using the thione (88a).

Our preparation of (75) began with the hydrolysis of the chloro adduct (88a) with $^{18}$O-labelled lithium hydroxide, made from lithium metal in $\text{H}_2^{18}$O (99 atom %) to give the product (108) (Figure 2.17). This conversion, taking nearly 6 hours at room temperature, was essentially quantitative as determined by $^{31}$P n.m.r. spectroscopy ($\delta_{p} +75.2 \rightarrow +70.9$ p.p.m.).

The freeze-dried lithium salt (108) then underwent P–N bond cleavage in acidic conditions with adenosine-5'-monophosphoric acid (AMP free acid) (44a) in dimethylformamide (DMF). Unfortunately AMP free acid was found to be only partially soluble in DMF whilst other solvents like acetonitrile and dioxan proved to be no better. Dimethyl
FIGURE 2.16 The synthetic route to (Sp)-[$\gamma$-\textsuperscript{16}O, \textsuperscript{17}O, \textsuperscript{18}O]ATP devised by Blattler and Knowles.
FIGURE 2.17 Proposed synthesis of ADPβSβ¹⁸O (75) via the ephedrine route.
sulphoxide (DMSO) caused substantial loss of sulphur from the thione (108), presumably by oxidation. Nevertheless, despite this limited solubility in DMF, the heterogeneous reaction mixture was stirred rapidly at 60°C for 8 hours and gave a reasonable amount of the expected pyrophosphate (109) as its mono-triethylammonium salt after purification by ion-exchange chromatography through A-25 Sephadex resin. The yield (70%) was based on the amount of the adenine chromophore ($\lambda_{\text{max}} = 260$ nm, extinction coefficient ($\epsilon$) = $15 \times 10^3$) present, determined spectrophotometrically.

![Diagram](image)

(109) had a $^1$H n.m.r. spectrum that showed, as expected, no P-NMe coupling and it also indicated that the ephedrine moiety was still attached; the low-field $^{31}$P n.m.r. spectrum showed two well separated doublets (+46.1 p.p.m., -10.8, $J = 35.6$ Hz), indicative of a pyrophosphate structure (Figure 2.18). The doublet at +46.1 p.p.m. is assigned to the $\beta$-phosphorus atom, as phosphorus atoms attached to sulphur generally resonate at a lower field in comparison with oxygen equivalents. (109) was further characterised by high resolution mass spectrometry.

The presence of sulphur in the thiophosphoryl moiety of (109) prevented hydrogenolysis of the benzylic ester (C–O bond cleavage) and the presence of the adenosine (Ad) group prevented the use of trimethylsilyl iodide. However, reductive cleavage of the ephedrine group with sodium (15 eq) in liquid ammonia proved successful in this case to give ADP$\beta$S$\beta^{18}$O (75) which was purified by ion-exchange chromatography; its position of elution from the column suggested a molecule with three negative charges. The low-field $^{31}$P n.m.r. spectrum of (75) again showed the presence of a pyrophosphate because of the P–P coupling. Removal of the ephedrine framework meant that the $^{31}$P n.m.r. chemical
$^{31}\text{P}$ n.m.r. chemical shift ($\delta$) / p.p.m.

**FIGURE 2.18** The low-field $^{31}\text{P}$ n.m.r. spectrum (36.3 MHz) of pyrophosphate (109).
shift of the β-phosphorus of (75) appeared more upfield than that of (109), as removal of the ephedrine moiety changes the bond order to sulphur.

The yield for the production of (75) was moderate (53%) and there were fears that this yield was being diminished by accompanying reduction of the adenine ring (Figure 2.17), thus decreasing the intensity of the chromophore. Decreasing the contact time (4 minutes to 1 minute) and the amount of sodium used only had the effect of leading to incomplete reduction (9 equivalents gave 41% yield, 6 equivalents gave 23%). However, adenine reduction could be discounted as the UV spectrum of the prepared ADPβSβ<sup>18</sup>O (75) still showed only the one characteristic peak for the adenine chromophore (λ<sub>max</sub> = 260 nm).

Since the conversion of (88a) to (108) is known to proceed with retention of configuration at phosphorus,<sup>131,151</sup> the ring opening of (108) to (109) involves inversion of configuration and C–O bond cleavage of (109) to (75) has no stereochemical consequence at phosphorus, the product (75) was assumed to have the Sp configuration at the β-phosphorus. Indeed to confirm this, further characterisation of ADPβSβ<sup>18</sup>O was made by determining whether it was a substrate for the enzyme, pyruvate kinase, which can stereoselectively catalyse the phosphorylation of the pro-S oxygen of Pβ in ADPβS (75a) with phosphoenolpyruvate (PEP) (111) to give predominantly the Sp diastereoisomer of ATPβS (110a);

![Chemical Structure](image)

however this stereoselectivity is not total; the ratio is usually 6:1, Sp/Rp.<sup>152</sup> On the other hand, acetate kinase catalyses the phosphorylation of the pro-R oxygen in (75a).<sup>153</sup> Clearly, treatment of our prepared <sup>18</sup>O-labelled ADPβS (75) with pyruvate kinase will give
18O-labelled ATPβS either with 18O in the Pβ-O-Pγ bridge or in the non-bridging position at Pβ depending on the enantiomeric purity at Pβ. Since 18O directly bonded to phosphorus causes an upfield (isotopic) shift on the 31P n.m.r. resonance of the phosphorus154-156 by an amount that increases with increasing bond order141,157,158 (Figure 2.19, see Chapter 3 for a more detailed discussion), the position of the 18O can be readily established and hence the absolute configuration of [β-18O]ADPβS (75a) determined.

Incubation of (75) with PEP in the presence of pyruvate kinase under typical assay conditions for 25 hours followed by ion-exchange chromatography gave a compound whose elution position from the column was indicative of a compound with four negative charges. The high-field 31P n.m.r. spectrum of this colourless oil shows three sets of peaks (Figure 2.20). This clearly shows that the ADPβS has been phosphorylated to give the triphosphate, ATPβS, which was isolated in 77% yield from (75). The following coupling pattern is seen:

- Pα : couples with Pβ ⇒ doublet (J = 27.6 Hz)
- Pβ : couples with both Pα and Pγ ⇒ doublet of doublets (J = 27.6, 28.9 Hz)
- Pγ : couples with Pβ ⇒ doublet (J = 28.9 Hz)

Although Pγ shows small duplicate resonances that correspond to a small amount of
FIGURE 2.20 Expanded high-field $^{31}$P n.m.r. spectrum (121.5 MHz) of pure $\text{ATP}\beta\gamma\text{S}^{18}$O (110). Spectral parameters: sweep width 5494 Hz, pulse width 5 $\mu$sec, acquisition time 3 sec, broad-band proton decoupling, Gaussian multiplication (line-broadening - 1 Hz, Gaussian broadening 0.15 Hz).
unlabelled ATPβS arising from residual $^{16}$O in the $H_2^{18}$O used together with any dilutions that occurred during synthesis, the spectrum does not clearly confirm which phosphorus resonance suffers an isotopic shift. This assignment was confirmed by adding unlabelled ATPβS (prepared the same way) to the labelled sample and re-recording the $^{31}$P n.m.r. spectrum (Figure 2.21). The Pβ and Pγ resonances are clearly split, the size of these isotopic shifts (2.7 Hz in each case) being proof that the $^{18}$O is in the βγ-bridge, while Pα remains a doublet. It therefore follows that the ADPβSβ$^{18}$O derived from our synthetic route has the Sp configuration (Figure 2.17), as expected.

It is worth mentioning that the Pγ resonance of ATPβSβ$^{18}$O shows an extra set of lines upfield (Figure 2.20). This could arise as a result of the presence of ADP, as the Pβ of ADP resonates in a very similar place to that of Pγ of ATPβS. One has to argue that the ADP is arising after purification because ATPβS would be well resolved from ADP on an ion-exchange column. Therefore one could argue that trace ADP arises from breakdown and loss of sulphur from ATPβS.

b) Attempted synthesis of adenosine-5'-[γ-thio]triphosphate (ATPγS) (107a)

Having successively prepared ADPβS using the stereocontrolled reactions of (88a), one could extend this and make other nucleotide analogues, namely ATPγS (107a). The proposed reaction scheme is shown in Figure 2.22.

However, this synthesis was not found to be as straightforward as before. Adenosine-5'-diphosphoric acid (ADP free acid) (105) was found to be more insoluble in DMF than AMP. The heterogeneous mixture containing ADP and the lithium salt (108) in DMF was rapidly stirred at high temperature (70°C); but monitoring of this mixture by $^{31}$P n.m.r. spectroscopy gave no evidence for the formation of a triphosphate.

ADP free acid does not dissolve in most solvents and in fact is only really soluble in water and dimethyl sulphoxide which were unsuitable for our study. $N,N'$-dimethylpropyleneurea (DMPU), which is claimed to be an excellent replacement solvent for the carcinogenic hexamethylphosphoramide (HMPA), failed to dissolve the ADP.
FIGURE 2.21 Expanded high-resolution $^{31}$P n.m.r. spectrum (121.5 MHz) of ATPβSβ$^{18}$O (110) mixed with authentic ATPβS (ca. 1:1), in 0.1 M-Tris buffer, pH 9.5, containing D$_2$O (100%) and EDTA (10 mM). Spectral parameters: sweep width 6097 Hz, pulse width 5 μsec, acquisition time 2 sec, broad-band proton decoupling, Gaussian multiplication (line-broadening - 0.7 Hz, Gaussian broadening 0.3 Hz).
FIGURE 2.22 Proposed synthesis of ATPγS (107a) via the ephedrine route.
Conclusion

Isotopically chiral samples of (Rp)-4-nitrophenyl $^{16}$O,$^{18}$O thiophosphate and ethyl $^{16}$O,$^{18}$O thiophosphate have been synthesised via the ephedrine route. The syntheses have been improved from that reported in the preliminary work by Cullis et al. In particular, we have managed to obtain the X-ray crystallographic structure of the 4-nitrophenyl derivative (92), one of the intermediate compounds in the synthetic route to 4-nitrophenyl $^{16}$O,$^{18}$O thiophosphate.

Furthermore, the synthetic approach has been extended to nucleoside diphosphates, providing a more straightforward synthesis of ADPβSβ$^{18}$O than that reported by Frey (see Figure 2.4).
CHAPTER 3
Configurational Analysis of Isotopically Chiral Thiophosphate Monoesters
Introduction

In the previous chapter the syntheses of isotopically chiral phosphate and thiophosphate monoesters were reviewed and a new general method for thiophosphate esters was described. However, in order to investigate the stereochemical course of the (thio)phosphoryl transfer reaction that these chiral materials may undergo, an independent method for determining the absolute configuration of the monoester and the quantitative assessment of the enantiomeric excess at phosphorus is needed.

Several groups who synthesised isotopically-chiral phosphate esters concurrently developed configurational analysis methods for them. These methods will be reviewed here. To date, three different methods have been employed to analyse the absolute chirality of \([^{16}\text{O},^{17}\text{O},^{18}\text{O}]\text{phosphate monoesters}\), namely, chiroptical methods, mass spectrometry and \(^{31}\text{P}\) n.m.r. spectroscopy.

Without question the easiest and most direct method for investigating the chirality of \([^{16}\text{O},^{17}\text{O},^{18}\text{O}]\text{phosphate monoesters}\) would be to use a chiroptical method. Lowe and co-workers\(^{129,159}\) have reported that (Sp)-methyl \([^{16}\text{O},^{17}\text{O},^{18}\text{O}]\text{phosphate}\) (32) possesses a measurable circular dichroic spectrum. However, it was found to be too small to be of any significance in the stereochemical analysis of such chiral esters.

\[
\begin{align*}
\text{MeO} & \quad \text{O} \\
\text{P} & \quad \text{O} \\
\text{MeO} & \\
\end{align*}
\]

Barron\(^{160}\) reported that Laser Raman optical activity of isotopically chiral phosphates could be used as a method of analysis, but this method still awaits technical development.

Mass spectrometry method

Two mass spectral methods for determining the configuration of oxygen chiral phosphate esters have been reported. The first by Knowles et al.\(^{49,128}\) is based on metastable ion mass spectrometry and was used in the configurational analysis of \([^{16}\text{O},^{17}\text{O},^{18}\text{O}]\text{phosphate}\)
monoesters. The second method was developed by Stec\textsuperscript{161} in the configurational analysis of \(^{16O,18O}\)-chiral cyclic phosphate esters and is based on the fragmentation patterns of isomeric benzyl esters.

The principle of Knowles's method is shown in Figure 3.1. The strategy is based on the conversion of a pro-prochiral phosphate monoester to a prochiral phosphate diester so that the isotopic identity of the resulting diastereotopic oxygens can be established by virtue of their chemical non-equivalence. To generate a phosphate diester that is diastereomeric at phosphorus demands the presence of a second conventional chiral centre in the phosphate monoester. This was achieved by the generation of the phosphate monoester of (S)-propane-1,2-diol, (58). Alkaline phosphatase, in the presence of (S)-propane-1,2-diol (112), catalyses the transfer of a phosphoryl group from essentially any phosphate monoester to the diol. It has been shown that this transfer proceeds with overall retention of configuration at phosphorus.\textsuperscript{116,119} The oxygen atoms of the monoester (58) are randomly activated for ring closure reaction by reacting it with diphenylphosphoimidazol to give the mixed anhydride; this ring closure is shown to occur with inversion of configuration\textsuperscript{49,128} to give a mixture of three isotopomeric five-membered ring cyclic phosphates, i.e. \(^{16O,17O,-}\), \(^{17O,18O-}\) and \(^{16O,18O-}\) diastereoisomers (113a,b,c). Methylation of the isotopomorphic mixture with diazomethane converts the diesters into a mixture of \textit{syn} and \textit{anti} methyl triesters in which the exocyclic oxygens are chemically distinguished. The \textit{syn} and \textit{anti} isomers are diastereomeric and can be separated chromatographically.

The triesters that would arise from either the (Rp) or (Sp)-[\(^{16O,17O,18O}\)]phosphate monoester are shown in Figure 3.2. Comparison of structures of identical isotopic composition shows that the stereochemical information is contained in the identity of the oxygen isotope that is methylated. If the \textit{syn} triesters obtained from both the Rp and Sp phospho samples of propanediol are compared we see that in the \(^{16O,18O}\)-labelled cyclic methyl ethers, the ester derived from the Rp monoester is methylated on \(^{16O}\), whereas the ester derived from the Sp monoester is methylated on \(^{18O}\). However, because each mixture contains equal amounts of species that are methylated on each isotope of oxygen, normal
FIGURE 3.1 The transformation involved in the configurational analysis of isotopically chiral phosphate monoesters developed by Knowles et al.
FIGURE 3.2  Stereochemical analysis of isotopically chiral $[^{18}O, ^{17}O, ^{18}O]$ phosphate monoesters by a mass spectral method devised by Knowles et al.
spectral techniques cannot be used to determine the site of methylation. Knowles has shown that metastable ion mass spectrometry can be used, and allows the origin of the \([-\text{OMe}]^+\)-derived fragment to be determined by relating daughter and granddaughter peaks to the parent ion. This information is necessary for configurational assignments. For example, the \([-^{18}\text{OMe}]^+\)-derived fragment from the analysis of the syn triester which came from the Sp starting material, would arise from the \(M+2\) molecular ion whereas the corresponding \([-^{18}\text{OMe}]^+\)-derived from this analysis of the syn isomer (from the Rp starting material) would originate from the \(M+3\) molecular ion.

The fact that the cyclic triesters are hydrolytically labile and the syn and anti isomers need to be separated physically before analysis makes this method technically quite difficult. Besides, the method is not general as it is suitable only for compounds which give metastable peaks. For this reason, the mass spectral technique has now been superseded by a \(^{31}\text{P}\) n.m.r. method which is discussed in the next section.

\(^{31}\text{P}\) n.m.r. spectroscopy method

High-field \(^{31}\text{P}\) n.m.r. spectroscopy is now considered the most important and reliable method for the configurational analysis of \([^{16}\text{O},^{17}\text{O},^{18}\text{O}]\)phosphate esters. The application of such a method to the configurational analysis of isotopically chiral phosphate esters is based on two observations that have been made about the effect of oxygen isotopes directly bonded to phosphorus on the \(^{31}\text{P}\) n.m.r. resonances.

\(^{18}\text{O}\) effect: The laboratories of Cohn,\(^{155}\) Lutz\(^{156}\) and Lowe\(^{154}\) independently reported that \(^{18}\text{O}\) directly bonded to phosphorus exerts an upfield shift on the \(^{31}\text{P}\) n.m.r. resonance of phosphorus to which it is directly attached. This effect is relatively small, approximately 0.02 p.p.m. (~3 Hz) per bond between \(^{18}\text{O}\) and \(^{31}\text{P}\) in inorganic phosphate, but is easily measurable on high-field n.m.r. spectrometers.

Initial reports showed that the magnitude of this isotopic shift was approximately additive increasing as the number of \(^{18}\text{O}\)s attached to phosphorus increased. For instance, inorganic phosphate, randomly labelled with 50% enriched \(^{18}\text{O}\), gives five well resolved \(^{31}\text{P}\) n.m.r. resonances as shown in Figure 3.3.
Further investigations by Lowe\textsuperscript{141,157} and Cohn\textsuperscript{158} demonstrated that the size of this isotopic perturbation on the chemical shift is dependent on the order of the bond between $^{18}\text{O}$ and $^{31}\text{P}$, with increased bond order resulting in a larger isotopic perturbation. For example, in the series inorganic phosphate, monomethyl phosphate, dimethyl phosphate and trimethyl phosphate, the $^{18}\text{O}$ isotopic shift resulting from labelling of one phosphoryl oxygen were 0.020, 0.024, 0.029 and 0.035 p.p.m. respectively.\textsuperscript{157} Cohn obtained similar data in the examination of various species of labelled adenine nucleotides.\textsuperscript{158}

$^{17}\text{O}$ effect: The second important observation concerning the effect of oxygen isotopes on the $^{31}\text{P}$ n.m.r. spectral properties of phosphate esters was reported independently by Tsai\textsuperscript{162} and Lowe.\textsuperscript{157} $^{18}\text{O}$, like $^{16}\text{O}$, has a nuclear spin quantum number of zero and therefore does not effect the relaxation of the phosphorus nucleus. However, $^{17}\text{O}$ has a nuclear spin quantum number of $\frac{5}{2}$ and possesses a comparatively large nuclear electric quadrupole moment. This means that the direct spin-spin coupling of this quadrupolar oxygen nucleus to the dipolar $^{31}\text{P}$ nucleus leads to the rapid and effective relaxation of the $^{31}\text{P}$ nucleus which results in the $^{31}\text{P}$ n.m.r. resonance being generally broad. This quadrupolar effect can obscure the one bond coupling which can be of the order of 100 MHz for some phosphate esters and nucleotides,\textsuperscript{163} however in some cases, for example $\text{P}^{17}\text{OCl}_3$, the $^{31}\text{P}^{17}\text{O}$ coupling can be clearly seen, appearing as six equally spaced lines of equal intensity for this molecule.\textsuperscript{164} Thus, in phosphate esters and nucleosides, the only effect that $^{17}\text{O}$ has on directly bonded $^{31}\text{P}$ nuclei is extensive line broadening.

The combined effects of $^{17}\text{O}$ and $^{18}\text{O}$ substitution on $^{31}\text{P}$ n.m.r. are exploited in the stereochemical analysis of [ $^{16}\text{O}^{17}\text{O}^{18}\text{O}$ ]phosphate esters. The first application was des-
scribed by Knowles and Buchwald\textsuperscript{165} and employs the same strategy and model as their previously described mass spectral method (Figure 3.1). This \textsuperscript{31}P n.m.r. configurational analysis again requires the [\textsuperscript{16}O,\textsuperscript{17}O,\textsuperscript{18}O] phosphoryl unit to be transferred to the propane-1,2-diol enzymatically, cyclised onto the chiral framework and then methylated to give the resulting isotopomeric triesters. As with the mass spectral method, \textit{syn} and \textit{anti} isotopomers are produced (Figure 3.1). However, in contrast to the metastable ion mass spectral method of analysis, this \textsuperscript{31}P n.m.r. analysis does not require the separation of the \textit{syn} and \textit{anti} triesters since these are well separated in terms of the \textsuperscript{31}P n.m.r. resonances. Methylation produces single and double bond character in the exocyclic P–O bonds, and this allows the application of \textsuperscript{18}O isotopic shifts on the resolved \textsuperscript{31}P n.m.r. resonances of the isomeric triesters to ascertain the configuration of the \textsuperscript{16}O,\textsuperscript{18}O-containing ester. The triesters containing \textsuperscript{17}O are not observable in the \textsuperscript{31}P n.m.r. spectrum due to the quadrupolar effect of this nucleus and this therefore simplifies the analysis method.

The predicted \textsuperscript{31}P n.m.r. spectra of the mixture of \textit{syn} and \textit{anti} cyclic triesters derived from labelled samples of 1-phospho-(S)-propane-1,2-diol that are Rp or Sp are shown in Figure 3.4. It must be noted that the [\textsuperscript{17}O] water used in the synthesis of phosphate monoester (58) contains \textsuperscript{16}O,\textsuperscript{17}O,\textsuperscript{18}O in the ratio of 1:2:1.

Figure 3.5 shows the actual spectrum observed. The 1\textsuperscript{st} and 5\textsuperscript{th} peaks are assignable to the unlabelled triesters and the 4\textsuperscript{th} and 8\textsuperscript{th} are due to the doubly labelled ones. Stereochemical information is derived from the 2\textsuperscript{nd}, 3\textsuperscript{rd}, 6\textsuperscript{th} and 7\textsuperscript{th} peaks. The absolute configuration and the enantiomeric excess is determined by the relative intensity of these peaks.

The configurational analysis devised by Lowe \textit{et al}.\textsuperscript{166} is conceptually similar to Knowles's except that it uses glucose or adenosine in place of propane-1,2-diol as the chiral framework.

\textbf{Literature configurational analysis of inorganic [\textsuperscript{16}O,\textsuperscript{17}O,\textsuperscript{18}O] thiophosphate}

The stereochemical course of phosphoryl transfers (chemical or enzymic) to water can only be investigated by employing thiophosphate analogues incorporating all three stable
FIGURE 3.4 The predicted $^{31}$P n.m.r. spectra of the products from ring closure and methylation of the diastereoisomers of [(16O, 17O, 18O)phospho-(S)-1,2-propanediol, assuming that the H$_2^{17}$O used in the synthesis of (58) contained 16O, 17O and 18O in the ratio of 1:2:1.

The analysis developed by Webb and Trentham is shown in Figure 3.6. The key to this analysis involves the stereospecific enzymatic incorporation of the isotopically chiral oxygen isotopes. Such reactions produce inorganic [(16O, 17O, 18O)thiophosphate (77).

The configurational analysis of this species has also been conducted by a $^{31}$P n.m.r. method, developed independently by Webb,^138 Tsai^139 and Lowe.^140

The analysis developed by Webb and Trentham is shown in Figure 3.6. The key to this analysis involves the stereospecific enzymatic incorporation of the isotopically chiral
FIGURE 3.5 Predicted and observed $^{31}$P n.m.r. spectra (121.5 MHz) of the mixture of methyl esters prepared from the diastereoisomers of $[1\cdot^{16}O,^{17}O,^{18}O]$-phospho-(S)-1,2-propanediol.
i) Glyceraldehyde phosphate dehydrogenase, glyceraldehyde phosphate, NAD⁺;
ii) MgADP, phosphoglycerate kinase;
iii) Adenylate kinase MgAMP;
iv) Phosphoglycerate kinase Mg²⁺, glycerate 1,3-bisphosphate.

**FIGURE 3.6** Configurational analysis of (Rp)-inorganic [¹⁶O, ¹⁷O, ¹⁸O]thiophosphate (77) devised by Trentham and Webb.
inorganic thiophosphate \((77)\) into the \(\beta\) position of the product \(\text{ATP}\beta\text{S}\). The isotopic chirality is deduced by \(^{31}\text{P}\) n.m.r. spectroscopy relying on the ability to distinguish between \(\beta,\gamma\)-bridging and \(\beta\)-non-bridging \(^{18}\text{O}\) in \(\text{ATP}\beta\text{S}\) (Figure 3.6). A shortcoming of this method is the observed significant loss of label from the inorganic thiophosphate during incorporation into \(\text{ATP}\beta\text{S}\) as well as the quality of the \(^{31}\text{P}\) n.m.r. spectra.

A more recent method by Lowe and Arnold\(^{140}\) for the stereochemical analysis of chiral inorganic \([^{16}\text{O},^{17}\text{O},^{18}\text{O}]\) thiophosphate has been reported which is closely analogous to that developed for \([^{16}\text{O},^{17}\text{O},^{18}\text{O}]\) phosphate monoesters\(^{129,130}\). The thiophosphate \((77)\) is reacted with \((S)\)-2-iodo-1-phenyl ethanol \((114)\) (Figure 3.7) and the \(S\)-alkylated product stereospecifically cyclized and then methylated to give a mixture of cis and trans \(4-(S)\)-phenyl-2-methoxy-2-oxo-1,3,2-thiaoxaphospholane. The \(^{31}\text{P}\) n.m.r. spectrum reveals only \(^{16}\text{O},^{18}\text{O}\) species \((^{17}\text{O}\) species are not observable as \(^{17}\text{O}\) directly bonded to phosphorus causes broadening of the \(^{31}\text{P}\) n.m.r. resonances\). The analysis is based upon the greater \(^{18}\text{O}\) isotopic shift when in a \(P=O\) bond compared to a \(P-O-R\) bond. The precise enantiomeric excess can be determined by comparing the ratio of the peak intensities with those calculated from the known isotopic compositions of the \((\text{Rp})\) and \((\text{Sp})\)-\([^{16}\text{O},^{17}\text{O},^{18}\text{O}]\) inorganic thiophosphate. This analysis involves low loss of label.

**Literature configurational analysis of \([^{16}\text{O},^{18}\text{O}]\) thiophosphate esters**

The configurational analyses of monosubstituted \([^{16}\text{O},^{18}\text{O}]\) thiophosphates so far reported are either not general or impractical. For instance, two methods specific for nucleotides such as adenosine-\(5'\)-\([^{16}\text{O},^{18}\text{O}]\) thiophosphate have been reported.

Frey and Sheu\(^{137}\) have shown that adenylate kinase (also known as myokinase) in the presence of pyruvate kinase and phosphoenolpyruvate (PEP) catalyses the phosphorylation of adenosine-\(5'\)-\([^{16}\text{O},^{18}\text{O}]\) thiophosphate \((69,70)\), (Figure 3.8), on oxygen to give stereospecifically the \(S\) diastereoisomer of \(\text{ATP}\alpha\text{S}\) \((115,116)\). Therefore \((\text{Sp})\)-\([^{16}\text{O},^{18}\text{O}]\) AMPS \((69)\) leads to \((\text{Sp})\)-\([^{16}\text{O},^{18}\text{O}]\) \(\text{ATP}\alpha\text{S}\) \((115)\) with \(^{18}\text{O}\) exclusively in the non-bridging position whereas \((\text{Rp})\)-\([^{16}\text{O},^{18}\text{O}]\) AMPS \((70)\) leads to \((\text{Sp})\)-\([^{16}\text{O},^{18}\text{O}]\) \(\text{ATP}\alpha\text{S}\) \((116)\) with \(^{18}\text{O}\) in the bridging position; as a result these triphosphates can be distinguished by high-
FIGURE 3.7 Configurational analysis of isotopically chiral inorganic thiophosphate devised by Arnold and Lowe based upon the analysis developed for $^{16}O$, $^{17}O$, $^{18}O$ phosphate monoesters.
FIGURE 3.8 Enzymatic configurational analysis of nucleoside \[^{16}\text{O},^{18}\text{O}\] thiophosphates devised by Sheu and Frey.

field \(^{31}\text{P}\) n.m.r. spectroscopy. However, as this analysis method rests on the specificity of adenylate kinase it is not applicable to other systems.

The other more recent configurational analysis of nucleoside thiophosphates is based on a combination of a chemical and enzymic method, devised by Cummins and Potter.\(^{167}\) Methylation of 2'-deoxyadenosine-5'-\[^{16}\text{O},^{18}\text{O}\] thiophosphate (117), (Figure 3.9), gave two diastereoisomeric triesters (118,119) whose absolute configurations can be assigned by relating them to the corresponding \(O\)-methyl nucleoside thiophosphate diester. The latter
FIGURE 3.9  Chemical configurational analysis of (deoxy)nucleoside $[^{16}\text{O},^{18}\text{O}]$-thiophosphates reported by Cummins and Potter.

have been assigned on the basis of the known stereoselectivity of snake venom phosphodiesterase.$^{168}$

The only configurational analysis that is applicable to both alkyl and aryl thiophosphate monoesters has been reported by Cullis et al.$^{142}$ in conjunction with the general synthesis of $O$-substituted $[^{16}\text{O},^{18}\text{O}]$thiophosphates. This analysis relies upon the conversion of the
conventional prochiral centre into diastereoisomeric pyrophosphates by reacting the O-
substituted \([^{16}\text{O},{}^{18}\text{O}]\text{thiophosphate (90a)}\) with cis 2-chloro-1,3,2-oxazaphospholidin-2-one (54), derived from (-)-ephedrine (Figure 3.10).

The location of the \(^{18}\text{O}\) within the two epimers was established by the size of the \(^{18}\text{O}\)-isotopic shift induced on the \(^{31}\text{P}\) n.m.r. resonances thus assigning the absolute configuration of the initial \([^{16}\text{O},{}^{18}\text{O}]\text{thiophosphate monoesters (90a)}\). Two main problems are apparent with this analysis. Firstly, there is some loss of stereochemical control in the derivatisation reaction leading to four epimers instead of two. Normally exocyclic displacement reactions of these oxazaphospholidine-2-ones (54) proceed stereospecifically with retention of configuration. However, in the presence of nucleophilic catalysts such as pyridine, \(N\)-methylimidazole and \(N,N\)-dimethylaniline, epimerisation of the chloro compound (54) occurs prior to its reaction with the thiophosphate (90a) thus giving 4 epimers (120, 121, 122, 123). This problem has been eliminated by using tertiary amines like tributylamine under anhydrous conditions. In this case, the product was shown to be principally the two diastereoisomers (120, 121).

The second problem encountered with this analysis concerns the chiral auxiliary (54). As this is a phosphorus centre, this introduces a phosphorus-phosphorus coupling in the \(^{31}\text{P}\) n.m.r. spectrum thus reducing the sensitivity by a factor of two. These problems make this configurational analysis by Cullis et al. impractical. An alternative and simpler general analysis is needed.

**Development of a new and simple configurational analysis of isotopically chiral thiophosphate monoesters**

In order to study the stereochemical course of thiophosphoryl transfer reactions of \([^{16}\text{O},{}^{18}\text{O}]\text{thiophosphate monoesters}\), which may involve a monomeric thiometaphosphate intermediate, a stereochemical analysis is needed which will assign the absolute configuration of both the starting material and products of the reaction (see overleaf).

The analysis, like those before, will be based on \(^{31}\text{P}\) n.m.r. spectroscopy and must be able to distinguish between the pro-R/S oxygens in an O-substituted thiophosphate
FIGURE 3.10 Configurational analysis of simple [$^{16}$O, $^{18}$O]thiophosphate monoesters by a high-field $^{31}$P n.m.r. method devised by Cullis et al.
In order to generate diastereotopic oxygens in a thiophosphate monoester, the simplest strategy would be to attach a chiral auxiliary (R*) to the sulphur atom, shown below.

This can be easily achieved as alkylation usually occurs selectively on sulphur. Derivatising the diastereotopic oxygen atoms of the diester (124) will then give a pair of diastereoisomers (125a) and (125b). If the absolute configuration of these triesters can be unambiguously assigned this could be used to determine the absolute configuration of the original isotopically chiral thiophosphate monoester (29); in one diastereoisomer (125a) the $^{18}$O would appear in the bridging position while in the other diastereoisomer (125b) it would appear in the non-bridging position. These should be distinguishable by high-field $^{31}$P n.m.r. spectroscopy provided the diastereomeric separation between the unlabelled
diastereoisomers of (125a) and (125b) is large enough to show the P-\(^{18}\)O and P-\(^{18}\)O isotopic shifts clearly.

Ideally, the proposed configurational analysis must take into account the following important points:

i) It should be an efficient method involving minimum loss of isotopic label.

ii) The analysis should not involve any displacement reactions at the phosphorus centre of the labelled compound as this would introduce some stereochemical ambiguity.

iii) The derivatised materials should be stable and readily distinguishable by \(^{31}\)P n.m.r. spectroscopy.

iv) The method should be applicable to both alkyl and aryl thiophosphate monoesters.

Our development of a new configurational analysis for thiophosphate monoesters concentrated on \(O\)-4-nitrophenyl thiophosphate (89) and \(O\)-ethyl thiophosphate (90) as these are starting material and the product respectively of the solvolysis reaction under investigation, below.

\[
\begin{align*}
\text{(89)} & \quad \delta p +42.2 \text{ p.p.m.} \\
\text{(90)} & \quad \delta p +48.2 \text{ p.p.m.}
\end{align*}
\]

are starting material and the product respectively of the solvolysis reaction under investigation, below.

\[
\begin{align*}
\text{O}_2\text{N}-\text{O-P-S} & \quad \text{EtO-P-S} \\
\text{O} & \quad \text{O} \\
\text{EtOH} & \quad \text{EtOH}
\end{align*}
\]

**Configurational analysis of 4-nitrophenyl thiophosphate and ethyl thiophosphate**

**S-Alkylation:**

In a recent report,\(^{170}\) myrtenyl bromide (\(R^2\)Br; 126) was used as a chiral alkylating agent to probe the chirality of \(^{18}\)O-labelled phosphinic acids (127). We thought this terpene-derived halide would be an ideal chiral auxillary for the configurational analysis of the thiophosphates in our study.

Myrtenyl bromide was synthesized from the corresponding alcohol by bromination with phosphorus tribromide in a moderate yield (67%). Both 4-nitrophenyl thiophosphate and
ethyl thiophosphate were rapidly S-alkylated with (126) in the presence of triethylamine to give the diesters (128) and (132) respectively. S-Alkylation was confirmed by the expected large upfield shift of the $^{31}$P n.m.r. signal on going from the monoester to the diester. This was also verified by the $^1$H n.m.r. spectra of each diester which shows the methylene protons of the myrtenyl group coupling to the phosphorus ($J = 12$ Hz). (128) and (132) now have diastereotopic oxygen atoms due to the presence of the two asymmetric centres in the chiral auxiliary. O-Derivatising produces a pair of diastereoisomeric triesters in each case.

**O-Derivatisations:**

Individual methylation of the diesters (128) and (132) with dimethyl sulphate ($\text{Me}_2\text{SO}_4$) shifted the $^{31}$P n.m.r. resonances downfield in each case. This methylation gave a pair of
diastereoisomers (129a,b) and (133a,b) respectively, Figure 3.11, which in each case were distinguishable only by high-field $^{31}$P n.m.r. (121.5 MHz), see Figures 3.12 and 3.13. At this stage, it is impossible to assign the absolute configurations of (129a) and (129b), and also that of (133a) and (133b).

The diastereoisomers of (129) differed in chemical shift by 0.078 p.p.m. (9.4 Hz at 121.5 MHz); however for (133) the diastereomeric separation was only 0.031 p.p.m. (3.7 Hz) and did not appear to vary significantly with solvent (Table 3.1). Ideally, the diastereomeric separation should exceed the $^{18}$O double bond isotopic shift (0.05 p.p.m., ca. 6 Hz at 121.5 MHz for phosphates of this type) in order to make n.m.r. interpretation straightforward and

<table>
<thead>
<tr>
<th>Compound</th>
<th>Diastereomeric separation in Hz at 121.5 MHz</th>
</tr>
</thead>
<tbody>
<tr>
<td>(129)</td>
<td>9.4 (CDCl$_3$)</td>
</tr>
<tr>
<td>(131)</td>
<td>12.0 (CDCl$_3$)</td>
</tr>
<tr>
<td>(133)</td>
<td>3.7 (CDCl$_3$)</td>
</tr>
<tr>
<td>&quot;</td>
<td>3.9 (CD$_3$OD)</td>
</tr>
<tr>
<td>&quot;</td>
<td>3.5 (C$_6$D$_6$)</td>
</tr>
<tr>
<td>(134)</td>
<td>4.4 (CDCl$_3$)</td>
</tr>
<tr>
<td>&quot;</td>
<td>6.3 (C$_6$D$_6$)</td>
</tr>
<tr>
<td>(135)</td>
<td>8.2 (CDCl$_3$)</td>
</tr>
</tbody>
</table>
FIGURE 3.11 Sequence in the derivatisation of \( O \)-substituted thiophosphate monoesters.
FIGURE 3.12  Methylation of phosphate diester (128) monitored by high-field $^{31}$P n.m.r. spectroscopy (121.5 MHz)
FIGURE 3.13 Methylation of phosphate diester (132) monitored by high-field $^{31}$P n.m.r. spectroscopy (121.5 MHz)
unambiguous when labelled thiophosphates are taken through the analysis sequence. This small separation of the diastereoisomers of (133) is presumably due to the modest difference between methyl and ethyl in conferring chirality, therefore other O-derivatising reagents were sought to increase this discrimination.

Diphenylidazomethane (Ph\textsubscript{2}CN\textsubscript{2}) was found to increase the discrimination between the diastereoisomers of (134) (Figure 3.11) when it was used to alkylate the diastereotopic oxygens of (132). Although the diastereoisomers of (134) showed this larger diastereomeric separation (0.052 p.p.m., 6.3 Hz, C\textsubscript{6}H\textsubscript{6}), it was still too close to the P=\textsuperscript{18}O isotopic shift for phosphate esters.

Use of benzoyl chloride (PhCOCl) to derivatise the diastereotopic oxygens in (132) gave diastereomeric acyl thiophosphates (135) (Figure 3.11) with an ideal diastereomeric separation of 0.068 p.p.m. (8.2 Hz at 121.5 MHz). Furthermore, derivatisation of the 4-nitrophenyl thiophosphate diester (128) gave diastereomeric triesters (131a,b) whose chemical shift differed by 12 Hz (summary of these results are shown in Table 3.1). Therefore, benzoylation was chosen as our O-derivatising method for the 4-nitrophenyl thiophosphate and ethyl thiophosphate diesters.

Interestingly, the yields in each step of the configurational analysis were essentially quantitative, as judged by \textsuperscript{31}P n.m.r. spectroscopy, such that the analysis could be performed as a "one-pot" reaction without need of purification steps. However, as the acyl triester, formed at the end of the analysis, is unstable and susceptible to hydrolysis over a period of time, its \textsuperscript{31}P n.m.r. spectrum was recorded without delay.

Having established the conditions for the configurational analysis of unlabelled O-substituted thiophosphates, the next task was to apply it to \textsuperscript{18}O-labelled samples.

**Configurational analysis of Rp-4-nitrophenyl [\textsuperscript{16}O,\textsuperscript{18}O]thiophosphate (89a)**

(Rp)-4-Nitrophenyl [\textsuperscript{16}O,\textsuperscript{18}O]thiophosphate (89a), prepared by our general route (Chapter 2), was taken through the analysis sequence under the same conditions as its unlabelled counterpart. The \textsuperscript{31}P n.m.r. shown in Figure 3.14 illustrates the configurational analysis of (89a). This configurational analysis of (89a) and its subsequent \textsuperscript{31}P n.m.r.
spectrum gave us evidence that the stereocontrolled synthesis of (89a) involved high incorporation of $^{18}$O label and little isotope washout. Therefore, some unlabelled triester (131) was added to the labelled sample to provide a reference for the assignment of peaks.

\[
\begin{align*}
\text{(89a)} & \\
\end{align*}
\]

Since the absolute configuration follows from the synthesis, the absolute configurations of (131a) and (131b) can be assigned on the basis of the observation of a double bond $^{18}$O isotopic shift on the downfield diastereoisomer and a single bond $^{18}$O shift on the upfield diastereoisomer. Therefore the downfield isomer has the Rp configuration. The assignments are shown in Figure 3.14.

**Configurational analysis of (Rp)-ethyl $[^{16}$O,$^{18}$O ]thiophosphate (90a)**

(Rp)-ethyl $[^{16}$O,$^{18}$O ]thiophosphate (90a) was also configurationally analysed by the S-alkylation, O-benzoylation method. Some unlabelled acyl triester was added to aid

\[
\begin{align*}
\text{(90a)} & \\
\end{align*}
\]

assignment and we can see from Figure 3.15 that the high-field $^{31}$P n.m.r. spectrum of the analysis of (90a) has a similar pattern to the previous one. Again from the isotope shifts, the downfield isomer has the Rp configuration.

**Conclusion**

S-alkylation, then O-benzoylation followed by high-field $^{31}$P n.m.r. analysis offers a straightforward method for the determination of the absolute configuration of $[^{16}$O,$^{18}$O ]-thiophosphate monoesters. This method is general and is conceptually and experimentally simpler than the analysis reported by Cullis et al.\textsuperscript{142}
FIGURE 3.14 The high-field $^{31}$P n.m.r. spectrum (proton-decoupled, 121.5 MHz) of the triesters derived by S-alkylation followed by $O$-benzyolation of (Rp)-4-nitrophenyl [${}^{16}$O, ${}^{18}$O]thiophosphate (89a).
FIGURE 3.15 The high-field $^{31}$P n.m.r. spectrum (proton-decoupled, 121.5 MHz) of the triesters derived by S-alkylation followed by O-benzylation of (Rp)-ethyl [16O, 18O]thiophosphate (90a).
CHAPTER 4
Stereochemical Study: Solvolysis of Rp-4-Nitrophenyl $[^{16}\text{O},^{18}\text{O}]$Thiophosphate Monoester
Introduction

[\textsuperscript{16}O,\textsuperscript{17}O,\textsuperscript{18}O]phosphate (28) and [\textsuperscript{16}O,\textsuperscript{18}O]thiophosphate (29) substrates have been utilized extensively to determine the stereochemical course of many enzyme-catalysed phosphoryl\textsuperscript{89,98,99} and thiophosphoryl\textsuperscript{94,96} transfer reactions. Esters of (28) have been used to study the stereochemical course of some simple chemical phosphoryl transfer reactions in order to probe the lifetime of the monomeric metaphosphate intermediate (2). Current stereochemical,\textsuperscript{50,51} kinetic\textsuperscript{58,60} and thermodynamic\textsuperscript{74} studies (discussed in detail in Chapter 1) agree that there is no activation barrier to the reaction of monomeric metaphosphate in aqueous solutions. In contrast, stereochemical studies on analogous phosphoryl transfer reactions in aprotic and less nucleophilic protic solvents have found them to proceed with extensive racemisation\textsuperscript{61,62,69-71} which suggests that the metaphosphate may have an appreciable lifetime.

Hitherto, few studies have been conducted on simple chemical thiophosphoryl transfer reactions pertinent to the lifetime of the related monomeric thiometaphosphate intermediate (48). Such studies are of interest since:

a) The stereochemical course of enzyme-catalysed thiophosphoryl transfer reactions has
often been assumed to be the same as for the natural phosphoryl transfer reaction and it would be relevant to determine whether these reactions are indeed stereochemically equivalent.

b) Thiophosphate monoesters are found to react more rapidly than corresponding phosphate monoesters suggestive of a dissociative mechanism.\(^{85}\) In view of the recent conclusions about phosphoryl transfer in aqueous solutions, there could be mechanistic differences between simple phosphoryl and thiophosphoryl transfer reactions which need exploring.

As with phosphoryl transfer reactions, there are at least four basic mechanisms possible for thiophosphoryl transfer (see Figure 1.1, Chapter 1). It seems likely the mechanism will either conform to a dissociative or preassociative pathway, see Figure 4.1. Racemisation or partial racemisation would imply a reaction involving a long-lived monomeric thiometaphosphate intermediate whereas inversion of configuration at phosphorus could indicate a preassociative reaction (stepwise or concerted) or at the limit an associative reaction.

**Literature study of simple thiophosphoryl transfer reactions**

In one of the first studies of chemical thiophosphoryl transfer reactions, Benkovic and his co-workers\(^ {84}\) studied the hydrolysis of labelled 4-nitrophenyl thiophosphate monoanion (89a) (Figure 4.2) and found that the product, inorganic \[^{16}O,^{17}O,^{18}O\] thiophosphate (77), was 30-40% racemic which suggests the fleeting existence of a monomeric thiometaphosphate intermediate in aqueous solution. However, there is a large amount of uncertainty in this result because of the shortcomings of the configurational analysis of Trentham and Webb\(^ {138}\) arising from the use of enzymes and concomitant loss of label (see Chapter 3).

In a preliminary study, Cullis and Iagrossi\(^ {143}\) investigated the stereochemical course of the ethanolysis of labelled 4-nitrophenyl thiophosphate dianion (89a). This work showed the product, ethyl \[^{16}O,^{18}O\] thiophosphate (90a), to be \(~80%\) racemic, indicating a comparatively long-lived thiometaphosphate intermediate. However, this work requires a more detailed stereochemical study to establish, in particular, the effect of:
Reaction:

\[
\text{RO} \quad \overset{\text{S}^-}{\text{P}} \quad \text{O}^- \quad \xrightarrow{\text{Nu}^-} \quad \text{RO}^- \quad + \quad \text{Nu} \quad \overset{\text{S}^-}{\text{P}} \quad \text{O}^- 
\]

R = alkyl, aryl

Mechanisms:

1. Dissociative mechanism via thiometaphosphate intermediate

2. Preassociative mechanism (and at the limit associative)

Stereochemical course: racemisation

Stereochemical course: inversion

FIGURE 4.1 Proposed mechanisms for the solvolysis of thiophosphate monoester.
The stereochemical course of solvolysis of 4-nitrophenyl \(^{16}O,^{18}O\) thiophosphate (89a):

**Development and analysis of the stereochemical course**

In order to establish the conditions of the thiophosphoryl transfer reactions, unlabelled 4-nitrophenyl thiophosphate (89) was synthesized by hydrolysing the corresponding dichloride (136) which was prepared by the method of Tolkmith,\(^ {172}\) as shown in Figure 4.3. The sodium salt of (89) can be stored as an aqueous solution and used without purification.
The triethylammonium (Et₃NH) salt of (89) was obtained by passing it through an A-25 Sephadex ion-exchange column (HCO₃⁻ form).

\[
\text{O}_2\text{N} - \text{OH} + \text{P(S)}\text{Cl}_3 \xrightarrow{\text{Pyridine, CH}_2\text{Cl}_2} \text{O}_2\text{N} - \text{O} - \text{PCl}_2 \quad (136)
\]

\[
\text{NaOH(aq) [4eq]}, \text{dioxan}
\]

\[
\text{O}_2\text{N} - \text{O} - \text{P} - \text{S}^- \quad 2\text{Na}^+
\]

\[
(89)
\]

**The stereochemical course of the ethanolysis of (Rp)-4-nitrophenyl [¹⁶O,¹⁸O]thiophosphate (dianion)**

The bis-(tetra-n-butylammonium) (Bu₄N) salt of (Rp)-4-nitrophenyl [¹⁶O,¹⁸O]thiophosphate (89a) was prepared from the corresponding triethylammonium salt by titrating with 2 equivalents of tetra-n-butylammonium hydroxide. However, we found that when we tried to isolate the bis-Bu₄N salt of (89a) completely free of water, it decomposed into inorganic thiophosphate (77) and 4-nitrophenolate (137) (see below). The dianion although stabilised by solvation in water reacts extremely rapidly (10⁷ times faster) in non-aqueous solvents and as a gum. (89a) was freed of triethylamine by repeated co-evaporation with dry ethanol without taking the residue to dryness.

\[
\text{O}_2\text{N} - \text{O} - \text{P} - \text{S}^- \xrightarrow{\text{H}_2\text{O}} \text{O}_2\text{N} - \text{O}^- + \text{HO-PCl}_2 \quad (137)
\]

\[
(77)
\]

The dianion (89a) was solvolyzed in neat ethanol (concentration of starting material ~7.5 mM) at 45°C for approximately 1 hour. After this time, two singlet resonances were seen in
the $^{31}$P n.m.r. spectrum of the reaction mixture, one at $+40.9$ p.p.m. and the other at $+43.2$ p.p.m. This latter peak was assigned to the product ethyl $[^{16}$O,$^{18}$O]thiophosphate (90a) by proton-coupled $^{31}$P n.m.r. which shows the characteristic triplet splitting pattern for methylene proton coupling to phosphorus. The resonance at higher field remained a singlet and its chemical shift was consistent with the starting material (89a). The reaction was judged to have gone to 50% completion and the products were separated by ion-exchange chromatography (A-25 Sephadex resin). Ethyl thiophosphate was the first to elute from the column followed by the starting material. Both of these fractions were fully characterised by $^1$H and $^{31}$P n.m.r. spectroscopy and derivatisation ($S$-alkylation with myrtenyl bromide and $O$-methylolation with dimethyl sulphate followed by chromatography allowed characterisation by high resolution mass spectrometry).

Both the reisolated starting material (89a) and the product (90a) were subjected to our configurational analysis ($S$-alkylation with myrtenyl bromide and $O$-benzoylation with benzoyl chloride, described in Chapter 3) and the high-field $^{31}$P n.m.r. spectra were recorded.

The high-field $^{31}$P n.m.r. spectrum of the isolated ethyl $[^{16}$O,$^{18}$O]thiophosphate (90a) after configurational analysis (Figure 4.4) shows double bond $^{18}$O isotopic shifts on both the downfield and the upfield unlabelled diastereoisomers and comparing with Figure 3.15 (Chapter 3), one can conclude that the product was essentially completely racemic. As the configurational analysis does not depend on the absolute intensities of the peaks but on the relative intensities, the enantiomeric excess in the product can then be quantified directly from these relative intensities of the resonances. Inspection of Figure 4.4 shows that the intensities of the peaks of the four labelled triesters are virtually the same, therefore one can
Figure 4.4. The high-field $^3$P- n.m.r. spectrum (proton-decoupled, 121.5 Hz) of ethyl 1$^{16}$O, 18$^O$ O-jithiophosphate (90a) isolated from the ethanolysis of the dianion of (89a); derivatised by S-alkylation with myrcenyl bromide and O-benzylation with benzyol chloride.
conclude that the ethyl \( \text{[}^{16}\text{O},^{18}\text{O}\text{]} \) thiophosphate obtained from the solvolysis reaction was \(~100\%\) racemic. This means that \((\text{Rp})\) and \((\text{Sp})\)-ethyl \( \text{[}^{16}\text{O},^{18}\text{O}\text{]} \) thiophosphate were produced in equal amounts during the solvolysis reaction. The reisolated starting material (89a) after configurational analysis was approximately 95\% \( \text{Rp} \) (Figure 4.5) which is identical to the material as synthesised indicating that the starting material did not racemise during the reaction and that it is configurationally stable under the conditions of the solvolysis reaction.

In the previous study,\(^{143}\) it was unclear whether the mono- or dianion was being studied since the triethylammonium salt was used. This also meant that any free tertiary amine could have acted as a nucleophilic catalyst (below), offering a trivial explanation for the observed racemisation. The use of the quaternary bis-(tetra-\(n\)-butylammonium) salt clearly addresses both of these problems.

In theory, the product, ethyl thiophosphate, could have racemised during the solvolysis reaction by further attack of ethanol as shown below. To explore this, a control experiment was performed in which \((\text{Rp})\)-ethyl \( \text{[}^{16}\text{O},^{18}\text{O}\text{]} \) thiophosphate (90a) (prepared earlier, see Chapter 2) was heated in ethanol under more severe conditions (60°C, 3 hours) than the solvolysis reaction. Configurational analysis of the reisolated ethyl thiophosphate showed that it had not undergone any stereochemical changes. The spectrum is identical to that
**FIGURE 4.5** The high-field $^{31}$P n.m.r. spectrum (proton-decoupled, 121.5 Hz) of thiophosphate (89a) reisolated from the ethanolysis of the dianion of (89a); derivatised by S-alkylation with myrtenyl bromide and O-benzoylation with benzoyl chloride.
established in the analysis of (Rp)-ethyl $^{16}$O,$^{18}$O thiophosphate. Therefore, this shows that the product is also configurationally stable under the conditions of the solvolysis reaction.

From the above control experiments it is clear that the racemisation of configuration in the product must have arisen during the thiophosphoryl transfer step itself.

Similarly, with a higher concentration of starting material, 75 mM, the solvolysis was shown by $^{31}$P n.m.r. analysis to proceed with a similar rate and configurational analysis of the product (90a) also showed the reaction to have gone with the same stereochemistry. These collective results tend to rule out the involvement of any bimolecular processes and provide definitive evidence that the thiophosphoryl transfer process goes via a dissociative pathway. In fact, the observation of complete racemisation during the thiophosphoryl transfer reaction of the dianion of (89a) provides evidence for a free, symmetrically solvated thiometaphosphate intermediate (48) which can be attacked by ethanol from either face.

![Diagram](image)

The stereochemical course of the ethanolysis of (Rp)-4-nitrophenyl $^{16}$O,$^{18}$O thiophosphate (monoanion)

The next study involved the use of a different ionized state of the starting material (89a). The mono-triethylammonium salt of (89a) was solvolysed in neat ethanol, the presence of the monoanion was inferred from the $^{31}$P n.m.r. chemical shift [monoanion +45.4 p.p.m., dianion +40.9 p.p.m.]. The reaction was left at room temperature for 4 hours.
The solvolysis reaction after 4 hours had gone to 50% completion as judged by \( ^{31}\text{P} \) n.m.r. spectroscopy. After ion-exchange chromatography of the mixture, the reisolated starting material (89a) and the product (90a) were subjected to configurational analysis as before. (89a) was again found to be identical in configuration to the material as synthesised thus showing that the starting material was configurationally stable under the conditions of the solvolysis reaction. The \( ^{31}\text{P} \) n.m.r. spectrum of the product (90a) (Figure 4.6) after configurational analysis is similar to the spectrum obtained from the dianion experiment (Figure 4.4) except that the level of racemisation is lower. There is clearly more Sp (90a) than Rp enantiomer, in fact the ratio of the intensities of the resonances for the labelled triesters indicates that the product (90a) is 80% racemic with 20% excess Sp arising from inversion of configuration. The usual control experiment showed (90a) to be configurationally stable under the reaction condition and as a result the observed racemisation again arises during the thiophosphoryl transfer steps and supports a long-lived thiometaphosphate intermediate.

The extensive racemisation observed in the solvolysis of (89a) both as its mono- and dianion provides evidence that a monomeric thiometaphosphate ion is generated via a dissociative process which can live long enough to escape the solvent cage completely to allow racemisation. A reaction mechanism which accommodates this point is proposed in Figure 4.7. This can also help to explain why the monoanion undergoes incomplete racemisation (~80%) during the ethanolysis in comparison to the dianion (~100%). In the case of the dianion, solvent separation of the 4-nitrophenolate anion and the thiometaphosphate ion on electrostatic grounds will be more favoured than the solvent separation of the 4-nitrophenol from thiometaphosphate ion in the monoanion case. In the case of the
FIGURE 4.6 The high-field $^{31}\text{P}$ n.m.r. spectrum (proton-decoupled, 121.5 Hz) of ethyl [${}^{16}\text{O}, {}^{18}\text{O}$]thiophosphate (90a) isolated from the ethanolation of the monoanion of (89a); derivatised by $S$-alkylation with myrtenyl bromide and $O$-benzoylation with benzoyl chloride.
**Dianion**

\[
\begin{array}{c}
\text{O}_2\text{N} - \text{O} - \text{P} - \text{S}^-
\end{array}
\xrightleftharpoons{\text{r.l.s.}}
\begin{array}{c}
\text{O}_2\text{N} - \text{PO}^-
\end{array}
\]

(89a)

\[
\begin{array}{c}
\text{EtO} - \text{P} - \text{S}^-
\end{array}
\xrightarrow{\text{EtOH}}
\begin{array}{c}
\text{EtOH}
\end{array}
\rightarrow
\begin{array}{c}
\text{S}^-
\end{array}
\]

\[
\begin{array}{c}
\text{PO}^-
\end{array} + \begin{array}{c}
\text{O}_2\text{N} - \text{O}^-
\end{array}
\]

(90a)

**Monoanion**

\[
\begin{array}{c}
\text{O}_2\text{N} - \text{O} - \text{P} - \text{S}^-
\end{array}
\xrightleftharpoons{\text{r.l.s.}}
\begin{array}{c}
\text{O}_2\text{N} - \text{PO}^+
\end{array}
\]

(89a)

\[
\begin{array}{c}
\text{EtO} - \text{P} - \text{S}^-
\end{array}
\xrightarrow{\text{EtOH}}
\begin{array}{c}
\text{EtOH}
\end{array}
\rightarrow
\begin{array}{c}
\text{S}^-
\end{array}
\]

\[
\begin{array}{c}
\text{PO}^-
\end{array} + \begin{array}{c}
\text{O}_2\text{N} - \text{OH}^-
\end{array}
\]

(90a)

**FIGURE 4.7** The dissociative pathway for the ethanolyis of 4-nitrophenyl thiophosphate (dianion and monoanion).
dianion, this allows the nucleophile to attack the electrophilic intermediate from either side. It is important to point out that racemisation could also occur if the intermediate is long-lived enough to tumble within the solvent cage in which it was generated.

For the monoanion (89a), 20% of reaction occurred with inversion of configuration for which a preassociative (concerted or stepwise) mechanism would seem to be the likely explanation where there may be some assistance from the incoming nucleophile (below), or shielding of one face of the monomeric thiometaphosphate by the neutral leaving group.

\[
\begin{align*}
\text{preassociative complex} \\
\begin{array}{c}
\text{O}_2\text{N} - \text{O} - \text{P} - S^- \\
\text{H} \quad \text{HOEt}
\end{array}
\end{align*}
\rightarrow
\begin{align*}
\text{inversion} \\
\begin{array}{c}
\text{O}_2\text{N} - \text{OH} - \text{P} - S^- \\
\text{HOEt}
\end{array}
\end{align*}
\]

Ethyl thiophosphate

The monoprotonated species of (89a) was found to require milder conditions for solvolysis than the dianion; this faster reaction rate is ascribed to the rapid loss of the neutral leaving group from the zwitterion (Figure 4.7), in agreement with the work of Benkovic and his collaborators.\(^4\)

The stereochemical course of the solvolysis of (Rp)-4-nitrophenyl \([^{16}\text{O},^{18}\text{O}]\)thiophosphate (dianion) in aqueous alcohols

As mentioned in Chapter 1, Knowles et al. found that the methanolysis of the phenyl \([^{16}\text{O},^{17}\text{O},^{18}\text{O}]\)phosphate monoanion or 2,4-dinitrophenyl \([^{16}\text{O},^{17}\text{O},^{18}\text{O}]\)phosphate dianion in aqueous methanol proceed with complete inversion of configuration (within experimental error) at phosphorus. Therefore, as an interesting comparison, we conducted the stereochemical course of the solvolysis of (Rp)-4-nitrophenyl \([^{16}\text{O},^{18}\text{O}]\)thiophosphate (89a) in aqueous ethanol.
The disodium salt of (89a) was solvolysed in ethanol-water (1:1.5 mol ratio) at a nominal pH of 6.8 for 90 minutes at 60°C. Monitoring by $^{31}$P n.m.r. spectroscopy showed that ethyl [$^{16}$O,$^{18}$O]thiophosphate, inorganic [$^{16}$O,$^{18}$O]thiophosphate (77) (identified by spiking the n.m.r. sample with authentic compound) and remaining starting material were present in roughly equal amounts.

Under these experimental conditions, (89a) is predominately present as the dianion since the "apparent" pKa, determined by recording the $^{31}$P n.m.r. chemical shift of (89a) in ethanol-water (1:1.5 mol ratio) at various pHs (Figure 4.8), was calculated to be ~4.8. However, in a series of reactions, raising the pH by one unit reduced the rate of reaction markedly which seems to suggest that the solvolysis in ethanol-water at pH 6.8 proceeds through the monoanion which is in agreement with our earlier observations and in accord with the work of Benkovic et al.}

In previous studies, solvolyses of phosphate monoesters in aqueous alcoholic media have usually given a reasonable correlation (though rarely perfect) between the mole fraction of alcohol in the solvent and the mole fraction of alkyl phosphate in the product. The data has always been interpreted in terms of the intermediacy of a reactive and non-selective electrophile. In our study the product ratio of ethyl thiophosphate to inorganic thiophosphate is not exactly equal to the mole ratios of ethanol to water, with ethyl thiophosphate slightly more favoured over inorganic thiophosphate. This data suggests that although monomeric thiometaphosphate intermediate is a powerful electro-
FIGURE 4.8 Determination of the apparent second "pKa" of 4-nitrophenyl thiophosphate (89) in ethanol / water.
phile, it shows some selectivity between nucleophiles.

The ethyl $[^{16}O,^{18}O]$ thiophosphate was isolated and its chirality determined as before by the configurational analysis method. Comparison of the peak intensities from the $^{31}$P n.m.r. spectrum (Figure 4.9) shows that the thiophosphoryl transfer had proceeded with extensive racemisation (~70%) with 30% excess inversion of configuration, arising from the formation of excess (Sp)-ethyl $[^{16}O,^{18}O]$ thiophosphate. The normal control experiments showed that the racemisation arises during the thiophosphoryl transfer reaction. This result is in marked contrast to the corresponding phosphoryl transfer reactions in aqueous alcoholic media which proceed stereospecifically.$^{50}$

In a parallel study, David Wilkins, of this Department, conducted the solvolysis of (89a) in ethanol / $^{17}$O-water under identical conditions. The product, inorganic $[^{16}O,^{17}O,^{18}O]$ thiophosphate (77), was configurationally analysed using the method of Lowe and Arnold$^{140}$ (see Chapter 3) and was found to have suffered a similar degree of racemisation (~70%). It is interesting that in the thiophosphoryl case, one can determine the hydrolysis and alcoholysis stereochemistries in a single reaction.

**Conclusion**

The results of our stereochemical study of the solvolysis of (Rp)-4-nitrophenyl $[^{16}O,^{18}O]$ thiophosphate are summarized in Table 4.1.

From the three solvolysis reactions we have shown substantial racemisation of configuration during thiophosphoryl transfer independent of:

i) ionized state (monoanion, dianion),

ii) counter ion (tetrabutylammonium, triethylammonium, sodium),

iii) solvent (neat ethanol, aqueous ethanol),

iv) concentration of the thiophosphate monoester.
FIGURE 4.9  The high-field $^{31}$P n.m.r. spectrum (proton-decoupled, 121.5 Hz) of ethyl [¹⁶O,¹⁸O]thiophosphate (90a) isolated from the solvolysis of the dianion of (89a) in ethanol / water; derivatised by S-alkylation with myrtenyl bromide and O-benzoylation with benzoyl chloride.
TABLE 4.1

<table>
<thead>
<tr>
<th>IONIZED STATE</th>
<th>COUNTER ION</th>
<th>SOLVENT</th>
<th>STEREOCHEMISTRY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dianion</td>
<td>Bu$_4^+$N</td>
<td>EtOH</td>
<td>100% racemisation</td>
</tr>
<tr>
<td>Monoanion</td>
<td>Et$_3$NH</td>
<td>EtOH</td>
<td>80% racemisation</td>
</tr>
<tr>
<td>Monoanion</td>
<td>Na$^+$</td>
<td>EtOH / H$_2$O</td>
<td>70% racemisation</td>
</tr>
</tbody>
</table>

More importantly, the observation of complete racemisation of configuration in the solvolysis of the dianion in neat ethanol provides the first direct evidence for a free, symmetrically solvated monomeric thiometaphosphate intermediate (48) in hydroxylic solvent. The existence of a long-lived thiometaphosphate in the solvolysis reaction of (89a) in aqueous ethanol is in sharp contrast to the analogous phosphoryl transfer reactions in aqueous alcoholic medium in which the putative metaphosphate intermediate (2) is so reactive that the reaction follows a concerted preassociative mechanism.$^{50,51,58,60,74}$ It is clear, therefore, that the thiometaphosphate ion is kinetically more stable than the metaphosphate ion in aqueous solution to allow the liberated species to be formed under favourable conditions. This apparent increased stability of (48) over (2) is in accord with the work of Roesky et al.$^{86}$ (Chapter 1) who isolated trithiometaphosphate (PS$_3^-$) as its tetraphenylarsonium salt and can be attributed to the larger sulphur atom being better able to disperse the negative charge than the oxygen atom.

Soon after our work in the area of thiophosphoryl transfer reactions, Lowe and Harnett$^{173}$ reported results that agree with the observations in this Chapter. They studied the hydrolysis of deoxyadenosine-5'-[β-$^{17}$O, thio]diphosphate (138) in $^{18}$O-water (Figure
4.10). Hydrolysis of the dianion (pH 4.5) gave dAMP (139) and inorganic thiophosphate

\[
\text{dAMP} + \overset{\text{H}_2\overset{\text{O}}{\text{O}}}{} \overset{\text{pH} \sim 4.5}{\text{50 °C / 1.5 h}} \text{dAMP} + \overset{\text{P} - \text{O}}\text{O}
\]

Result: 50% racemisation
50% inversion

FIGURE 4.10 Stereochemical course of the thiophosphoryl transfer reaction of dADP (138) investigated by Harnett and Lowe.

(77) with 50% racemisation and 50% inversion of configuration at phosphorus implying a liberated thiometaphosphate intermediate of finite lifetime. It is interesting to note that the hydrolysis at pH 7.2, where the trianion is present, was found to occur with complete inversion of configuration at phosphorus which rules out any 'free' intermediate. It is thought this trianion may be hydrolysed by a preassociative concerted mechanism with an
'exploded' transition state where the departure of the dAMP dianion requires assistance from the solvent nucleophile.

Our study also confirms the results of Breslow and Katz who reported that thiophosphate monoesters react more rapidly via a dissociative reaction than the corresponding phosphate esters. This and our observations establish a clear mechanistic difference between phosphoryl and thiophosphoryl transfer reactions in aqueous solutions which may lead one to question the widespread use of thiophosphoryl transfer reactions to probe the mechanism of enzyme-catalysed phosphoryl transfer reactions.

Thiophosphate analogues of natural substrates have been used for many years to probe enzyme-catalysed phosphoryl reactions simply because it is easier to generate chirality at a thiophosphoryl centre involving use of one less oxygen isotope and separation of diastereoisomers of thio-nucleotides is much simpler than those of oxy-nucleotides. In the vast majority of cases, it is found that the thiophosphate analogues follow the same stereochemical course as the natural substrates in enzyme-catalysed reactions (Chapter 1). Most of them proceed with inversion of configuration of the transferred (thio)phosphoryl moiety, the simplest interpretation of this result is that the (thio)phosphoryl transfer proceeds by a single associative in-line transfer between the two substrates in the ternary complex. It would therefore seem valid to use thiophosphate analogues as stereochemical probes. But the similarity between the course of enzymic phosphoryl and thiophosphoryl reactions may perhaps be expected on the grounds that an enzyme-active site is unlikely to be able to provide more than one catalytic path for a given reaction.
CHAPTER 5
Kinetic Study: Effect of Pressure on the Rate of Hydrolysis of 2,4-Dinitrophenyl Thiophosphate Dianion
Introduction

We have seen in the previous chapter how stereochemical methods can provide information about the mechanism of the solvolysis of thiophosphate monoesters thus giving an insight into the lifetime of the thiometaphosphate intermediate. In the past, thermodynamic, kinetic and positional isotope exchange (P.I.X.) methods as well as stereochemical ones have been used as tests for the intermediacy of metaphosphate in the solvolysis reactions of phosphate esters (Chapter 1). In the light of these studies and to complement our earlier stereochemical study, we thought it would be interesting to study thiophosphoryl transfer reactions via a kinetic method, and in particular use the activation parameter, $\Delta V^*$ (volume of activation), to distinguish between dissociative and associative mechanisms.

**Volume of activation $\Delta V^*$ as a test of reaction mechanism**

The volume of a reacting system may increase or decrease in passing to the transition state depending on the competition between bond breaking and bond making processes. In an associative (bimolecular) pathway bond making and bond breaking proceed together with bond formation usually dominating thus involving a shrinkage at the transition state, whereas in a dissociative (unimolecular) mechanism bond breakage is complete before bond making thus involving an expansion in the transition state. The activation parameter, $\Delta V^*$ (volume of activation) is a quantity which characterises a reaction and from which much may be learned about the reaction mechanism. This parameter $\Delta V^*$, which is derived from transition state theory, represents the difference between the partial molar volume of the transition state and that of the reactants in solution (Equation 5.1).

$$\text{Volume of activation } (\Delta V^*) = \Delta V^\dagger (\text{transition state}) - \Delta V^\dagger (\text{initial state})$$

**Equation 5.1**

The activation volume can be measured by means of the effect of hydrostatic pressure on the rate of reaction and subsequent application of Equation 5.2. One can see from Equations 5.1 and 5.2 that for an associative process, which involves a volume shrinkage in the transition state, $\Delta V^*$ will be negative and applying an external pressure on the system...
where:  
\[ \delta \ln k = - \frac{\Delta V^+}{RT} \]  
\[ \text{Equation 5.2} \]

will increase the rate of reaction. However, for a dissociative process, as it involves an expansion of the transition state, \( \Delta V^+ \) will be positive and pressure will severely retard the reaction (Figure 5.1).

Typical values of \( \Delta V^+ \) vary between +25 and -25 cm\(^3\) mol\(^{-1}\).\(^{178,179}\) For example, Diels-Alder reactions and other concerted processes (below) show large negative values of \( \Delta V^+ \) consistent with simultaneous bond formation at both ends of the system in contrast to non-concerted processes which would show more positive values.

\( \Delta V^+ = -24 \text{ cm}^3 \text{ mol}^{-1} \)

Diels-Alder reaction (dimerisation of cyclopentadiene)

\( \Delta V^+ = -15 \text{ cm}^3 \text{ mol}^{-1} \)

Claisen rearrangement (of allyl phenyl ether)

The formation of dichlorocarbene (\(\text{CCI}_2\)) in the base promoted hydrolysis of chloroform has a \( \Delta V^+ \) of +16 cm\(^3\) mol\(^{-1}\) thus indicating a dissociative process.\(^{175}\)
For an **associative process**

- contraction of transition state,
- $\Delta V^\pm$ is negative,
- increasing pressure accelerates the reaction.

**FIGURE 5.1** Physical changes on transition state formation for an associative and a dissociative process and how volume of activation may be visualized.

For a **dissociative process**

- expansion of transition state,
- $\Delta V^\pm$ is positive,
- increasing pressure retards the reaction.
It must be pointed out that the magnitude and size of $\Delta V^\ddagger$ not only depends on bond cleavage and formation but also on whether charges are being formed, dispersed or neutralized. Table 5.1 shows the principal mechanistic features that are important in making estimates of $\Delta V^\ddagger$, and their contributions. These values are only approximate and individual structures and conditions will largely determine $\Delta V^\ddagger$. The above table indicates that for a dissociative process involving the generation of charge, $\Delta V^\ddagger$ is surprisingly negative. Despite the fact that dissociation of the substrate would tend to increase the volume, electrostriction of the solvent around the developing ions has the opposite effect, which is usually dominant thus contracting the transition state. In that respect, for reactions that generate charge in the transition state, electrostrictive effects can cloud and sometimes totally obscure the interpretation of activation volumes. On the whole, despite this one anomaly, the magnitude and size of $\Delta V^\ddagger$ for a reaction can still be a good mechanistic indicator. For instance, typical $S_N2$ displacement reactions in water lead to activation volumes of -5 to -10 cm$^3$ mol$^{-1}$ and $S_N1$ reactions lead to activation volumes which exceed +10 cm$^3$ mol$^{-1}$.74

<table>
<thead>
<tr>
<th>Mechanistic feature</th>
<th>Contribution, cm$^3$ mol$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bond cleavage</td>
<td>+10</td>
</tr>
<tr>
<td>Bond formation</td>
<td>-10</td>
</tr>
<tr>
<td>Synchronous displacement</td>
<td>-5</td>
</tr>
<tr>
<td>Ionic dissociation</td>
<td>$&lt; -20$ (solvent-dependent)</td>
</tr>
<tr>
<td>Charge neutralisation</td>
<td>+20</td>
</tr>
<tr>
<td>Charge concentration</td>
<td>-5</td>
</tr>
<tr>
<td>Charge dispersal</td>
<td>+5</td>
</tr>
</tbody>
</table>
**Effect of pressure on the rate of hydrolysis of phosphate monoesters**

A recent kinetic study by le Noble et al. investigated the rate of hydrolysis of 2,4-dinitrophenyl phosphate dianion (31) as a function of pressure in order to yield information on the mechanism of these reactions of phosphates. Other types of studies on the hydrolysis of (31) have produced conflicting evidence, some of which support and some disfavor a monomeric metaphosphate intermediate. The liberation of 2,4-dinitrophenoxide (140) from the phosphate dianion was found to be accelerated by pressure with $\Delta V^+ = -4.8 \text{ cm}^3 \text{ mol}^{-1}$ (Figure 5.2). This result is inconsistent with a free metastaphosphate intermediate since bond cleavage would give rise to an expansion in the transition state (i.e. positive $\Delta V^+$) which would be expected to be further amplified by the effects of the greater charge delocalisation at the transition state (electrostriction effects). In such plots as Figure 5.2(a), the curvature often seen may hide the incursion of a small contribution from a second mechanism, if that is the case an Arrhenius plot of rate against temperature will then show a curvature. Figure 5.2(b) shows that such a plot is accurately linear over the whole range thus ruling out a significant contribution from a second mechanism. As a result one can conclude that this reaction occurs by a nucleophilic attack of water at the phosphorus atom, with loss of phenoxide ion, through an associative transition state (as shown overleaf). This data is consistent with the presently held view that the monomeric metaphosphate is such a reactive electrophile that it cannot exist in aqueous solvent whereas in aprotic and less nucleophilic solvents it may have a finite lifetime. Our stereochemical studies on the thiophosphoryl transfer reactions of 4-nitrophenyl thiophosphosphate (Chapter 4) suggest a dissociative pathway even in aqueous solvents and that monomeric thiometaphosphate may be sufficiently kinetically and thermodynamically stable to
a) Pseudo-first order rate constant as a function of pressure (1 MPa = 10 bar) for the hydrolysis of 2,4-dinitrophenyl phosphate (31) at pH 12 (43.2 °C) and

b) Arrhenius plot for the hydrolysis at atmospheric pressure, reported by le Noble et al.

FIGURE 5.2
participate as an intermediate even in reactions in aqueous solution. By analogy with the work of le Noble,\textsuperscript{74} we would predict that thiophosphoryl transfer reactions in aqueous solution will show a volume of activation with the opposite sign to that reported for the corresponding phosphoryl transfer reaction.

**Kinetic study on the hydrolysis of 2,4-dinitrophenyl thiophosphate dianion (141)**

Kinetic study was conducted on the hydrolysis of 2,4-dinitrophenyl thiophosphate dianion (141), similar experiments on the hydrolysis of 4-nitrophenyl thiophosphate (89) were initially performed but the reaction was found to be too slow to allow kinetics at high pressure to be easily determined.\textsuperscript{183} (141) Was found to be very much more reactive such that it proved necessary to generate this in situ.

**Synthesis of 2,4-dinitrophenyl thiophosphoryl dichloride (142)**

The synthesis of 2,4-dinitrophenyl thiophosphoryl dichloride (142) was based on a method by Tolkmith\textsuperscript{172} who had prepared the corresponding mono-nitro compound. The synthesis of (142) is shown in Figure 5.3.

Reaction of lithium 2,4-dinitrophenoxide (140) with thiophosphoryl chloride over 24 hours and concentration of the mixture gave a brown oil from which (142) was isolated by
FIGURE 5.3 Synthesis of 2,4-dinitrophenyl thiophosphoryl dichloride (142).

Repeated extraction with hydrocarbon solvents in 57% yield (m.p. 55-57°C, δp +54.2). It was only possible to purify (142) by recrystallisation as it is known compounds of this type readily decompose on silica chromatography. In fact (142) was found to be unstable at room temperature over a number of hours and had to be stored at -30°C. The low yield of (142) was in part due to the formation of other by-products as suggested by the 31P n.m.r. spectrum of the residual oil which could conceivably be di- and trisubstituted products of (142) and for the above reason it was not possible to separate them.

Rate of hydrolysis of 2,4-dinitrophenyl thiophosphate dianion (141) at atmospheric pressure

Preliminary experiments showed that the hydrolysis of the dichloride (142) to 2,4-dinitrophenyl thiophosphate (141) in excess aqueous sodium hydroxide was complete within the first 15 minutes, as judged by 31P n.m.r. spectroscopy (Figure 5.4); the resonance at +54.2 p.p.m. for the dichloride was rapidly replaced by a singlet at +50.6 p.p.m. for 2,4-dinitrophenyl thiophosphate (141). After leaving the reaction for a further 3 hours, the
FIGURE 5.4 The hydrolysis of 2,4-dinitrophenyl thiophosphoryl dichloride (142) in NaOH(aq) monitored by $^{31}$P n.m.r. spectroscopy.
resonance for the thiophosphate was replaced by a broad singlet which was identified as inorganic thiophosphate (77) by spiking the n.m.r. mixture with an authentic sample of (77). The liberation of 2,4-dinitrophenoxide (140) was obvious by the appearance of a bright yellow colour during the course of the reaction. This was also later confirmed spectro-photometrically at 400 nm.

On the basis of this preliminary experiment, the rate of hydrolysis of the thiophosphate (141) at atmospheric pressure was investigated by following the release of (140) at 400 n.m. The hydrolysis was performed under pseudo-first order conditions employing an excess of base (aq. NaOH). Figure 5.5 shows the UV spectrum of the base hydrolysis of (141) monitored by repeat scanning (t = 4 min.) and it clearly shows the release of (140) with time (bottom curve represents the start of the monitoring process). The first three curves show the initial hydrolysis of the dichloride (142) while the later curves seem to pass through at least two clear isosbestic points (X and Y), that is, wavelengths at which the absorbance does not change with time during the course of the reaction. It is generally accepted that occurrence of an isosbestic point in reaction requires that the changes in the concentrations of the various components be linearly related, although cases are known where changes in molar absorption coefficients with solvent composition and temperature may produce isosbestic points. However, this complication is unlikely in kinetic studies, and the presence of an isosbestic point may be taken as evidence that a single set of reactants gives a single set of products in constant proportions, in other words, the point can be a good indicator of 1:1 conversion. Therefore, Figure 5.5 suggests that the hydrolysis of the dichloride is complete before the hydrolysis of thiophosphate starts, i.e.

\[
\begin{align*}
A \xrightarrow{} B \xrightarrow{} C \\
\text{(dichloride)} \quad \text{(thiophosphate)}
\end{align*}
\]

and not,

\[
A \xrightarrow{} B + C
\]

The dark line in Figure 5.5 represents the end of hydrolysis of the thiophosphate.

A plot of In (absorbance) against time (see Figure E.2, Experimental section) gave good
**FIGURE 5.5** Monitoring of the hydrolysis of 2,4-dinitrophenyl thiophosphate dianion (141) by UV spectroscopy. (Scans at 4 minute intervals)
first order kinetics, the observed rate constant at atmospheric pressure was determined from the slope of this plot \([k_{a(\text{obs})} = 6.9 \times 10^{-4} \ \text{s}^{-1} \ (25^\circ \text{C})]\). As good first order kinetics were observed, the half-life \((\tau)\) of the reaction was calculated from the standard Equation 5.3 and found to be 16.7 minutes.

\[
\tau = \frac{\ln 2}{k}
\]

\textit{Equation 5.3}

**Rate of hydrolysis of 2,4-dinitrophenyl thiophosphate dianion (141) at high pressure**

The kinetics of the hydrolysis of (141) at elevated pressure were studied by the use of the apparatus built in the University of Leicester after the design of Professor D. R. Stranks. This apparatus, depicted in outline in Figure E.3 (see Experimental section) allows reaction mixtures to be pressurised up to 17 500 pounds per square inch (1.25 kbar). A typical run involved making up a solution of the appropriate composition of (142) and initiating the reaction by adding aqueous sodium hydroxide solution (excess). The reaction mixture was divided, the majority was utilized in the high pressure apparatus and the remaining solution was placed in a cuvette for the atmospheric pressure comparison run. Both solutions were monitored at 25°C; aliquots were withdrawn from the pressure system and monitored in the spectrophotometer. For this experiment, the range of pressures used were 0.36, 0.71, 0.89, 1.07 and 1.25 kbars.

Rate constants at high pressures \([k_{p(\text{obs})}]\) were again determined graphically (Table 5.2). The logarithm of the ratio of the rate constant at high pressure to the rate constant at atmospheric pressure \((k_p/k_a)\) was plotted against pressure (Figure 5.6) and the best straight line drawn through the origin and the other points. The error bars shown in Figure 5.6 represent our estimates for the 95% confidence limits, based on both our confidence in the best straight lines for the respective high pressure runs and on the reproducibility of the simultaneous atmospheric pressure run in each case. The slope was used to evaluate the activation volume according to Equation 5.2. This calculation indicates a volume of activation of +11 cm³ mol⁻¹. Although the error bars on the plot are significant, the sign of the volume of activation is clearly positive and as a consequence one can infer that the rate
TABLE 5.2 Rate constants at high pressure for the base hydrolysis of 2,4-dinitrophenyl thiophosphate (141) (25 °C).

<table>
<thead>
<tr>
<th>$p / \text{kbar}$</th>
<th>$10^4 k_{p(\text{obs})} / \text{s}^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.36</td>
<td>5.781</td>
</tr>
<tr>
<td>0.71</td>
<td>5.212</td>
</tr>
<tr>
<td>0.89</td>
<td>4.932</td>
</tr>
<tr>
<td>1.07</td>
<td>4.188</td>
</tr>
<tr>
<td>1.25</td>
<td>4.036</td>
</tr>
</tbody>
</table>

of release of 2,4-dinitrophenoxide from the thiophosphate dianion is reduced by increasing pressure. The sign is clearly different from that reported by le Noble et al.\textsuperscript{74} for the corresponding phosphate ester (-4.8 cm\textsuperscript{3} mol\textsuperscript{-1}).

Interpretation of volume of activation in terms of mechanism, particularly in cases where charge distribution changes from ground state to transition state is difficult. However, these two reactions are very similar and the fact that they exhibit different signs of volume of activation and the absolute magnitude of difference is large leads us to conclude that they proceed by different mechanistic pathways. This conclusion is not based upon interpreting the absolute values in either case.

Our result suggests a dissociative mechanism with substantial bond cleavage giving rise to an expansion at the transition state (below), such an expansion would be further
amplified by the effects of greater charge delocalisation (in the leaving group) at the transition state. As a result, one can envisage a relatively free thiometaphosphate intermediate in hydrolysis of (141), Figure 5.7, in agreement with our stereochemical study of

![Chemical reaction diagram]

**FIGURE 5.7** Hydrolysis of 2,4- dinitrophenyl thiophosphate dianion (141) via a dissociative pathway.
the thiophosphoryl transfer reactions of 4-nitrophenyl thiophosphate (Chapter 4).

On the whole, the difference in activation volumes for the reactions of 2,4-dinitrophenyl phosphate (31) and the corresponding thiophosphate (141) is large (16 cm$^3$ mol$^{-1}$) and gives good support to the claim that this parameter is a good mechanistic indicator.$^{74}$

Conclusion

The sign and magnitude of the volume of activation (+11 cm$^3$ mol$^{-1}$) determined for the base hydrolysis of 2,4-dinitrophenyl thiophosphate dianion indicates that the thiophosphoryl transfer reaction to water follows a dissociative mechanism. This observation is consistent with our earlier stereochemical study and provides further direct evidence for the participation of thiometaphosphate ion as an intermediate in the hydrolysis of aryl thiophosphate dianion in aqueous solution. This kinetic study adds more weight to claims that phosphoryl and thiophosphoryl transfer reactions follow different and distinct mechanistic pathways in aqueous solution, a finding which may be pertinent to the widespread use of the latter to probe the mechanism of enzyme-catalysed phosphoryl transfer reactions.
CHAPTER 6
Stereochemical Study: Intramolecular Thiophosphoryl Transfer Reaction of Rp-2-(Hydroxymethyl)-4-Nitrophenyl \[ ^{16}\text{O}^{18}\text{O} \text{Thiophosphate} \]
Introduction

The results of the many stereochemical studies of enzyme-catalysed nucleophilic substitution at phosphorus (Chapter 1) have shown that the reactions are stereospecific: the majority proceeding with inversion and a few with retention of configuration of the transferred phosphoryl (or thiophosphoryl) moiety. Inversion is thought to support a single associative in-line transfer whereas retention involves a double in-line transfer via a phosphoenzyme intermediate.

However, in the absence of evidence of in-line geometry or the participation of a phosphoenzyme, stereochemical results may be mechanistically ambiguous. For instance, a dissociative mechanism occurs with inversion of configuration at phosphorus only if the generated metaphosphate (or thiometaphosphate) is prevented from tumbling within the active site (of the enzyme) before capture by the intended nucleophile. Also, a preassociative stepwise mechanism results in inversion of configuration only if the preassociated nucleophile is constrained to approach in-line with respect to the leaving group. It is pertinent to ask the question: can a dissociative or a preassociative stepwise mechanism occur with retention of configuration? If the nucleophile is constrained to attack from the same side as the leaving group, the stereochemical outcome would be retention. This hypothetical "front-side" displacement within an active site of an enzyme can be envisaged as in Figure 6.1. One way in which "front-side" displacement could be favoured is if normal in-line displacement is hindered by shielding of one face possibly by non-reactive amino acid residues of the enzyme.

We thought it would be interesting to test this argument by using a chemical model in which a nucleophile is constrained to attack phosphorus "adjacent" to the leaving group. One of the simplest ways of ensuring this is to have the nucleophile (Y) covalently attached to the leaving group (X), in an apparently intramolecular fashion as depicted in Figure 6.2. In this case it is impossible for the nucleophile to approach the phosphorus atom in an in-line geometry.

Thiophosphate monoesters were chosen in our model study since we know from our
FIGURE 6.1 Enzyme catalysed "front-side" displacement leading to retention of configuration at phosphorus.
s stereochemical studies (Chapter 4) that thiophosphates react largely through a dissociative mechanism in solvent systems ranging from pure organic solvents to aqueous organic solvents. The participation of a relatively long-lived thiometaphosphate intermediate (48) increases the chances of detecting the reaction mechanism depicted in Figure 6.2. However, with a sufficiently stable reactive intermediate other pathways may compete as shown in Figure 6.3. The observation of any excess retention of configuration would provide evidence for a dissociative "front-side" displacement. Phosphate monoesters, on the other hand, react with some dissociative character in organic solvents but the reactions become increasingly associative in character in aqueous media. Another advantage of studying reactions of thiophosphates is that, for isotopically labelled samples, their configurational analysis, developed during the course of this work, is much easier and more straightforward than that of isotopically chiral phosphates. But ultimately we would wish to study the reactions of phosphates as they represent the closest model to enzyme-catalysed reactions.

In the general realm of phosphate chemistry, there are numerous cases where nucleophilic substitution reactions of phosphate di- and triesters proceed with retention of configuration especially in those that involve exocyclic displacement at phosphorus held in a five-membered ring. The ring helps to stabilize the phosphorane intermediate which then pseudorotates before the expulsion of the leaving group. This concept of pseudorotation has been pioneered in the extensive work by Westheimer and is discussed in
FIGURE 6.3 Possible mechanistic pathways for reaction of thiophosphate with covalently attached nucleophile.
detail in Chapter 1. In contrast, there are very few cases of retention of configuration occurring in the displacement reactions of phosphate monoesters. One notable case is the study by Knowles and co-workers who examined the intramolecular phosphate exchange reaction of 2-phosphopropane-1,2-diol (143). By a stereochemical method involving the use of oxygen isotopes, they found that the acid-catalysed rearrangement of 2-phosphopropane-1,2-diol (143) to 1-phosphopropane-1,2-diol (58) occurs predominantly with retention of configuration (72%) confirming an intramolecular "adjacent" associative mechanism forming a penta-coordinate intermediate (144) that undergoes pseudorotation to allow expulsion of the leaving group from an apical position (Figure 6.4a). Indirect migration of the phosphoryl group via a cyclic diester which undergoes ring opening with water (Figure 6.4b) is also observed, however, the contribution of this pathway is minimal under the reaction conditions (0.5 M HClO₄, 85°C). The direct migration via an adjacent addition-elimination mechanism is not surprising, especially under the acidic conditions as protonation of the oxygen atoms helps in two key ways. Firstly, it makes the phosphorus centre more electrophilic thereby more susceptible to nucleophilic attack and secondly, it allows the penta-coordinate intermediate (144) formed initially (Figure 6.4a) to undergo pseudorotation, as it places a hydroxy group in an apical position which is very favourable whereas in the absence of acid, the extremely low apicophilicity of the oxy anion presents a barrier to pseudorotation and as a consequence the first formed intermediate returns to starting material; this kinetic and stereochemical study of Knowles provides two important observations:

i) the demonstration of retention via pseudorotation employing chiral phosphate analysis, and,

ii) direct evidence for a pseudorotation mechanism in the reaction of a phosphate monoester.

One could argue that the thiophosphoryl transfer in our study might also go via an adjacent addition-elimination, however, we would not expect this from our knowledge of nucleophilic substitution reactions of thiophosphate dianions which have fully dissociative character (Chapter 4).
FIGURE 6.4 Pathways for the isomerisation of 2-phosphopropane-1,2-diol to 1-phosphopropane-1,2-diol studied by Knowles et al.

2-(Hydroxymethyl)-4-nitrophenyl thiophosphate (145) was chosen as our model for "front-side" displacement. It resembles in reactivity the thiophosphate (89) used in our earlier stereochemical study (Chapter 2-4); and has an hydroxymethyl group in the ortho position which is strategically placed on the same side as the leaving group.
Strategy

As thiophosphoryl transfer reactions occur more rapidly in organic solvents than in aqueous solvents (due to desolvation of the thiophosphate in the former case), tert-butanol would seem to be an ideal choice of solvent for our investigation. The reaction of monomeric metaphosphate (and thiometaphosphate) with t-butanol is also known to occur with full racemisation of configuration implying that solvent does not capture the reactive intermediate within the dissociation ion pair.\(^\text{70}\)

In order to follow the stereochemistry of this reaction successfully two problems need to be addressed:

i) synthesis of isotopically chiral (145) of known absolute configuration, and

ii) configurational analysis of product(s) to determine its (their) absolute configuration (Figure 6.5).

These problems can be addressed by our earlier stereochemical study of the solvolysis of 4-nitrophenyl thiophosphate (Chapter 4). In this earlier study, we synthesized isotopically chiral (Rp)-4-nitrophenyl \([^{16}\text{O},^{18}\text{O}]\)thiophosphate (89a) of high enantiomeric purity (>90%). This chirality was achieved by exploiting the stereocontrolled displacement reactions of 2-substituted 1,3,2-oxazaphospholidine-2-thiones (Figure 2.8, Chapter 2), which have established precedent in the work of Inch et al.\(^\text{131}\) Also, in our earlier study, we devised a configurational analysis for 4-nitrophenyl \([^{16}\text{O},^{18}\text{O}]\)thiophosphate and ethyl \([^{16}\text{O},^{18}\text{O}]\)thiophosphate (S-alkylation, O-benzyolation) based on a \(^{31}\text{P}\) n.m.r. method (Chapter 3); as this analysis is general for any thiophosphate monoester, it can be used to analyse the product(s) in our present study. Therefore, on the basis of our earlier study, the synthesis of (Rp)-2-(hydroxymethyl)-4-nitrophenyl \([^{16}\text{O},^{18}\text{O}]\)thiophosphate (145a) is pro-
FIGURE 6.5  Analysis of the stereochemical course of the thiophosphoryl transfer reaction of thiophosphate (145a).

posed as shown in Figure 6.6. One can see that the primary alcohol in 2-hydroxy-5-nitrobenzyl alcohol (146) needs to be protected as this potential nucleophile can give side-products in the steps leading to the ultimate product (145a). The choice of protecting group is also important in that it needs to be base labile as one of the synthetic steps involves the use of trifluoroacetic acid. We chose the acetyl ($\text{CH}_3\text{CO}^-$) moiety as the protecting group, as like most other esters, it is reasonably stable under acidic conditions. The acetyl group can be introduced under mild conditions (acetic anhydride/pyridine) and can be removed under mild conditions (aqueous sodium hydroxide at room temperature). Our initial objective was therefore to synthesize the benzyl acetate (146a).
FIGURE 6.6 The proposed synthesis of (Rp)-2-(hydroxymethyl)-4-nitrophenyl [\(^{16}\text{O},^{18}\text{O}\)]thiophosphate (145a).
Synthesis of 2-hydroxy-5-nitrobenzyl acetate (146a)

(146a) was synthesized in a number of steps (Figure 6.7) and began with the reduction of the readily available starting material, 2-hydroxy-5-nitrobenzaldehyde (147). Sodium borohydride reduction of the aldehyde group in a mixture of methanol and dioxan at around 15-20°C proceeded smoothly to give the corresponding alcohol (148) in good yield (82%). The presence of the (primary) alcohol functionality was confirmed by $^1$H n.m.r., infra-red spectroscopy and mass spectrometry. (148) was purified by recrystallisation a number of times from water which gave hard yellow crystals. Our next aim was to protect both

\[
\begin{align*}
\text{OH} & \quad \text{CHO} \\
\text{NO}_2 & \quad \text{NaBH}_4 \\
\text{OH} & \quad \text{OH} \\
\text{NO}_2 & \quad (148)
\end{align*}
\]

\[
\text{Ac}_2\text{O} / \text{pyridine}
\]

\[
\begin{align*}
\text{OH} & \quad \text{OAc} \\
\text{NO}_2 & \quad \text{aq. Na}_2\text{CO}_3 \\
\text{OAc} & \quad \text{acetone} \\
\text{NO}_2 & \quad (149)
\end{align*}
\]

**FIGURE 6.7** Synthetic route to 2-hydroxy-5-nitrobenzyl acetate (146a).

hydroxy groups of (148) with a base labile group, preferably with an acetyl group. Acetylation was successfully achieved by stirring (148) in a mixture of acetic anhydride (6.5 eq) and pyridine at room temperature over a couple of hours, to give an almost quantitative yield (92%) of the di-acetylated product (149). Di-acetylation was confirmed
by $^1$H n.m.r. spectroscopy which showed no residual hydroxyl group but instead the expected two singlet resonances due to the methyl groups of the acetyl function [$\delta$(CDCl$_3$) 2.1 (-CH$_2$OC(O)CH$_3$), 2.4 (ArOC(O)CH$_3$)]; the presence of the two carbonyl groups in (149) was confirmed by infra-red spectroscopy. The next step in the synthetic sequence involved the selective removal of the phenolic acetyl group in (149). Aqueous sodium carbonate (in acetone) was found to be ideal in removing only the phenolic acetyl group and leaving the one protecting the primary alcohol intact. The phenolic acetyl group is selectively removed because the phenolate is a much better leaving group leading to a resonance-stabilized anion. However, one equivalent of base was found to be insufficient in deprotecting the phenolic acetyl group completely, as work-up not only yielded the desired product (146a) but also some starting material (149) as confirmed by t.l.c. and $^1$H n.m.r. spectroscopy. A larger amount of base (3 eq) was used and the hydrolysis monitored by t.l.c. for the disappearance of (149). Acid work-up followed by flash chromatography gave (146a) which recrystallised from water. The structure was confirmed by $^1$H n.m.r. and infra-red spectroscopy which showed that the benzylic acetyl group was still intact and that no signal attributable to the phenolic acetyl group was present. The structure of (146a) was further confirmed by high resolution mass spectrometry. A very small amount of the completely deprotected compound (148) was also isolated [separate from (146a)] from the silica column (<5%).

**Synthesis of (Rp)-2-(hydroxymethyl)-4-nitrophenyl [16O,18O]thiophosphate (145a)**

The Rp $^{18}$O-labelled enantiomer (145a) was synthesized using the stereocontrolled displacement reactions of cis (4S,5R)-2-chloro-3,4-dimethyl-5-phenyl-1,3,2-oxazaphospholidine-2-thione (88a) (Figure 6.8) to generate isotopic chirality at phosphorus. Having already synthesized (Rp)-4-nitrophenyl [16O,18O]thiophosphate (89a) in this way, we used our knowledge of experimental and reaction conditions gained in that study to prepare (145a).

(88a) Was prepared from (-)-ephedrine (53) and thiophosphoryl chloride (91) and isolated as the major epimer by flash chromatography (previously discussed in Chapter 2).
FIGURE 6.8 The synthesis of (Rp)-2-(hydroxymethyl)-4-nitrophenyl [\( ^{16}\text{O},^{18}\text{O} \)]-thiophosphate (145a).
The benzyl acetate (146a) was reacted with the chloro-adduct (88a) in the presence of triethylamine with dioxan as the solvent. Quite vigorous reaction conditions were employed (60°C, 50h.) as we know that associative nucleophilic substitution reactions of trisubstituted thiophosphates are very slow. Monitoring of the reaction by $^{31}$P n.m.r. spectroscopy showed the gradual appearance of just one product ($\delta +75.2 \rightarrow +77.2$ p.p.m.) which after work-up was purified by flash chromatography in 66% yield. The $^1$H n.m.r. spectrum of the purified product showed the presence of the oxazaphospholidine ring protons and the protons from the aryl group (146a); more importantly, it showed that the acetyl protecting group was still intact. Inch et al. have assigned the configurations of the phosphoramidic chlorides and esters (below) on the observed deshielding of protons H-4 and H-5 in a 1,3 cis relationship of the (thio)phosphoryl group. However, in our case, we cannot make assignments on this basis as we only have one epimer, but we know that the ester we isolated (150) has the cis configuration (spatial relationship between phenyl and aryl group designates configuration) since exocyclic displacement reactions at phosphorus held in a five-membered ring proceed with retention of configuration because of the strong preference for the ring to be placed axial-equatorial in the penta-coordinate intermediate.

This has been clearly shown in our own work (Chapter 2) in which 4-nitrophenolate reacted with cis (88a) exclusively with retention of configuration which was unambiguously proved by the X-ray crystal structure of the product. It is interesting to note that while the methylene protons in (146a) are magnetically equivalent, they are magnetically non-equivalent (diastereotopic) in (150) and one can see the AB splitting pattern in the $^1$H n.m.r. spectrum of (150).

$^{18}$O Label was introduced by hydrolyzing the P–N bond in H$_2^{18}$O [(98 atom %), diluted with H$_2^{16}$O 7:3 v/v] under acidic conditions (trifluoroacetic acid, 3.5 eq) to give the acyclic
thiophosphate diester (151). This acid-catalysed ring opening is (by analogy with the acid-catalysed alcoholysis reaction\(^ {17,131} \)) presumed to proceed by in-line displacement leading to inversion of configuration at phosphorus (as shown in Figure 6.8). (151) was formed in quantitative yield as judged by \( ^{31} \text{P} \) n.m.r. spectroscopy (\( \delta_p +77.2 \rightarrow +52.3 \) p.p.m.); the \( ^{1} \text{H} \) n.m.r. spectrum of (151) showed, as expected, no coupling between phosphorus and the \( N \)-methyl group and also that the acetyl group was still intact. The fact that the diester (151) has a conventional chiral centre at phosphorus which is attached to a chiral auxiliary (i.e. the ephedrine moiety) means that if both diastereoisomers had been produced, this would have been visible in the \( ^{31} \text{P} \) n.m.r. spectrum.

In order to release the isotopically chiral thiophosphate from (151), cleavage of the benzylic C–O bond (in the ephedrine moiety) is required. In general, there are many reagents which can effect C–O bond cleavage, however, in our case hydrogenolysis (using \( \text{H}_2/\text{Pd/C} \)) is inappropriate as the sulphur would poison the metal catalyst and the use of sodium in liquid ammonia is not wise as this can lead to Birch reduction of the nitrophenyl group and cleavage of the acetate group. Thus, the only suitable reagent that can cleave C–O bonds is a trialkylsilyl halide and in particular, trimethylsilyl iodide (TMSiI).\(^ {147} \) However, in our earlier study (Chapter 2), we found that after the initial reaction of (93)

\[
\begin{align*}
\text{Ph} & \quad \text{Me} \\
\text{O} & \quad \text{P} \quad \text{S} \\
\text{Me} & \quad \text{NH} \\
\text{Me} & \quad \text{OR}
\end{align*}
\]

(93)

\( R = \text{Ph}, \text{Ar} \)

with TMSiI, further reaction with TMSiI to effect C–O bond cleavage is very slow. This is because a P=S bond is much less reactive than a P=O bond in the initial silylation reaction.\(^ {148} \) The reluctance shown by thiophosphates to react with trialkylsilyl halides is due to the very low nucleophilicity of the P=S group towards silicon whereas the P=O
group has an exceptionally high nucleophilicity towards silicon as the resulting Si–O bond is very strong. This problem can be overcome by selectively methylating the sulphur prior to the addition of TMSil. This ensures that the silylation goes through the P=O bond intermediate (Figure 2.13, Chapter 2). The S-methylation of (151) [with methyl iodide (20 eq) at room temperature] shifted the $^{31}$P n.m.r. signal upfield ($\delta_{p} +52.3 \rightarrow +26.7$ p.p.m.) and the reaction was complete within an hour in a quantitative yield. C–O bond cleavage of (152) with 4 equivalents of TMSil was found to be complete in 2.5 hours, ($\delta_{p} +26.7 \rightarrow +36.4$ p.p.m.). This C–O bond cleavage reaction involves attack of the iodide ion on carbon (Figure 6.9) to eventually release the bis-(trimethylsilyl) ester (153) and, as such, this does not affect the stereochemistry at phosphorus.

The bis-(trimethylsilyl) ester (153) spontaneously hydrolysed on stirring in a mixture of aqueous sodium bicarbonate and mercaptoethanol to give the thiophosphate (154a). This was purified by ion-exchange chromatography on DEAE-Sephadex using triethylammonium bicarbonate (TEAB) eluant (50-250 mM, pH 7.6): the thiophosphate (154a) eluted at 210 mM buffer. Importantly, the $^1$H n.m.r. spectrum of this isolated product (154a) confirmed that the acetyl protecting group was still attached to the molecule. Although (154a) was isolated in a reasonable yield [73%, based on (150)], we expected a higher yield in view of the fact that each of the steps up to the hydrolysis of the bis-(trimethylsilyl) ester proceeded essentially quantitatively. As well as isolating (154a) from the ion-exchange column, a small amount of another compound was also isolated, eluting much earlier (80 mM buffer) than (154a). Its $^{31}$P ($^1$H-decoupled) n.m.r. chemical shift ($\delta_{p} +7.6$ p.p.m., singlet) suggested it was a P=S–X compound rather than a P=S one. It is conceivable that a small amount of free iodine present in the reaction mixture could have oxidized a small amount of (154) into the disulphide (155). Oxidation of thiophosphates to the corresponding disulphides has previously been reported by Eckstein\textsuperscript{94} using a variety of oxidizing agents, particularly hydrogen peroxide. Disulphides can be reduced back to the thiophosphates by mercaptoethanol or even sodium borohydride. However, neither of these reagents had any effect on the unknown compound, its $^{31}$P n.m.r. chemical shift remained

- 171 -
FIGURE 6.9 Mechanism of C—O bond cleavage of (151) with TMSiI to give thiophosphate (154a).
unchanged. Its $^1$H n.m.r. spectrum was not helpful in determining its structure as it was swamped by peaks from the triethylammonium counter ion. It is possible, and potentially very worrying that a small amount of (154) could undergo cleavage of the acetate group by iodide ion to give (156) which could conceivably cyclise to produce (157). It should be possible to confirm this structure as one should see phosphorus coupling to the methylene protons. However, no clear coupling pattern was discernible in the $^{31}$P ($^1$H-coupled) n.m.r. spectrum of the unknown compound as the signal-to-noise ratio was very low. The formation of a small amount of this unidentified by-product cannot be avoided as the minimum amount of TMSiI needed for completion of reaction is used.

Removal of the acetyl group from (154a) was achieved by hydrolysis in aqueous sodium hydroxide (4 eq) at room temperature. It is interesting to note that it was possible to follow this reaction by $^{31}$P n.m.r. spectroscopy (36.3 MHz; $\delta$ +44.2 → +44.1 p.p.m.) despite the
fact that one might have expected the starting material (154a) and product (145a) to have an identical $^{31}$P n.m.r. chemical shift. The hydrolysis was complete within 2 hours and the product was purified by ion-exchange chromatography under the same conditions as before. (145a) was isolated as the bis-triethylammonium ($\text{Et}_3\text{NH}$) salt (86%) and its $^1$H n.m.r. spectrum confirmed that the acetyl group had been removed. (145a) as its $\text{Et}_3\text{NH}$ salt is found to be quite stable in water and can be handled in this medium without any fear of decomposition.

As there is good precedent for the stereochemical courses of each of the key reactions just described from the work of Inch and his co-workers, the absolute configuration of $^{18}$O-labelled (145a) follows from the synthesis and has the Rp configuration. The fact that the P–N bond cleavage reaction gives only one diastereoisomer of (151) and that subsequent C–O bond cleavage has no stereochemical implications at phosphorus suggests that the product (145a) is of high enantiomeric purity.

Naturally, (145a) was taken through our configurational analysis, S-alkylation followed by O-benzoylation. However, the high-field $^{31}$P n.m.r. spectrum of the analysis of (145a) did not show the expected pattern as observed in Figure 3.14 (Chapter 3). Instead, the pattern shown in Figure 6.12 was observed. On the basis of the chemical shift and the $^{18}$O-labelling pattern, the products are assigned to the cyclic structures (158) and (159) arising from cyclisation during the configurational analysis as a result nucleophilic attack at phosphorus by the hydroxy group, with displacement of the benzoate group, as shown overleaf.

Although the cyclised products were not fully characterised, we believe they cannot have any other structure and besides, the cyclisation nicely explains the pattern observed in Figure 6.12. Resonances a and b must be assigned to the unlabelled triesters as resonance b is the only one to show a normal isotopic shift; resonance b shows a double bond $^{18}$O isotopic shift.

If the thiophosphate (145a) had been produced as a mixture of enantiomers, then we would also have expected to see $^{18}$O in the diastereoisomer of the cyclic structure (159).
fact, a small amount of the other $^{18}$O-labelled diastereoisomer is also present (resonance d) and therefore by comparing the intensities of resonances c and d, one can see that the enantiomeric purity of the thiophosphate synthesized (145a) is ~90%. Assignments have been made assuming the cyclisation proceeds with inversion of configuration for which there are good literature precedents.$^{17,131}$

Having prepared an isotopically chiral sample of (145a) which we knew to be of high enantiomeric purity, we were now in a position to study its thiophosphoryl transfer reaction in $\text{tert}$-butanol from a stereochemical point of view.
FIGURE 6.12 The high-resolution $^{31}$P n.m.r. spectrum (proton-decoupled, 121.5 MHz) of starting material (145a); derivatised by S-alkylation with myrtenyl bromide and O-benzoylation with benzoyl chloride.
The thiophosphoryl transfer reaction of (Rp)-2-(hydroxymethyl)-4-nitrophenyl [\textsuperscript{18}O,\textsuperscript{16}O] thiophosphate (145a)

Before we could embark on the stereochemical study, we needed to convert the triethylammonium (Et\textsubscript{3}NH) salt of (145a) into its tetra-n-butylammonium (Bu\textsubscript{4}N) counterpart as there is always a remote possibility that the tertiary amine can be involved as a nucleophilic catalyst in the thiophosphoryl transfer reaction. The use of the Bu\textsubscript{4}N counterion clearly avoids this problem. The Et\textsubscript{3}NH salt of (145a) was converted into its bis-Bu\textsubscript{4}N salt by titrating an aqueous solution of (145a) with 2 equivalents of tetra-n-butylammonium hydroxide (Bu\textsubscript{4}NOH). Again, as in the case of the bis-Bu\textsubscript{4}N salt of 4-nitrophenyl thiophosphate (Chapter 2), we found that when the aqueous solution of bis-Bu\textsubscript{4}N (145a) was evaporated down to complete dryness, it decomposed into inorganic thiophosphate (P\textsubscript{i}S) (77) and 2-(hydroxymethyl)-4-nitrophenolate (148). To prevent any decomposition of (145a) in this way, solutions were concentrated but not taken to dryness. To make sure that (145a) had not decomposed before its reaction in tert-butanol, its UV spectrum was checked (\(\lambda_{\text{max}} 315 \text{ nm}\)).

\[
\text{O}_2\text{N} - \text{O} \quad \text{O} \quad \text{P} \quad \text{S} \quad \text{O} \quad \text{OH} \quad \text{OH}
\]

(145)

\[
\text{O}_2\text{N} - \text{O} \quad \text{P} \quad \text{S} \quad \text{O} \quad \text{OH} \quad \text{OH}
\]

(148)

(77)

The thiophosphoryl transfer reaction of \textsuperscript{18}O-labelled (145) was conducted by rapid dilution into a large volume of tert-butanol (100 ml). Immediately upon addition, the reaction mixture turned from very light yellow in colour to bright yellow indicating the release of 2-(hydroxymethyl)-4-nitrophenolate (148). After a minute of mixing, a UV spectrum of the
reaction mixture was recorded which clearly showed the release of the chromophore (148) 
($\lambda_{\text{max}}$ 415 nm). After stirring the reaction mixture for 3 minutes, all of the t-butanol was 
evaporated under reduced pressure and a $^{31}\text{P}$ n.m.r. spectrum of the resulting yellow oil was 
recorded which showed three main resonances (see Figure 6.13). At this stage, assign-

![Diagram](image)

$^{31}\text{P}$ n.m.r. chemical shift ($\delta$) / p.p.m.

**FIGURE 6.13** The $^{31}\text{P}$ n.m.r. spectrum (36.3 MHz) of the reaction mixture after the 
reaction of (145a) in Bu'OH.

ment of the peaks is impossible as $^{31}\text{P}$ n.m.r. resonances can show considerable variations 
with solvent and pH. These components of the reaction mixture were, however, separated 
by ion-exchange chromatography on DEAE-Sephadex resin using a linear gradient of 
triethylammonium bicarbonate eluant (50–250 mM). Components from the column were 
identified by using a programmable multiwavelength detector at 210 nm. The first fraction 
isolated from the column was identified as t-butyl [$^{16}\text{O},^{18}\text{O}$ ]thiophosphate (160) [$\delta_{\text{H}}$ 
(CD$_3$OH) 1.4 (9H, s); $\delta_{\text{p}}$ +44.7 (s)] which eluted at a buffer concentration of 180 mM. The 
next fraction (eluting at 210 mM) contained a mixture of inorganic [$^{16}\text{O}_2,^{18}\text{O}$ ]thiophos-
phate (77) [identified by spiking the $^{31}\text{P}$ n.m.r. sample with an authentic sample; $\delta_{\text{p}}$
(CD$_3$OD) +36.8 (br s)] and 2-(hydroxymethyl)-4-nitrophenoate (148).

Having isolated two of the three expected products of the thiophosphoryl transfer reaction, the third product was eventually isolated after further elution of the ion-exchange column with a much higher concentration of buffer (500–700 mM). The $^1$H-coupled $^{31}$P n.m.r. spectrum of this sample showed a triplet (6p +47.3, $J_{PH} = 9$ Hz), indicative of 2-hydroxy-5-nitrobenzyl [ $^{16}$O,$^{18}$O ]thiophosphate (161), the expected product of the intra-

\[
\begin{align*}
\text{O}_2\text{N} & - \text{OH} \\
\text{O} & - \text{P} \\
\text{O} & - \text{OMe}
\end{align*}
\]

molecular transfer reaction of (145a). Derivatisation of (161) [S-alkylation with myrtenyl bromide ($R^*$Br) followed by $O$-methylation, see below] and followed by flash silica chromatography gave a much cleaner sample whose $^1$H n.m.r. spectrum [6$_H$ 5.2 (2H, br d, $J_{PH} = 9$ Hz)] further confirmed the structure (161).

\[
\begin{align*}
\text{O}_2\text{N} & - \text{OH} \\
\text{O} & - \text{P} \\
\text{O} & - \text{OMe}
\end{align*}
\]

The products of the thiophosphoryl transfer reaction, (160) and (161) were subjected to our newly developed configurational analysis described in Chapter 3. The $O$-substituted [ $^{16}$O,$^{18}$O ]thiophosphates can be configurationally analysed by $^{31}$P n.m.r. spectroscopy after S-alkylation with myrtenyl bromide followed by $O$-derivatisation with benzoyl chloride. For instance, when a mixture of 4-nitrophenyl [ $^{16}$O ]thiophosphate and (Rp)-4-nitrophenyl [ $^{18}$O ]thiophosphate (89) is taken through the analysis sequence, the resulting unlabelled diastereoisomeric Rp and Sp triesters (131a,b) are distinguishable by high-field $^{31}$P-(broad-band proton decoupled) n.m.r. (see Figure 3.14, Chapter 3). Since the absolute
configuration of the original monoester (89) follows from the synthesis, the absolute configurations of the diastereoisomeric triesters (131a,b) can be assigned on the basis of the magnitude of the $^{18}$O shift on each triester. On that basis, we see (Figure 3.14) a double bond $^{18}$O shift on the downfield diastereoisomer and a single bond $^{18}$O shift on the upfield diastereoisomer. Therefore, the downfield isomer has the Rp configuration. Similarly, in the configurational analysis of (Rp)-ethyl [$^{16}$O,$^{18}$O]thiophosphate, the downfield $^{31}$P n.m.r. resonance is assigned to the Rp diastereoisomer (see Figure 3.15, Chapter 3).

On the basis of the configurational analysis of (Rp)-4-nitrophenyl [$^{18}$O]thiophosphate and (Rp)-ethyl [$^{18}$O]thiophosphate, Figure 6.14 shows the expected high-field $^{31}$P n.m.r.
FIGURE 6.14 Expected high-field $^{31}$P n.m.r. spectra of (A) an (Rp)-[$^{18}$O]phosphate monoester, derivatised by $S$-alkylation with myrtenyl bromide and $O$-benzylation with benzoyl chloride and (B) an (Sp)-[$^{18}$O]phosphate monoester, derivatised as in (A).
spectra of a general $[^{18}\text{O}]$phosphate monoester, Rp and Sp. The closeness of the resonances will depend on the separation between the unlabelled Rp and Sp diastereoisomers (a and d). For instance, if this diastereomeric separation is less than the $\text{P}[^{18}\text{O}]$ isotopic shift ($\sim 5.7$ Hz) then the various isotopomers of the unlabelled diastereoisomers will overlap, making interpretation less straightforward.

The proposed configurational analysis of the products (160) and (161) and the expected high-field $^{31}\text{P}$ n.m.r. spectrum are shown in Figure 6.15 and 6.16a respectively.

**Configurational analysis of the products**

The stereochemical analysis of the t-butyl $[^{16}\text{O},^{18}\text{O}]$thiophosphate product (160) is shown in Figure 6.17. It shows that the unlabelled diastereoisomers are well separated ($\Delta \delta_{p} = 9$ Hz) which means that the various isotopomers do not overlap. Since two $^{18}\text{O}$ shifts are clearly evident on each of the unlabelled diastereoisomers, the product (160) obtained was largely racemic. In fact, the amount can be quantified from the relative intensities of the peaks (due to the isotopomers) in Figure 6.17 (note: configurational analysis does not depend on absolute intensities) and shows that (160) was completely racemic. As the reaction was conducted under mild conditions, one can safely assume that the product (160) is configurationally stable under these conditions and therefore that the racemisation arises during the thiophosphoryl transfer reaction. The spectrum in Figure 6.17 is identical to the one obtained after the configurational analysis of ethyl $[^{16}\text{O},^{18}\text{O}]$thiophosphate which was isolated from the ethanolysis of 4-nitrophenyl $[^{16}\text{O},^{18}\text{O}]$thiophosphate (see Figure 3.14, Chapter 3).

The other product, 2-hydroxy-5-nitrobenzyl $[^{16}\text{O},^{18}\text{O}]$thiophosphate (161), was subjected to the same configurational analysis, the resulting high-field $^{31}\text{P}$ n.m.r. spectrum is shown in Figure 6.18. One can immediately see that this spectrum does not conform to the pattern normally seen for thiophosphates analysed previously and differs from that predicted (see Figure 6.16b). Closer inspection reveals that the separation between the unlabelled diastereoisomers (resonances a' and d') is only $\sim 1.2$ Hz. As a $\text{P}[^{18}\text{O}]$ isotopic shift is around 5.2 Hz this means that the resonances of the various isotopomers of the
Retention (160) Inversion

Configurational analysis

i) R*Br
ii) PhCOCl

Retention

Inversion

Downfield Rp triesters

Upfield Sp triesters

Expected \(^{31}\text{P}\) n.m.r. (\(^{1}\text{H}\)-decoupled) if Retention = Inversion

a = unlabelled Rp triester
d = unlabelled Sp triester

Isotopic shifts

\(R^*\)

FIGURE 6.15 Proposed configurational analysis of tert-Butyl [\(^{16}\text{O}, ^{18}\text{O}\)]thiophosphate (160) and the expected \(^{31}\text{P}\) n.m.r. spectrum upon analysis.
FIGURE 6.16a Proposed configurational analysis of 2-hydroxy-5-nitrobenzyl [\textsuperscript{16}O, \textsuperscript{18}O]-thiophosphate (161).
Expected $^{31}$P n.m.r. ($^1$H-decoupled) if:

i) Retention $>$ Inversion

ii) Retention $<$ Inversion

\( a' \) = unlabelled Rp triester  
\( d' \) = unlabelled Sp triester

FIGURE 6.16b Expected $^{31}$P n.m.r. spectra of (161) upon configurational analysis.
FIGURE 6.17 The high-resolution $^{31}$P n.m.r. spectrum (proton-decoupled, 121.5 MHz) of $r$-butyl [16O, 18O]thiophosphate (160) isolated from the reaction of (145a) in $r$-butanol; derivatised by $S$-alkylation with myrtenyl bromide followed by $O$-benzoylation with benzoyl chloride.
FIGURE 6.18 The high-resolution $^{31}$P n.m.r. spectrum (proton-decoupled, 121.5 MHz) of 2-hydroxy-5-nitrobenzyl [$^{16}$O, $^{18}$O]thiophosphate (161) isolated from the reaction of (145a) in t-butanol; derivatised by S-alkylation with myrtenyl bromide followed by O-benzoylation with benzoyl chloride.
unlabelled diastereoisomers overlap, leading to a more complex spectrum.

If we look again at the predicted $^{31}$P n.m.r. ($^1$H-decoupled) spectra in Figure 6.16b, in the case where retention > inversion (spectrum i) if the separation between the unlabelled diastereoisomers (resonances a' and d') is small and that the Rp diastereoisomer (resonance a') is downfield (which we expect from our previous analysis of other thiophosphates) as below,

\[
\text{Rp diastereomeric triesters}
\]

\[
\text{Sp diastereomeric triesters}
\]

superimposing these lines gives a spectrum as below,

\[
\text{which is identical to the observed spectrum (Figure 6.18).}
\]

However, if we also consider the case where inversion > retention (Figure 6.16b, spectrum ii), then if the separation between the unlabelled diastereoisomers is again small
but that the Sp diastereoisomer is downfield, as below,

\[ Rp \text{ diastereomeric triesters} \]

\[ Sp \text{ diastereomeric triesters} \]

superimposing these lines gives a spectrum as below,

which is also identical to the observed spectrum (Figure 6.18). However, we have established precedent in all our previous configurational analysis of thiophosphates that the Rp diastereoisomer is always downfield and therefore that the spectrum in Figure 6.18 can be interpreted as arising from an excess of retention of configuration in the product (161). The assignments of the diastereoisomers are shown in Figure 6.18. This enantiomeric excess in the product (161) can be quantified from the stereochemically informative peaks (b', c', e' and f') and on the basis of this, 65% racemisation with 35% excess retention of configuration in the product is observed.
The inorganic thiophosphate (77) also isolated from the reaction, however, cannot provide any stereochemical information as it is not isotopically chiral.

**Conclusion**

The stereochemical results of the thiophosphoryl transfer reaction of (145a) in t-butanol are summarized below:

\[
\text{(145a)} \quad 0 \\
\text{BuOH} \quad \xrightarrow{\text{BuOH}} \quad \text{(146)} \quad \text{(161)}
\]

65% racemisation
35% excess retention

The observation that the thiophosphoryl group transfer from (145a) to t-butanol proceeds with complete racemisation is consistent with a dissociative (elimination-addition) reaction that proceeds through a liberated, symmetrically solvated thiometaphosphate intermediate (48). This planar intermediate can be attacked by the nucleophile equally from either face (see overleaf). This result is not surprising considering we have already shown that the ethanolysis of the dianion of (Rp)-4-nitrophenyl [\[^{16}O,^{18}O\]thiophosphate (89a) proceeds with complete racemisation (Chapter 4). This study provided the first direct evidence for a freely solvated monomeric thiometaphosphate intermediate in a hydroxylic solvent. We have also shown through a kinetic study (Chapter 5) that this intermediate has a finite lifetime in aqueous media. However, its oxy-counterpart, monomeric metaphosphate (2), is found to be too unstable (short-lived) to exist as a fully liberated species in water and most other hydroxylic solvents, t-butanol being a notable exception. In fact, Ramirez and Maracek\(^{48}\) have suggested that the phosphorylation of sterically hindered nucleophiles such as t-butanol is diagnostic of the intermediacy of monomeric metaphosphate. By analogy, thiophosphorylation of t-butanol in our latest study therefore provides evidence for the involvement of a thiometaphosphate species.
The partial racemisation observed in the other product (161) also indicates a dissociative thiophosphoryl transfer reaction via a relatively long-lived thiometaphosphate intermediate. Moreover, the accompanying excess retention of configuration in (161) suggests that the thiophosphoryl group is trapped by the internal hydroxyl nucleophile (intramolecular process) involving a caged thiometaphosphate intermediate rather than by the hydroxyl group of another molecule of (145a) (intermolecular process). If the thiophosphoryl transfer reaction to give (161) did involve an intermolecular pathway, then one would have expected the product (161) to be completely racemic as such a process would involve a "free" intermediate. Besides, as the reaction was conducted under dilute conditions employing a high concentration of \( t \)-butanol, any contribution from an intermolecular pathway would be minimal.

Thiophosphoryl transfer via an intramolecular "adjacent" associative pathway involving pseudorotation (see Figure 6.4) seems very unlikely as it would involve a high-energy trigonal bipyramidal intermediate because of the placement of anions in an apical position.
Besides, approach of a nucleophile at the phosphorus centre of a monosubstituted thiophosphate ester is severely limited on electrostatic grounds by the negative charges on the thiophosphate.

In seeking to demonstrate a front-side displacement reaction of (145a), the observation of excess retention of configuration in the product (161) provides good evidence that the internal nucleophile attacks the phosphorus atom from the same side as the leaving group.

Because of the proximity of the nucleophile to the phosphorus centre, one might have expected a higher level of excess retention. The level observed reflects the stability of the monomeric thiometaphosphate intermediate which as a consequence has time to tumble within the solvent cage before capture by the internal nucleophile (see below).
Although we envisage the racemisation and excess retention of configuration in the product to occur via a dissociative mechanism, the stereospecific component of the reaction could equally accord with a preassociative stepwise mechanism in which the rate-limiting transition state is predominately dissociative but the formation of the product requires the assembled presence of the acceptor nucleophile.

In conclusion, this study has shown that a dissociative (or a preassociative stepwise) mechanism can occur with retention of configuration if the nucleophile is constrained to attack the phosphorus centre on the same side as the leaving group. This provides the first example of a formally dissociative reaction proceeding with net retention of configuration.

On the basis of this model thiophosphoryl transfer reaction, it is apparent that for enzyme-catalysed reactions, in the absence of evidence for in-line geometry or the participation of a phosphoenzyme, stereochemical evidence alone may be ambiguous since a dissociative pathway can proceed with inversion, racemisation or retention! Although the dissociative pathway for phosphoryl transfer in water appears to be excluded, phosphoryl and thiophosphoryl transfer reactions in non-aqueous media appear to follow similar dissociative mechanisms. Whether an enzyme active site should be considered an aqueous environment or not is debatable.
EXPERIMENTAL
**General Experimental Details:**

**Materials and methods**

Unless otherwise stated all solvents, chemicals and enzymes were obtained from:
- Aldrich Chemical Company Ltd. (Gillingham, Dorset), Sigma Chemical Company Ltd. (Poole, Dorset) or Fisons Scientific Apparatus Ltd. (Loughborough).

**Solvents**

Solvents were purified following the methods of Perrin and Armarego.\(^\text{187}\)

Anhydrous methanol and ethanol were obtained by heating under reflux over magnesium and iodine and distillation under nitrogen.

Tetrahydrofuran (THF) and dioxan were purified by passing through an alumina column followed by distillation from sodium and benzophenone; the dried solvents were stored under nitrogen.

Acetonitrile, tert-butanol, dichloromethane and ethyl acetate were distilled from calcium hydride and then stored over 4Å molecular sieves.

Analytical grade acetone was used throughout.

N\(_2\)N-Dimethylformamide (DMF) was stirred with barium oxide and then distilled under reduced pressure (15 mmHg). It was then stored over 4Å molecular sieves.

Chloroform was shaken four or five times with half its volume of water, then dried over anhydrous calcium chloride for at least 24 hours, and then distilled.

Sodium-dried benzene was used throughout.

Diethyl ether (ether) was dried over sodium wire and then purified by reflux and distilling from lithium aluminium hydride. The solvent was stored over 4Å molecular sieves.

Light petroleum (40-60°C fraction) was dried over sodium wire and then distilled.

Pyridine and triethylamine were refluxed over potassium hydroxide pellets, then distilled and stored over 4Å molecular sieves.

Acetic anhydride was stored over phosphorus pentoxide, decanted and then distilled under reduced pressure (20 mmHg). It was stored under nitrogen.
De-ionized water was obtained from the Milli-Q reagent grade water system.

**Chemicals**

Adenosine-5'-diphosphate (free acid) was obtained from Boehringer Mannheim.

$^{18}$O-enriched water (98 atom %) was obtained from Amersham International.

Thiophosphoryl chloride was purified by distillation under reduced pressure (20 mmHg) and then stored under nitrogen.

**Instrumentations and method**

Melting points were determined using a Kofler hot stage apparatus and are reported uncorrected.

The pHs of aqueous solutions were measured with a Radiometer pH meter with a single glass electrode.

Ultra-Violet (UV) spectra were obtained using a Shimadzu UV-240 spectrophotometer.

Infra-Red (IR) spectra were recorded with a Perkin-Elmer 298 spectrometer.

Accurate and Fast Atom Bombardment (FAB) mass spectrometry was performed by the SERC Mass Spectrometry Centre, University College of Swansea, [Chemical Ionisation (C.I.) was carried out using ammonia]. Standard mass spectra [Electron Ionisation (E.I.)] were obtained with a Micromass 16B spectrometer.

Microanalysis was performed by Butterworth Laboratories, Teddington, Middlesex.

Routine $^1$H n.m.r. spectra were recorded with a Varian EM-390 (90 MHz) spectrometer. High-field $^1$H n.m.r. spectra were obtained using a Bruker AM-300 (300 MHz) spectrometer. For all $^1$H n.m.r. spectra, tetramethylsilane (TMS) was used as an internal standard.

Routine $^{31}$P n.m.r. spectra ($^1$H-decoupled unless otherwise stated) were recorded at 36.3 MHz with a JEOL JNM-FX 90Q spectrometer and high-field spectra were recorded at 121.5 MHz with the Bruker AM-300 spectrometer; positive chemical shifts are downfield from external standard tetrahydroxyphosphonium ion [P(OH)$_4^+$].

Flash chromatography was carried out according to the method of Still et al.$^{188}$ using
silica (Merck & Co., Kiesel 60, 230-400 mesh). Thin layer chromatography (t.l.c.) was conducted on precoated aluminium sheets (60F-254) with a 0.2 mm layer thickness manufactured by Merck & Co.

Anion ion-exchange chromatography was carried out using DEAE-Sephadex A-25 resin (Pharmacia Fine Chemicals) using stated linear concentration gradients of triethyl-ammonium bicarbonate buffer (TEAB) which was prepared by adding the appropriate volume of triethylamine to the appropriate volume of water and then bubbling gaseous carbon dioxide through the mixture at 20°C until it reached pH ~7.6. The eluate from the column was monitored continuously using a spectrophotometer attached to the column. The buffer was removed from product fractions by the addition and then evaporation under reduced pressure of three or four aliquots of methanol.
Preparation of 4-nitrophenyl [\(^{16}\)O]thiophosphate (89) via the ephedrine route

![Chemical structure of 4-nitrophenyl (89)](image)

Synthesis of cis (4S,5R)-2-chloro-3,4-dimethyl-5-phenyl-1,3,2-oxazaphospholidine-2-thione (88a).

\[
\text{Me} \quad \text{Ph} \quad \text{OH} + \quad \text{P(S)Cl}_3 \quad \text{Et}_3\text{N} \quad \text{Me} \quad \text{Me} \quad \text{Cl} \quad \text{S} \\
\text{cis} \quad \text{trans}
\]

(88a) was prepared by the method of Inch et al.\(^{131}\) A solution of thiophosphoryl chloride (91) (4.1 ml, 41 mmol) in benzene (25 ml) was slowly added to a suspension of (−)-ephedrine hydrochloride (53) (8.2 g, 50 mmol) in triethylamine (34.5 ml, 247 mmol) and benzene (150 ml). The white suspension was stirred under nitrogen for 15 hours at room temperature and then poured into an excess of water. The benzene layer was isolated and the aqueous layer extracted with more benzene (3 x 50 ml), the combined extracts were dried over Na\(_2\)SO\(_4\), filtered and concentrated. The resulting light brown oil was purified by silica flash chromatography [light petroleum – chloroform (3:1), \(R_f = 0.42\)]. The product-containing fractions were evaporated and the resulting off-white solid was recrystallised from di-isopropyl ether to give the cis epimer (88a) as shiny white crystals (5.7 g, 53%), m.p. 124-126°C (Lit.,\(^{131}\) 125-128°C); \(^1\)H n.m.r. \(\delta(300\text{ MHz, CDCl}_3) 0.85\text{ p.p.m. (3H, d, } J = 7\text{ Hz, CH}_3)\), 2.85 (3H, d, \(3J_{PH} = 15\text{ Hz, NCH}_3\)), 3.84 (1H, dqn, \(J = 6.7\text{ Hz, } 3J_{PH} = 29\text{ Hz, H-4}\)), 5.81 (1H, br d, \(J = 6.7\text{ Hz, H-5}\)), 7.32 (5H, s, Ph); \(^{31}\)P n.m.r. \(\delta(36.3\text{ MHz, CDCl}_3) +75.2\text{ p.p.m. (s).}\)

Concentration of the mother liquor gave the trans epimer (88b) (0.7 g, 6%), m.p. 57°C (Lit.,\(^{131}\) 58°C); \(^1\)H n.m.r. \(\delta(300\text{ MHz, CDCl}_3) 0.79\text{ p.p.m. (3H, d, } J = 7\text{ Hz, CH}_3\)), 2.25 (3H,
\( \text{d, } ^3J_{\text{PH}} = 18 \text{ Hz, NCH}_3 \), 3.72 (1H, dqn, \( J = 7 \) Hz, \( ^3J_{\text{PH}} = 13 \) Hz, H-4), 5.60 (1H, dd, \( J = 7 \) Hz, \( ^3J_{\text{PH}} = 6.7 \) Hz, H-5), 7.20 (5H, m, Ph); \(^{31}\text{P n.m.r. } \delta(36.3 \text{ MHz, CDCl}_3) +80.3 \text{ p.p.m. (s).}

**Synthesis of cis (4S,5R)-2-(4-nitrophenoxy)-3,4-dimethyl-5-phenyl-1,3,2-oxazaphospholidine-2-thione (92)**

\[ \text{Ph} \]  
\[ \text{Me} \]  
\[ \text{Me} \]  
\[ \text{Me} \]  
\[ \text{N} \]  
\[ \text{Ph} \]  
\[ \text{O} \]  
\[ \text{Cl} \]  
\[ \text{P} \]  
\[ \text{S} \]  
\[ \text{Me} \]  
\[ \text{O} \]  
\[ \text{NO}_2 \]  
\[ (88a) \]

\[ \text{Me} \]  
\[ \text{N} \]  
\[ \text{Ph} \]  
\[ \text{O} \]  
\[ \text{P} \]  
\[ \text{S} \]  
\[ \text{Me} \]  
\[ \text{O} \]  
\[ \text{NO}_2 \]  
\[ (92) \]

To a solution of 4-nitrophenol (0.29 g, 2.1 mmol) in THF (15 ml) was added triethylamine (0.29 ml, 2.1 mmol) followed by the chloro derivative \( (88a) \) (0.5 g, 1.9 mmol) in THF (20 ml) over 5 minutes at 20°C under nitrogen. The mixture was then heated to 60°C for 48 hours after which time it was allowed to cool to 20°C. The THF was diluted with a large volume of water and extracted with dichloromethane (3 x 40 ml) and the combined organic extracts were washed with water (2 x 50 ml), dried over MgSO\(_4\), filtered and evaporated under reduced pressure to give a pale yellow oil which was purified by flash chromatography [dichloromethane – light petroleum (3:2), \( R_f = 0.35 \)]. This gave the required product (92) as a clear oil (4.85 g, 83%) which was recrystallised from di-isopropyl ether to give large colourless crystals, m.p. 61-62°C; \(^1\text{H n.m.r. } \delta(300 \text{ MHz, CDCl}_3) 0.85 \text{ p.p.m. (3H, d, } J = 6.6 \text{ Hz, CH}_3\), 2.90 (3H, d, \( ^3J_{\text{PH}} = 12.3 \) Hz, N-CH\(_3\)), 3.80 (1H, dqn, \( J = 6.3 \) Hz, \( ^3J_{\text{PH}} = 18 \) Hz, H-4), 5.8 (1H, dd, \( J = 6.3 \) Hz, \( ^3J_{\text{PH}} = 3 \) Hz, H-5), 7.25 (7H, br m, Ph, Ar), 8.10 (2H, d, \( J = 10 \) Hz, Ar); \(^{31}\text{P n.m.r. } \delta(36.3 \text{ MHz, CDCl}_3) +77.2 \text{ p.p.m. (s).}

**P-N bond cleavage of (92) using trifluoroacetic acid/water**

\[ \text{Ph} \]  
\[ \text{Me} \]  
\[ \text{Me} \]  
\[ \text{Me} \]  
\[ \text{N} \]  
\[ \text{PH} \]  
\[ \text{O} \]  
\[ \text{P} \]  
\[ \text{S} \]  
\[ \text{Me} \]  
\[ \text{O} \]  
\[ \text{NO}_2 \]  
\[ (92) \]

\[ \text{H}_1 \]  
\[ \text{H}_2 \]  
\[ \text{NH} \]  
\[ \text{P} \]  
\[ \text{S} \]  
\[ \text{Me} \]  
\[ \text{O} \]  
\[ \text{NO}_2 \]  
\[ (93) \]
The P–N bond cleavage of the cis diester amidate (92) is based on the method reported by Knowles et al.\textsuperscript{51} To a solution of (92) (1 g, 2.75 mmol) in dry dioxan (2 ml) was added a solution of trifluoroacetic acid (0.64 ml, 8.25 mmol, 3 eq) in water (0.8 ml) and the mixture was stirred at 20°C. After 1 hour, the \( ^{31}P \) n.m.r. spectrum of the mixture showed no starting material remained (\( \delta p +77.2 \rightarrow +55.2 \) p.p.m.). Removal of the solvent by evaporation under reduced pressure was followed by co-evaporation of the residue from dry dioxan (6 x 3 ml) to give the diester (93) as its zwitterion. \( ^{1}H \) n.m.r. \( \delta(90 \text{ MHz, CCl}_3) \) 1.2 p.p.m. (3H, d, J = 7 Hz, \( \text{CH}_3 \)), 3.0 (3H, s, N\( \text{CH}_3 \)), 4.3 (1H, dq, J = 7 Hz, H-2), 5.1 (2H, br s, NH\( \text{H}_2 \)), 6.2 (1H, dd, J = 7 Hz, \( ^{3}J_{\text{PH}} = 3 \) Hz, H-1), 7.3 (7H, br m, Ph, Ar), 8.0 (2H, d, J = 9 Hz, Ar); \( ^{31}P \) n.m.r. \( \delta(36.3 \text{ MHz, CCl}_3) +55.2 \) p.p.m. (s).

\textbf{S-Methylation of (93) using methyl iodide}

\[ \begin{array}{c}
\text{Ph} \quad \text{O} \quad \text{S} \\
\text{Me} \quad \text{NH} \quad \text{Me} \\
\text{Me} \\
\text{O} \\
\text{NO}_2
\end{array} \xrightarrow{\text{MeI}} \begin{array}{c}
\text{Ph} \quad \text{O} \quad \text{SMe} \\
\text{Me} \quad \text{NH} \quad \text{Me} \\
\text{SMe} \\
\text{O} \\
\text{NO}_2
\end{array} \]

(93) \quad (100)

The diester (93) as its zwitterion was dissolved in chloroform (2 ml) and DMF (0.1 ml) and treated with methyl iodide (3.4 ml, 55 mmol, 20 eq) at 20°C. After 1 hour, a pale yellow solution was obtained and \( ^{31}P \) n.m.r. spectroscopy showed no starting material, (\( \delta p +55.2 \rightarrow +27.8 \) p.p.m.). The solvent and excess methyl iodide were evaporated under reduced pressure to leave a yellow oil which was dried by co-evaporation with chloroform (2 x 5 ml). The S-methyl derivative (100) was isolated as a yellow foam. \( ^{1}H \) n.m.r. \( \delta(90 \text{ MHz, CCl}_3) \) 1.2 p.p.m. (3H, d, J = 7 Hz, \( \text{CH}_3 \)), 2.3 (3H, d, \( ^{3}J_{\text{PH}} = 28 \) Hz, \( \text{SCH}_3 \)), 3.0 (3H, s, N\( \text{CH}_3 \)), 4.3 (1H, dq, J = 7 Hz, H-2), 5.1 (2H, br s, NH\( \text{H}_2 \)), 6.3 (1H, dd, J = 7 Hz, \( ^{3}J_{\text{PH}} = 3 \) Hz, H-1), 7.2 (7H, br m, Ph, Ar); \( ^{31}P \) n.m.r. \( \delta(36.3 \text{ MHz, CCl}_3) +27.8 \) p.p.m. (s).
C–O bond cleavage of S-methylated compound (100) using trimethylsilyl iodide

To a solution of the S-methyl derivative (100) (iodide salt) in chloroform (10 ml) was added trimethylsilyl iodide (1.34 ml, 9.63 mmol, 3.5 eq) at 20°C under nitrogen. The resulting dark brown mixture was stirred for 2 hours, by which time $^3P$ n.m.r. showed the reaction to be complete, ($\delta$ +27.8 → +41.4 p.p.m.). The solvent and excess trimethylsilyl iodide were evaporated under reduced pressure and the deep red oil obtained was dissolved in a solution of sodium bicarbonate (1.39 g, 16.5 mmol) in water (5 ml) and mercaptoethanol (0.3 ml). A bright yellow solution was obtained which was diluted into water (100 ml) and applied to an ion-exchange column (150 ml) using DEAE-Sephadex A-25 resin. The aqueous solution of the product was applied to the top of the column at a rate of 60 ml per hour. A linear gradient of increasing ionic strength aqueous triethylammonium bicarbonate (TEAB) buffer was applied to the column from 50 mM to 250 mM over 20 hours (total volume of eluant used 1000 ml). The eluate from the column was continuously monitored by a UV detector at $\lambda$ 310 nm and collected in fractions (10 ml).

One major peak was seen on the UV chromatogram. The appropriate fractions were combined and concentrated by evaporation under a high vacuum (using a cold-finger rotary evaporator). Final traces of TEAB buffer were removed by repeated co-evaporation with methanol (5 x 5 ml). 4-Nitrophenyl [¹⁶O]thiophosphate (89) (bis-triethylammonium salt) eluted at 190 mM buffer strength and was isolated as a yellow gum (0.83 g, 69%); $^1H$ n.m.r. $\delta$(90 MHz, CD$_3$OD) 1.3 p.p.m. (19H, t, $J$ = 7 Hz, NCH$_2$CH$_3$), 3.2 (12H, q, $J$ = 7 Hz, NCH$_2$CH$_3$), 7.5 (2H, d, $J$ = 9 Hz, Ar), 8.1 (2H, d, $J$ = 9 Hz, Ar); $^{31}P$ n.m.r. $\delta$(32.3 MHz, CD$_3$OD) +40.2 p.p.m. (s).
Preparation of (Rp)-4-nitrophenyl [\(^{16}\text{O},^{18}\text{O}\)]thiophosphate (89a) via the ephedrine route

\[
\begin{align*}
\text{Me} & \quad \text{O} \quad \text{P} \quad \text{O} \\
\text{Ph} & \quad \text{Me} \quad \text{N} \quad \text{Me} \\
\text{O}_2\text{N} & \quad \text{P} \quad \text{O} \\
\text{O}_2\text{N} & \quad \text{P} \quad \text{O} \\
\text{SN} & \quad \text{O} \quad \text{Et} \\
\text{R} \quad \text{p} & \quad \text{O} \\
\end{align*}
\]

(92)

The above compound was prepared in the same way as the unlabelled material, using (92) (1 g, 2.75 mmol) as starting material and H\(_2\){\(^{18}\)O} (1 ml, 98 atom %). This gave the labelled thiophosphate (89a) (0.78 g, 65%) as the bis-triethylammonium salt.

Preparation of ethyl [\(^{16}\text{O}\)]thiophosphate (90) via the ephedrine route

\[
\begin{align*}
\text{EtO} & \quad \text{P} \\
\text{S} & \quad \text{O} \\
\text{O} & \quad \text{O} \\
\end{align*}
\]

(90)

Synthesis of cis (4S,5R)-2-ethoxy-3,4-dimethyl-5-phenyl-1,3,2-oxazaphospholidine-2-thione (103).

\[
\begin{align*}
\text{Me} & \quad \text{N} \quad \text{Me} \\
\text{Ph} & \quad \text{O} \quad \text{P} \quad \text{Cl} \\
\text{Me} & \quad \text{N} \quad \text{Me} \\
\text{S} & \quad \text{P} \quad \text{O} \quad \text{Et} \\
\text{EtOH} & \quad \text{O} \quad \text{P} \quad \text{O} \\
\text{Me} & \quad \text{N} \quad \text{Me} \\
\end{align*}
\]

(88a)

(103)

To a solution of the cis chloro adduct (88a) (1 g, 3.8 mmol) in ethanol (5 ml) was added a solution of sodium ethoxide [3.8 mmol, made from dissolving sodium (87 mg) in ethanol (0.22 ml)] in ethanol (5 ml) at 20°C. The mixture was stirred for 20 hours and then the reaction was quenched with water and extracted with chloroform (3 x 20 ml). The organic extracts were combined and dried over Na\(_2\)SO\(_4\), filtered and the solvent removed to give a clear oil which was purified by flash chromatography. Elution with chloroform – light petroleum (1:1) (R\(_f\) = 0.5) gave the title compound (103) (0.76 g, 74%) which recrystallised from propan-2-ol as colourless needles, m.p. 73-74°C (Lit.,\(^{131}\) 74-76°C); \(^1\)H n.m.r. \(\delta(90\)
MHz, CDCl₃) 0.8 p.p.m. (3H, d, J = 7 Hz, CH₃), 1.1 (3H, t, J = 7 Hz, OCH₂CH₃), 2.8 (3H, d, J = 12 Hz, NCH₃), 3.6 (1H, m, H-4), 3.8 (2H, m, OCH₂CH₃), 5.8 (1H, dd, J = 7 Hz, ³JPH = 3Hz, 5-H), 7.3 (5H, s, Ph); ³¹P n.m.r. 8(36.3 MHz) +83.1 p.p.m. (s), [¹H-coupled (m)].

P–N bond cleavage of (103) using trifluoroacetic acid/water

![Diagram](image)

The above compound (103) (136 mg, 0.5 mmol) was dissolved in dioxan (0.5 ml) and to this was added a solution of trifluoroacetic acid (116 μl, 1.5 mmol, 3 eq) in water (180 μl) at 20°C. The mixture was stirred for 1 hour, by which time ³¹P n.m.r. spectroscopy showed the reaction to be complete, (δp +83.1 → +59.5 p.p.m.). Solvent removal gave the product (104) as a white solid which was dried by co-evaporation of water with dioxan (5 x 3 ml).

C–O bond cleavage of (104) using sodium/liquid ammonia

![Diagram](image)

The above diester (104) as its zwitterion was dissolved in dioxan (5 ml) and added to a solution of sodium (46 mg, 2 mmol) in liquid NH₃ (10 ml) (distilled from sodium) at -78°C under nitrogen. After stirring the mixture for 5 minutes the reaction was quenched with an excess of ammonium chloride and the ammonia evaporated under a stream of nitrogen. The recovered white solid was dissolved in water (100 ml) and applied to a DEAE-Sephadex ion-exchange column (150 ml). A gradient of 50 mM - 200 mM–TEAB (total vol. 1000 ml, pH 7.6) was applied at a rate of 60 ml per hour and fractions collected with continuous
monitoring at 220 nm. Ethyl [\textsuperscript{16}O]thiophosphate (90) was eluted at 130 mM buffer concentration as the bis-triethylammonium salt (99 mg, 58%); \textsuperscript{1}H n.m.r. \(\delta(300\ \text{MHz, CD}_3\text{OD})\) 1.3 p.p.m. (22H, m, OCH\textsubscript{2}CH\textsubscript{3}, NCH\textsubscript{2}CH\textsubscript{3}), 3.4 (12H, q, \(J = 7\ \text{Hz, NCH}_2\text{CH}_3\)), 4.0 (2H, dq, \(J = 7\ \text{Hz, }^3J_P = 10\ \text{Hz, OCH}_2\text{CH}_3\)); \textsuperscript{31}P n.m.r. \(\delta(36.3\ \text{MHz, CD}_3\text{OD}) +42.8\ \text{p.p.m. (s), [}^1\text{H-coupled (t, }^3J_{PH} \sim 10\ \text{Hz}].\)

**Preparation of (Rp)-ethyl [\textsuperscript{16}O,\textsuperscript{18}O]thiophosphate (90a) via the ephedrine route**

(90a) was prepared according to the method for the unlabelled material, using (103) (136 mg, 0.5 mmol) and H\textsubscript{2}\textsuperscript{18}O (180 \mu l). This gave \textsuperscript{18}O-labelled ethyl thiophosphate (90a) (89 mg, 52%).

**Preparation of 4-nitrophenyl thiophosphate (89) (unlabelled)**

*Synthesis of 4-nitrophenyl thiophosphoryl dichloride (136)*

\[
\text{O}_2\text{N} - \text{OH} + \text{P(S)Cl}_3 \rightarrow \text{O}_2\text{N} - \text{O-PCl}_3
\]

This was prepared by the method of Tolkmith.\textsuperscript{172} Thiophosphoryl chloride (51 ml, 0.5 mol) was dissolved in dichloromethane (25 ml) and pyridine (8.4 ml, 0.1 mol) at 4-8°C, to which was added a solution of 4-nitrophenol (13.9 g, 0.1 mol) in ether (50 ml) over a period of 5 hours. The reaction mixture was stirred overnight at room temperature and the resulting mixture was filtered and the filtrate evaporated under reduced pressure. Cyclohexane was added to the resulting light orange oil and evaporated in the same manner. The residue was extracted with light petroleum (5 x 30 ml) and the hydrocarbon solution was evaporated to leave a crude white solid. Recrystallisation of this solid from hexane...
gave the dichloride product (136) as white needles (14.1 g, 52%), m.p. 52-54°C (Lit.,\textsuperscript{172} 53-54°C); \textsuperscript{1}H n.m.r. δ(90 MHz, CDCl\textsubscript{3}) 7.5 p.p.m. (d, J = 9 Hz, Ar), 8.2 (d, Ar); \textsuperscript{31}P n.m.r. δ(36.3 MHz, CDCl\textsubscript{3}) +52.8 p.p.m. (s).

**Hydrolysis of 4-nitrophenyl thiophosphoryl dichloride (136)**

\[
\begin{align*}
\text{O}_2\text{N} & \text{O} \quad \text{P} \quad \text{S} \\
\text{Cl} & \quad \text{Cl} \\
\rightarrow \\
\text{O}_2\text{N} & \text{O} \quad \text{P} \quad \text{O} \\
\text{S} & \\
\end{align*}
\]

4-Nitrophenyl thiophosphoryl dichloride (136) was hydrolysed by the method of Breslow and Katz.\textsuperscript{85} The dichloride (136) (1 g, 3.7 mmol) was dissolved in dioxan (6 ml), to which was added 2 M–NaOH (7.3 ml, 14.6 mmol, 4 eq) and the resulting bright yellow solution was stirred for 20 minutes. The required product was diluted into water (100 ml) and purified by ion-exchange chromatography (DEAE-Sephadex) (180 ml). The column was eluted with a linear gradient of TEAB buffer (50 mM - 250 mM, vol. 2000 ml) and the eluate monitored spectrophotometrically at 310 nm. The product eluted at 190 mM buffer strength, and the fractions combined and concentrated to give the product, 4-nitrophenyl thiophosphate (bis-triethylammonium salt) (89a) as a yellow gum (1.3 g, 83%); \textsuperscript{1}H n.m.r. δ(90 MHz, CD\textsubscript{3}OD) 1.3 p.p.m. (20H, t, J = 7 Hz, NCH\textsubscript{2}CH\textsubscript{3}), 3.2 (13H, q, J = 7 Hz, NCH\textsubscript{2}CH\textsubscript{3}), 7.5 (2H, d, J = 9 Hz, Ar), 8.1 (2H, d, J = 9 Hz, Ar); \textsuperscript{31}P n.m.r. δ(36.3 MHz, CD\textsubscript{3}OD) +42.3 p.p.m. (s).

**Preparation of ethyl thiophosphate (90) (unlabelled)**

\[
\begin{align*}
\text{EtOH} & + \text{P(S)Cl}_3 \\
& \xrightarrow{\text{NaOH}} \\
& \text{EtO}_2\text{P} \quad \text{S} \\
& \quad \text{O} \\
& \end{align*}
\]

Thiophosphoryl chloride (1.96 ml, 19.3 mmol) and dry ethanol (1.16 ml, 19.8 mmol) were dissolved in dry dioxan (20 ml). Triethylamine (2.8 ml, 20 mmol) was added to this
colourless solution at 0°C with vigorous stirring under nitrogen. The resulting white suspension was stirred for 2.5 hours at room temperature, after which time the mixture was quenched with 2 M-NaOH (40 ml). The mixture was stirred until a homogeneous solution was produced and then acidified to pH < 1 with 2 M-HCl. The product was extracted with ethyl acetate (4 x 40 ml) and the combined organic extracts were dried (MgSO₄) and evaporated under reduced pressure. The resulting colourless oil was dissolved in ether (20 ml) and the product precipitated out by addition of triethylamine (2.79 ml, 20 mmol) at 0°C. The product, mono-triethylammonium salt of ethyl thiophosphate (90), was isolated as a white solid by filtration (2.56 g, 58%); ¹H n.m.r. δ(90 MHz, CD₃OD) 1.2 p.p.m. (13H, m, OCH₂CH₃, NCH₂CH₃), 3.5 (7H, q, J = 7 Hz, NCH₂CH₃), 4.0 (2H, dq, J = 7 Hz, 3J₀H = 10 Hz, OCH₂CH₃); ³¹P n.m.r. δ(36.3 MHz, CD₃OD) +48.2 p.p.m. (s), ¹H-coupled (t, J₀H = 10 Hz).

Development of configurational analysis method for thiophosphate monoesters:
Preparation of myrtenyl bromide (126)

(126) was prepared by the method of Kay and Trippett. To a solution of (-)-myrtenol (162) (5 g, 33 mmol) in light petroleum (100 ml) under nitrogen stirred at 0°C was added a solution of phosphorus tribromide (3.1 ml, 33 mmol) in light petroleum (100 ml) over a period of 30 minutes. After allowing the reaction mixture to warm to room temperature over a 3 hour period, the colourless solution was washed with ice cold 0.8 M-potassium carbonate solution (3 x 40 ml). The organic layer was separated, dried (MgSO₄), filtered and evaporated under reduced pressure. The resulting light orange oil was Kugelröhr distilled to give the product (126) as a colourless oil (4.7 g, 67%), b.p. 109-111°C (at 2 mmHg); ¹H n.m.r. δ(90 MHz, CDCl₃) 0.8 p.p.m. (3H, s, CH₃), 1.2 (1H, d, J = 8 Hz), 1.3 (3H, s, CH₃), 1.9-2.5 (5H, m), 3.8 (2H, s, CH₂Br), 5.6 (1H, br s, R₂C=CRH).
S-Alkylation of thiophosphate monoesters:

**Preparation of S-myrtanyl-(4-nitrophenyl) thiophosphate (128)**

![Chemical structure](image)

4-Nitrophenyl thiophosphate (bis-triethylammonium salt) (89) (43.7 mg, 100 μmol) was dissolved in methanol (0.5 ml) and triethylamine (14 μl, 100 μmol). Myrtenyl bromide (126) (18 μl, 100 μmol) was added and the reaction mixture stirred for 5 minutes at room temperature by which time the reaction was shown to be complete by $^{31}$P n.m.r. spectroscopy, ($\delta$ +41.9 → +15.2 p.p.m.). Filtration and solvent removal gave the title compound (128) as the mono-triethylammonium salt. $^1$H n.m.r. $\delta$(90 MHz, CD$_3$OD) 3.4 p.p.m. (2H, d, $^3$J$_{PH}$ = 12 Hz, SCH$_2$); $^{31}$P n.m.r. $\delta$(36.3 MHz, CD$_3$OD) +15.2 p.p.m. (s).

**Preparation of S-myrtanyl-ethyl thiophosphate (132)**

![Chemical structure](image)

The above compound was prepared by the same method as the 4-nitrophenyl derivative (128) using ethyl thiophosphate (mono-triethylammonium salt) (90) (100 μmol). $^1$H n.m.r. $\delta$(90 MHz, CDCl$_3$) 3.4 p.p.m. (2H, d, $^3$J$_{PH}$ = 12 Hz, SCH$_2$), 3.9 (2H, dq, OCH$_2$CH$_3$); $^{31}$P n.m.r. $\delta$(36.3 MHz, CDCl$_3$) +18.2 p.p.m. (s).
**O-Derivatisation of S-alkylated thiophosphate diesters:**

**Preparation of O-methyl-S-myrtanyl-(4-nitrophenyl) thiophosphate (129)**

\[
\text{O}_2\text{N} - \text{O} - \text{P} \quad \text{Me}_2\text{SO}_4 \rightarrow \quad \text{O}_2\text{N} - \text{O} - \text{P} \\
\text{(128)} \quad \text{(129)}
\]

S-myrtanyl-(4-nitrophenyl) thiophosphate (mono-triethylammonium salt) (128) (47 mg, 100 µmol) was dissolved in DMF (0.2 ml) and dimethyl sulphate (0.19 ml, 2 mmol, 20 eq) along with triethylamine (55.6 µl, 400 µmol) were added. The mixture was stirred for 3 hours at room temperature, by which time the reaction was complete as judged by \(^{31}\text{P}\) n.m.r. analysis. The reaction mixture was poured into an excess of water and extracted with ether (3 x 8 ml). The combined ether extracts were dried (MgSO\(_4\)), filtered and evaporated under reduced pressure. The resulting light orange oil was purified by flash chromatography (chloroform - 1% methanol, R\(_f\) = 0.25) to give the methyl derivative (129) as a light yellow oil (32 mg, 86%); \(^1\text{H}\) n.m.r. \(\delta\)(300 MHz, CDCl\(_3\)) 0.8 p.p.m. (3H, s, MyrCH\(_3\)), 1.1 (1H, m, MyrH), 1.2 (3H, s, MyrCH\(_3\)), 2.0-2.4 (5H, m, MyrH), 3.5 (2H, m, SCH\(_2\)), 3.8 (3H, d, \(\text{J}_{\text{PH}} = 12.8\) Hz, OCH\(_3\)), 5.6 (1H, br s, R\(_2\)C=CRH), 7.4 (2H, d, J = 8.9 Hz, Ar), 8.2 (2H, d, J = 8.9 Hz, Ar); \(^{31}\text{P}\) n.m.r. \(\delta\)(121.5 MHz, CDCl\(_3\)) +27.23 p.p.m. (s), +27.31 (s), diastereomeric separation = 9.4 Hz (CDCl\(_3\)); m/z (C.I.) 401 ([M + NH\(_4\)]\(^{+}\); 100%), 384 ([M + H]\(^{+}\); 92), 135 (Myr\(^{+}\); 80), (Found: [M + H]\(^{+}\) 384.1035, C\(_{17}\)H\(_{22}\)O\(_3\)NPS + H\(^{+}\) requires 384.1034).

**Preparation of O-methyl-S-myrtanyl-ethyl thiophosphate (133)**

\[
\text{EtO} - \text{P} \quad \text{Me}_2\text{SO}_4 \rightarrow \quad \text{EtO} - \text{P} \\
\text{(132)} \quad \text{(133)}
\]

The above compound (133) was prepared by the same method as the 4-nitrophenyl derivative using 100 µmol of (132). The crude product was purified by flash chromatography (chloroform - 1% methanol, R\(_f\) = 0.45) to give the methyl derivative (133)
as a light yellow oil (24 mg, 83%); \(^1\)H n.m.r. δ(300 MHz, CDCl\(_3\)) 0.8 p.p.m. (3H, s, MyrCH\(_3\)), 1.1 (1H, d, J = 8.5 Hz, MyrH), 1.2 (3H, s, MyrCH\(_3\)), 1.3 (3H, t, J = 6.5 Hz, OCH\(_2\)CH\(_3\)), 2.0-2.4 (5H, m, MyrH), 3.4 (2H, br m, SCH\(_2\)), 3.7 (3H, d, 3\(^3\)J\(_{PH}\) = 12.7 Hz, OCH\(_3\)), 4.3 (2H, dq, J = 7.4 Hz, 3\(^3\)J\(_{PH}\) = 10.2 Hz, OCH\(_2\)CH\(_3\)), 5.5 (1H, br s, R\(_2\)C=CRH); \(^{31}\)P n.m.r. δ(121.5 MHz, CDCl\(_3\)) +27.03 (s), +27.00 (s), diastereomeric separation = 3.7 Hz (CDCl\(_3\)), 3.9 Hz (CD\(_3\)OD), 3.5 Hz (C\(_6\)D\(_6\)); m/z (C.I.) 308 ([M + NH\(_4\)]\(^+\); 5%), 291 ([M + H]\(^+\); 100), 135 (Myr\(^+\); 55), (Found: [M + H]\(^+\) 291.1184, C\(_{13}\)H\(_{23}\)O\(_3\)PS + H\(^+\) requires 291.1184).

**Preparation of O-diphenylmethyl-S-myrtanyl-ethyl thiophosphate (134)**

\[
\begin{align*}
\text{EtO-} & \stackrel{\text{PhCN}}{\longrightarrow} \text{PhCN}_2 \\
(132) & \quad \text{EtO-P} \quad \text{OCHPh}_2 \\
& \quad \text{S} \quad \text{O} \\
& \quad \text{Myr} \\
(134)
\end{align*}
\]

Diphenyldiazomethane\(^{189}\) (0.29 g, 1.5 mmol, 6 eq) was added to a stirred solution of S-myrtanyl-ethyl thiophosphate (mono-triethylammonium salt) (132) (89 mg, 250 μmol) in chloroform (4 ml) at room temperature. Monitoring by \(^{31}\)P n.m.r. spectroscopy showed the reaction to be complete after 75 minutes. Removal of the solvent by evaporation gave the title compound (134) as a light yellow oil. \(^{31}\)P n.m.r. δ(121.5 MHz, CDCl\(_3\)) +25.69 (s), +25.66 (s), diastereomeric separation = 4.4 Hz (CDCl\(_3\)), 6.3 Hz (C\(_6\)D\(_6\)).

**Preparation of O-benzoyl-S-myrtanyl-(4-nitrophenyl) thiophosphate (131)**

\[
\begin{align*}
\text{O}_2\text{N} & \stackrel{\text{PhCOCl}}{\longrightarrow} \text{O}_2\text{N} \\
(128) & \quad \text{O-P} \quad \text{OCOPh} \\
& \quad \text{S} \quad \text{O} \\
& \quad \text{Myr} \\
(131)
\end{align*}
\]

Triethylamine (58 μl, 400 μmol) and benzoyl chloride (48 μl, 400 μmol, 2 eq) were added to a stirred solution of S-myrtanyl-(4-nitrophenyl) thiophosphate (mono-triethyl-
ammonium salt) (128) (94 mg, 200 µmol) in chloroform (1.5 ml). The mixture was stirred for 10 minutes, by which time the reaction was shown to be complete by $^{31}$P n.m.r. analysis, ($\delta p +15.2 \rightarrow +21.7$ p.p.m.). The solvent was evaporated under reduced pressure and the resulting white solid was washed with ether (3 x 2 ml). The ether washings were then filtered and solvent removal gave the benzoylated product (131) as a colourless oil, (86 mg, 91%). The oil was taken up in CDCl$_3$ immediately for high-field n.m.r. analysis. $^{31}$P n.m.r. $\delta$(121.5 MHz, CDCl$_3$) +20.86 (s), +20.76 (s), diastereomeric separation = 12.0 Hz (CDCl$_3$); $^1$H n.m.r. (partial) $\delta$(90 MHz, CDCl$_3$) 7.4-8.3 (10H, m, Ph, Ar).

**Preparation of O-benzoyl-S-myrtenyl-ethyl thiophosphate (135)**

\[
\text{EtO-PO-S-O} \xrightarrow{\text{PhCOCl}} \text{EtO-PO-OCOPh}
\]

(132) \hspace{1cm} (135)

The corresponding ethyl derivative (135) was prepared in the same manner as the above 4-nitrophenyl derivative using 100 µmol of (132), to give (135) as a colourless oil (33 mg, 87%). The oil was immediately dissolved in CDCl$_3$ for high-field n.m.r. analysis. $^{31}$P n.m.r. $\delta$(121.5 MHz, CDCl$_3$) +23.47 p.p.m. (s), +23.40 (s), diastereomeric separation = 8.2 Hz (CDCl$_3$).

**Configuration analysis of 4-nitrophenyl [16O,18O]thiophosphate (89a)**

\[
\begin{align*}
\text{O}_2\text{N-PO-S-} & \quad \text{i) Br} \\
\text{O} & \quad \text{ii) PhCOCl}
\end{align*}
\]

(89a) \hspace{1cm} (131)

To a solution of 4-nitrophenyl [16O,18O]thiophosphate (89a) (21.9 mg, 50 µmol) in methanol (0.4 ml) was added triethylamine (7 µl, 50 µmol) followed by myrtenyl bromide (9 µl, 50 µmol). The reaction mixture was stirred at room temperature for 5 minutes by
which time the \( S\)-alkylation was shown to be complete by \( ^{31}\text{P} \) n.m.r. spectroscopy (\( \delta_p +42.4 \rightarrow +15.5 \) p.p.m.). Filtration followed by solvent removal gave the \( S\)-alkylated derivative of (89a) as a light yellow oil.

The \( S\)-alkylated derivative of (89a) was dissolved in chloroform (0.25 ml) and triethylamine (14 \( \mu l, 100 \) \( \mu mol \)). Benzoyl chloride (14.5 \( \mu l, 125 \) \( \mu mol \)) was added and the reaction mixture was stirred for 10 minutes by which time the \( O\)-benzylation was shown to be complete by \( ^{31}\text{P} \) n.m.r. spectroscopy (\( \delta_p +15.5 \rightarrow 21.4 \) p.p.m.). The solvent was removed and the white solid recovered was washed with ether (3 \( \times \) 1 ml), the ether washings were then filtered and solvent removal gave (131) as a clear oil which was dissolved in CDCl\(_3\) for high-field n.m.r.

\( ^{31}\text{P} \) n.m.r. \( \delta(121.5 \text{ MHz, CDCl}_3): +21.67 \text{ p.p.m. (s)}, +21.62 \text{ (s, } ^{18}\text{O isotopic shift = 5.7 Hz)}, +21.58 \text{ (s)}, +21.56 \text{ (s, } ^{18}\text{O isotopic shift = 2.1 Hz)}, \text{ (see Figure 3.14, Chapter 3).} \)

**Configurational analysis of ethyl \([^{16}\text{O},^{18}\text{O}]\)thiophosphate (90a)**

\[ \text{EtO} \begin{array}{c} \text{Br} \\ \text{(90a)} \end{array} \xrightarrow{\text{i)}} \text{PhCOCl} \begin{array}{c} \text{EtO} \\ \text{OCOPh} \end{array} \]

Ethyl \([^{16}\text{O},^{18}\text{O}]\)thiophosphate (90a) (20.6 mg, 60 \( \mu mol \)) was dissolved in methanol (0.3 ml) and triethylamine (8.4 \( \mu l, 60 \) \( \mu mol \)). Myrtenyl bromide (10.8 \( \mu l, 60 \) \( \mu mol \)) was added and the reaction mixture stirred for 5 minutes at room temperature by which time the \( S\)-alkylation was shown to be complete by \( ^{31}\text{P} \) n.m.r. spectroscopy (\( \delta_p +42.8 \rightarrow +18.6 \) p.p.m.). Removal of the solvent by evaporation gave the \( S\)-alkylated derivative of (90a) as the mono-triethylammonium salt.

The \( S\)-alkylated derivative of (90a) was dissolved in chloroform (0.25 ml) and triethylamine (16.8 \( \mu l, 120 \) \( \mu mol \)) and benzoyl chloride (17.4 \( \mu l, 150 \) \( \mu mol \)) was added. The reaction mixture was stirred for 10 minutes by which time the \( O\)-benzylation was shown to
be complete by $^{31}$P n.m.r. spectroscopy ($\delta p +18.6 \rightarrow 22.4$ p.p.m.). The chloroform was removed by evaporation under reduced pressure and the resulting white solid was washed with ether (3 x 1 ml); the ether washings were then filtered and solvent evaporated to give (135) as a clear oil which was dissolved in CDCl$_3$ for high-field n.m.r.

$^{31}$P n.m.r. $\delta$(121.5 MHz, CDCl$_3$): $+23.56$ p.p.m. (s), $+23.51$ (s, $^{18}$O isotopic shift = 5.7 Hz), $+21.58$ (s), $+23.49$ (s), $+23.47$ (s, $^{18}$O isotopic shift = 2.3 Hz), (see Figure 3.15, Chapter 3).

**Attempted preparation of bis-(tetra-n-butylammonium) salt of 4-nitrophosphoryl thiophosphate (89)**

\[ \begin{align*} 
\text{O}_2\text{N} & \quad \text{O} \quad \text{P} \quad \text{S} \\
\text{O}_2\text{N} & \quad \text{O} \quad \text{P} \quad \text{S} \\
(89) & \quad 2\text{Et}_3\text{NH} & \quad 2\text{Bu}_4\text{NOH} \\
\text{O}_2\text{N} & \quad \text{O} \quad \text{P} \quad \text{S} \\
(89) & \quad 2\text{Bu}_4\text{N} 
\end{align*} \]

1. To a solution of bis-triethylammonium salt of 4-nitrophosphoryl thiophosphate (89) (100 μmol) in water (0.5 ml) was added a 40% aqueous solution of tetra-n-butylammonium hydroxide (130 μl, 200 μmol). The solution was evaporated under high vacuum at room temperature to give a yellow oil which was dissolved in methanol (0.5 ml) for $^{31}$P n.m.r. analysis, ($\delta p +36.4$ p.p.m.), which was identified as inorganic thiophosphate.

2. The above preparation was repeated at 0°C and again only inorganic thiophosphate was recovered.

3. Preparation 1 was repeated up to the addition of tetra-n-butylammonium hydroxide and the aqueous layer was then extracted with chloroform (3 x 2 ml). $^{31}$P n.m.r. analysis of the chloroform layer did not give a phosphorus signal.
Ethanolysis of 4-nitrophenyl \([^{16}O]\) thiophosphate [bis-(tetra-\(n\)-butylammonium) salt] (89)

\[
\begin{align*}
\text{O}_2\text{N} & \quad \text{O} \\
\text{P} & \quad \text{S} \\
\text{O} & \quad \text{EtOH} \\
\text{O} & \quad \text{O}_2\text{N} \\
\text{H} & \quad \text{OH}
\end{align*}
\]

(89) 2Bu₄N

\[
\begin{align*}
\text{EtO} & \quad \text{P} \\
\text{S} & \quad \text{+} \\
\text{O}_2\text{N} & \quad \text{O}_2\text{N} \\
\text{H} & \quad \text{OH}
\end{align*}
\]

(90)

\section{a) 75 mM starting material concentration}

To a solution of the bis-triethylammonium salt of 4-nitrophenyl \([^{16}O]\) thiophosphate (89) (65 mg, 150 \(\mu\)mol) in ethanol (2 ml) was added a 40\% aqueous solution of tetra-\(n\)-butylammonium hydroxide (195 \(\mu\)l, 300 \(\mu\)mol, 2 eq). Half of the solvent was removed under reduced pressure and more ethanol (1 ml) was added to the solution. \(^{31}\text{P}\) n.m.r. analysis of this solution indicated that no decomposition had occurred (\(^{31}\text{P}\) n.m.r. +40.9 p.p.m.). The reaction mixture was heated at 45°C in a reacti-vial and monitored by \(^{31}\text{P}\) n.m.r. spectroscopy. After 60 minutes the reaction had gone to 50\% completion [\(\delta\)p 40.94 (starting material) and \(\delta\)p 43.16 (ethyl thiophosphate)], the reaction was quenched by the addition of water (50 ml) and then applied to an ion-exchange column (2 x 15 cm). A gradient of 50-250 mM-TEAB (vol. 1500 ml) was applied at a rate of 70 ml per hour. The eluate was continuously monitored by UV spectroscopy (\(\lambda\) 210 nm). Two main peaks were recorded on the UV chromatogram, each fraction was isolated as before.

\textit{First fraction:} off-white gum, eluted at 130 mM buffer concentration. This was identified as ethyl thiophosphate (bis-triethylammonium salt) (90) (23.7 mg, 46\%); \(^1\text{H}\) n.m.r. \(\delta\)(90 MHz, CD\(_3\)OD) 1.2 p.p.m. (21H, m, OCH\(_2\)CH\(_3\), NCH\(_2\)CH\(_3\)), 3.4 (12H, q, \(J = 7\) Hz, NCH\(_2\)CH\(_3\)), 4.1 (2H, dq, \(J = 7\) Hz, \(^3\text{J}_p = 10\) Hz, OCH\(_2\)CH\(_3\)); \(^{31}\text{P}\) n.m.r. \(\delta\)(36.3 MHz, CD\(_3\)OD) +43.8 p.p.m. (s), [\(^1\text{H}\) proton-coupled (t, \(^3\text{J}_{PH} \sim 10\) Hz)].

\textit{Second fraction:} the yellow gum, eluted at 180 mM buffer concentration, was identified as the reisolated starting material, 4-nitrophenyl thiophosphate (bis-triethylammonium salt) (89) (28 mg, 43\% recovery); \(^1\text{H}\) n.m.r. \(\delta\)(90 MHz, CD\(_3\)OD) 1.3 p.p.m. (18H, t, NCH\(_2\)CH\(_3\)), 3.2 (12H, q, \(J = 7\) Hz, NCH\(_2\)CH\(_3\)), 7.5 (2H, d, \(J = 9\) Hz, Ar), 8.1 (2H, d, \(J = 9\) Hz, Ar); \(^{31}\text{P}\)
n.m.r. δ(36.3 MHz, CD$_3$OD) +40.7 p.p.m. (s).

b) 7.5 mM starting material concentration

The above reaction was repeated using 20 ml of ethanol (to give 7.5 mM starting material concentration). The reaction mixture was heated at 45°C for a similar duration to give 50% reaction. Quenching and ion-exchange chromatography of the reaction mixture gave ethyl thiophosphate (24.2 mg, 47%) and 4-nitrophenyl thiophosphate (27.4 mg, 42% recovery) as the bis-triethylammonium salts.

Ethanolysis of (Rp)-4-nitrophenyl [¹⁶O,¹⁸O ]thiophosphate [bis-(tetra-n-butyl-ammonium)] salt (89a):

\[
\text{O}_2\text{N} \quad \text{O} \quad \text{P} \quad \text{S} \quad \text{EtO} \quad \text{O} \quad \text{N} \quad \text{O H}
\]

(89a) 2Bu$_4$N

(90a)

a) 75 mM starting material concentration

The procedure was the same as for unlabelled experiment, using (Rp)-4-nitrophenyl [¹⁶O,¹⁸O ]thiophosphate (150 μmol) and ethanol (2 ml).

The recovered starting material and isolated product were configurationally analysed (S-alkylation, O-benzoylation) using our established method. High-field $^{31}$P n.m.r. spectroscopy showed ethyl [¹⁶O,¹⁸O ]thiophosphate to be completely racemic and 4-nitrophenyl [¹⁶O,¹⁸O ]thiophosphate was found to be ~95% Rp with ~5% excess Sp which is as synthesised.

$^{31}$P n.m.r. δ(121.5 MHz, CDCl$_3$):

Ethyl [¹⁶O,¹⁸O ]thiophosphate, +23.26 p.p.m. (s), +23.24 (s, $^{18}$O isotopic shift = 2.2 Hz), +23.22 (s, $^{18}$O isotopic shift = 4.8 Hz), +23.21 (s, $^{18}$O isotopic shift = 2.1 Hz), +23.17 (s, $^{18}$O isotopic shift = 4.8 Hz), (see Figure 4.4, Chapter 4); 4-nitrophenyl [¹⁶O,¹⁸O ]thiophosphate, +21.42 p.p.m. (s), +21.37 (s, $^{18}$O isotopic shift = 5.6 Hz), +21.33 (s), +21.31 (s, $^{18}$O isotopic shift = 2.1 Hz), (see Figure 4.5, Chapter 4).
b) 7.5 mM starting material concentration

The same procedure as for unlabelled experiment was followed.

Analysis of the chirality of the ethyl \[^{16}O,^{18}O\]thiophosphate and 4-nitrophenyl \[^{16}O,^{18}O\]thiophosphate after separation gave the same result as the 75 mM concentration labelled experiment, described above.

**Ethanolysis of 4-nitrophenyl \[^{16}O\]thiophosphate (mono-triethylammonium) salt (89)**

\[
\begin{align*}
\text{O}_2\text{N} & \quad \text{O} \quad \text{P} \quad \text{S} \\
\text{O} & \quad \text{OH} \quad \text{Et}_3\text{NH}
\end{align*}
\]

Unlabelled 4-nitrophenyl thiophosphate (bis-triethylammonium salt) (150 µmol) was dissolved in ethanol (1.92 ml), and 1.4 M-ethanol/HCl solution (73.5 µl) (prepared by bubbling dry HCl gas through ethanol) was added. The reaction mixture was stirred at room temperature and after 4.5 hours, analysis of the \(^{31}\text{P}\) n.m.r. spectrum showed the reaction to be 50% complete (\(\delta\text{p} +45.4\ \text{p.p.m.},\ 4\)-nitrophenyl thiophosphate; +50.4, ethyl thiophosphate). The two components were separated by ion-exchange chromatography as before; ethyl \[^{16}O\]thiophosphate (24.6 mg, 48%), \(\delta\text{p} +43.2\ \text{p.p.m.}\) (s); 4-nitrophenyl \[^{16}O\]thiophosphate (28 mg, 43% recovery), \(\delta\text{p} +40.3\) (s).

**Ethanolysis of (Rp)-4-nitrophenyl \[^{16}O,^{18}O\]thiophosphate (mono-triethylammonium) salt (89a)**

\[
\begin{align*}
\text{O}_2\text{N} & \quad \text{O} \quad \text{P} \quad \text{S} \\
\text{O} & \quad \text{OH} \quad \text{Et}_3\text{NH}
\end{align*}
\]

The same procedure as for unlabelled experiment (above) was employed using (Rp)-4-nitrophenyl \[^{16}O,^{18}O\]thiophosphate (150 µmol). The reaction mixture was quenched and subjected to ion-exchange chromatography as before. The two components were configura-
tionally analysed in the usual way (S-alkylation, O-benzylation). Ethyl \([^{16}\text{O},^{18}\text{O}]\)thiophosphate was found to be 80% racemic with 20% excess Sp enantiomer and the reisolated starting material, 4-nitrophenyl \([^{16}\text{O},^{18}\text{O}]\)thiophosphate, was found to be identical to the material as synthesised.

\[31\text{P n.m.r. } \delta(121.5 \text{ MHz, CDCl}_3):\]

Ethyl \([^{16}\text{O},^{18}\text{O}]\)thiophosphate, +23.73 p.p.m. (s), +23.71 (s, \(^{18}\text{O}\) isotopic shift = 2.2 Hz), +23.69 (s, \(^{18}\text{O}\) isotopic shift = 4.7 Hz), +23.68 (s), +23.66 (s, \(^{18}\text{O}\) isotopic shift = 2.2 Hz), +23.64 (s, \(^{18}\text{O}\) isotopic shift = 4.8 Hz), (see Figure 4.6, Chapter 4); 4-nitrophenyl \([^{16}\text{O},^{18}\text{O}]\)thiophosphate, +21.67 p.p.m. (s), +21.62 (s, \(^{18}\text{O}\) isotopic shift = 5.8 Hz), +21.58 (s), +21.56 (s, \(^{18}\text{O}\) isotopic shift = 2.2 Hz).

**Determination of the apparent second pKa of 4-nitrophenyl thiophosphate (89) in aqueous ethanol**

In a typical experiment, 4-nitrophenyl thiophosphate (disodium salt) (30 \(\mu\)mol) was dissolved in water (3 ml) (pH of resulting solution \(~\)11.2). The pH of the solution was lowered by the addition of 0.2 M—HCl. The solution was then evaporated under reduced pressure to dryness. The resulting solid was dissolved in a mixture of water (0.25 ml) and ethanol (0.5 ml) (1:1.5 mol ratio) and then the \(31\text{P n.m.r.}\) chemical shift of this solution was recorded. This experiment was repeated for a series of pHs (Table E.1 overleaf).

From a plot of chemical shift against pH (Figure 4.8, Chapter 4), the apparent second pKa was determined to be approximately 4.8.
### TABLE E.1

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<th>$^{31}$P n.m.r. chemical shift / p.p.m.</th>
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**Solvolysis of 4-nitrophenyl [¹⁶O]thiophosphate (di-sodium salt) (89) in aqueous ethanol**

![Chemical reaction diagram]

The bis-triethylammonium salt of (89) (150 μmol) was dissolved in water (1 ml) and NaHCO₃ (33.6 mg, 300 μmol, 2 eq) was added. The resultant solution was stirred for 20 minutes, then the water was removed under high vacuum and the residue dried by co-evaporation of water with dry ethanol (3 x 2 ml). The dry residue was now dissolved in water (0.5 ml, 27.8 mmol, 1.5 eq w.r.t. ethanol) and ethanol (0.98 ml, 17 mmol) and the "nominal" pH was then adjusted to 6.8 with 1M-HCl. The reaction was heated at 60°C for 90 minutes by which time an equal proportion of starting material (δp +41.2 p.p.m.), ethyl thiophosphate (δp +43.9 p.p.m.) and inorganic thiophosphate (δp +38.1 p.p.m.) was observed by $^{31}$P n.m.r. spectroscopy, also present was a trace amount of inorganic...
phosphate (δp +1.2 p.p.m.). The reaction mixture was cooled to room temperature and
diluted with water (100 ml), it was then applied to a DEAE-Sephadex ion-exchange column
(2 x 21 cm). A gradient of 50-200 mM-TEAB (vol. 1500 ml) was applied at a rate of 65 ml
per hour. The eluate was continuously monitored at λ = 210 nm.

Ethyl thiophosphate (90) was eluted at 130 mM buffer concentration followed by and
separate from inorganic thiophosphate which was eluted at 150 mM buffer concentration;
also present at this buffer concentration was 4-nitrophenolate. Finally, 4-nitrophenyl
thiophosphate was eluted at 180 mM buffer concentration.

**Solvolysis of (Rp)-4-nitrophenyl \([^{16}O,^{18}O]\)thiophosphate (di-sodium salt) (89a) in aqueous ethanol**

\[
\begin{align*}
\text{NO}_2\text{C} & \begin{array}{c} \text{O} \\ \text{P} \end{array} \bigcirc \equiv \bigcirc & \begin{array}{c} \text{S} \\ \text{O} \end{array} \\ \text{H} \end{align*}
\]

Procedure was the same as for the unlabelled experiment described above.

The product, ethyl \([^{16}O,^{18}O]\)thiophosphate (90a), was configurationally analysed (S-
alkylation followed by O-benzoylation) and found to be ~70% racemic with 30% excess Sp
enantiomer. The recovered starting material, 4-nitrophenyl \([^{16}O,^{18}O]\)thiophosphate (89a),
after configurational analysis was found to have the same configuration as synthesised (i.e.
~95% Rp).

\[^{31}\text{P n.m.r. } \delta(121.5 \text{ MHz, CDCl}_3):\]

Ethyl \([^{16}O,^{18}O]\)thiophosphate, +24.04 p.p.m. (s), +24.02 (s, \(^{18}O\) isotopic shift = 2.3 Hz),
+24.00 (s, \(^{18}O\) isotopic shift = 4.6 Hz), +23.99 (s), +23.97 (s, \(^{18}O\) isotopic shift = 2.4 Hz),
+23.95 (s, \(^{18}O\) isotopic shift = 4.6 Hz), (see Figure 4.9, Chapter 4).
Stability of (Rp)-ethyl $^{16}$O,$^{18}$O thio phosphate (bis-triethylammonium salt) (90a) under solvolysis conditions

(Rp)-ethyl $^{16}$O,$^{18}$O thio phosphate (bis-triethylammonium salt) (51.6 mg, 150 µmol) was dissolved in ethanol (2 ml) and the mixture was stirred at 60°C for 90 minutes. After this time, the reaction mixture was diluted with water (100 ml) and isolated by ion-exchange chromatography using 50-200 mM-TEAB (vol. 1000 ml). After isolation and concentration of the major fraction, ethyl $^{16}$O,$^{18}$O thio phosphate, was obtained as an off-white gum (48.5 mg, 94%), $^{31}$P n.m.r. δ(36.3 MHz, CD$_3$OD) +43.5 p.p.m. (s), $^1$H-coupled (t, $J_{PH}$ ~10 Hz).

The reisolated ethyl $^{16}$O,$^{18}$O thio phosphate, after configurational analysis, was found to be 95% Rp, which is as synthesised.
Synthesis of adenosine-5'-[\beta-thio]triphosphate (110) [ATPβS] via the ephedrine route

\[
\begin{array}{c}
\text{AdO} \quad \text{P} \quad \text{O} \quad \text{P} \quad \text{O} \quad \text{P} \quad \text{O} \\
\text{O} \quad \text{O} \quad \text{O} \quad \text{O}
\end{array}
\]

(110)

The synthesis of (110) was achieved by a method analogous to that of Blättler and Knowles for ADP.\(^{132}\)

Hydrolysis of cis 2-chloro-3,4-dimethyl-5-phenyl-1,3,2-oxazaphospholidine-2-thione (88a)

\[
\begin{array}{c}
\text{Ph} \\
\text{Me}
\end{array}
\quad \text{P} \quad \text{S} \\
\text{Cl} \quad \text{N} \quad \text{Me}
\]

(88a)

\[
\begin{array}{c}
\text{Me} \\
\text{Ph}
\end{array}
\quad \text{P} \quad \text{S} \quad \text{O} \\
\text{Cl} \quad \text{N} \quad \text{Me}
\]

(108)

The purified chloro adduct (88a) (0.2 g, 0.75 mmol) was dissolved in dry dioxan (1.5 ml) and this colourless solution was added to aqueous lithium hydroxide [0.5 ml of a 3.4 M solution; prepared from lithium metal (11.9 mg, 1.17 mmol, 2.2 eq) and water (0.5 ml)]. This reaction mixture was stirred vigorously and heated in an oil bath at 35°C and the reaction monitored by \(^{31}\)P n.m.r. spectroscopy. Hydrolysis of the chloro adduct was complete after 5.5 hours (\(\delta p 75.2 \rightarrow 70.9 \text{ p.p.m.}\)). The reaction mixture was freeze-dried, and the resulting white solid was then redissolved in dry dioxan (2 ml) and freeze-dried twice to give the hydrolysed product, cis 2-oxy-3,4-dimethyl-5-phenyl-1,3,2-oxazaphospholidine-2-thione (108) as the lithium salt in essentially a quantitative yield.
P–N bond cleavage of 2-oxy-3,4-dimethyl-5-phenyl-1,3,2-oxazaphospholidine-2-thione (108)

The lithium salt of (108) (~0.75 mmol) was dissolved in dry DMF (4 ml) and to this solution was added adenosine-5'-monophosphate (44a) (free acid) (0.39 g, 1.12 mmol, 1.5 eq) (dried by co-evaporation with dry dioxan). The resulting partially heterogeneous reaction mixture was vigorously stirred at 60°C in an oil bath and the reaction monitored by 31P n.m.r. spectroscopy. After 8 hours, the 31P n.m.r. spectrum of the mixture showed no starting material. The reaction mixture was quenched with triethylamine (0.25 ml) and water (100 ml) and then applied to an ion-exchange column (1.5 x 30 cm) eluting with a linear gradient of 50-200 mM-TEAB, pH = 7.6 (vol. = 2000 ml). The flow rate of the buffer through the column was set at 70 ml per hour and the eluate monitored at λ = 260 nm.

The P–N bond cleaved product (109) was eluted at a buffer concentration of 60 mM. The appropriate fractions were combined and concentrated under a high vacuum and the residual TEAB was removed as before to leave the product (109) (mono-triethylammonium salt) as a colourless gum [0.54 mmol, 70% (based on the UV absorbance at 260 nm)]; λmax (H2O) 260 nm (ε 15000 dm³ mol⁻¹ cm⁻¹); 31P n.m.r. δ(121.5 MHz, CD3OD) +46.11 p.p.m. (d, JPP = 35.6 Hz, Pβ), -10.81 (d, Pα); m/z (FAB) 692 ([M + H]⁺; 10%), 591 ([M + H]⁺ - Et3N; 30), 444 ([M + H]⁺ - Et3N - ephedrine moiety; 25), 148 ([ephedrine moiety]⁺; 100), 136 (adenine⁺; 55), (Found: [M + H]⁺ 692.2397, C26H43N7O9P2S + H⁺ requires 692.2396).

Excess adenosine-5'-monophosphate (0.49 mmol) was also isolated from the ion-exchange column which eluted at a buffer concentration of 150 mM.
Compound (109) (0.2 mmol) was dissolved in methanol and transferred to a 3-neck round-bottom flask. The methanol was then removed under reduced pressure.

Ammonia (50 ml) was condensed at -78°C under dry nitrogen and dried by distillation from sodium. Sodium (69 mg, 3 mmol) was added to the freshly distilled ammonia. When the sodium had dissolved, the resulting dark blue solution was quickly siphoned into the vessel containing (109) via a glass tube. The reaction mixture was stirred at -78°C for 4 minutes and then quenched with ammonium chloride (0.6 g). The reaction vessel was removed from the cooling bath and the ammonia allowed to evaporate. The resulting pale yellow residue was dissolved in water (80 ml) and applied to an ion-exchange column (1.5 x 20 cm). The column was eluted with 50-900 mM-TEAB buffer (vol. = 2000 ml, pH = 7.7) at 70 ml per hour. The combined fractions containing the product (eluted at 390 mM buffer concentration) were evaporated under a high vacuum; the residual TEAB was removed as before to leave the product, ADPβS (75) as a light yellow gum (0.11 mmol, 55%); \( \lambda_{\text{max}} \) (H\(_2\)O) 260 nm; \(^{31}\)P n.m.r. \( \delta \) (121.5 MHz, D\(_2\)O, Et\(_3\)N) +36.40 p.p.m. (d, \( J_{\text{PP}} \text{OP} = 34 \) Hz, Pβ), -11.60 (d, Pα).

**Enzymic phosphorylation of ADPβS (75)**

\[
\begin{align*}
\text{ADPβS} \rightarrow & \text{PEP} + \text{ADP} \\
\end{align*}
\]
ADPβS (75) was phosphorylated by the procedure of Jaffe and Cohn. A solution containing 100 mM Hepes, 100 mM KCl, 12 mM magnesium acetate, 15 mM phosphoenolpyruvate and 0.25 mM EDTA was prepared and adjusted to pH 7.6 with concentrated sodium hydroxide solution. ADPβS (75) (72 µmol) was dissolved in this solution (7.2 ml) and incubated with pyruvate kinase (100 units) at 37°C for 24 hours.

The reaction was followed by monitoring the formation of pyruvate, one of the products of the enzyme catalysed reaction, by using NADH (β-nicotinamide adenine dinucleotide, reduced form); this co-enzyme functions as a hydrogen transfer agent NADH reduces pyruvate to lactate in a reaction catalysed by lactate dehydrogenase.

Pyruvate + NADH ➔ Lactate + NAD⁺

As NADH has a strong UV absorbance (extinction coefficient, ε = 6.22 x 10³), the enzyme assay was followed spectrophotometrically, (for a detailed method see reference 190).

At the end of the incubation period, the reaction mixture was diluted with water (50 ml) and subjected to ion-exchange chromatography. The column was eluted with a linear gradient of 50-900 mM-TEAB buffer (vol. = 2000 ml, pH = 7.6) at 70 ml per hour. The combined product-containing fractions were evaporated under a high vacuum and the residual TEAB removed as before to give the product ATPβS (110) as a colourless gum (55 µmol, 77%); λmax (H₂O) 260 nm; ³¹P n.m.r. δ[121.5 MHz, D₂O, 0.1 M Tris buffer, EDTA (10 mM), pH 9.3] +27.53 p.p.m. (dd, JpPO₆ = 27.3 Hz, JpPOP₇ = 28.8 Hz, Pβ), -7.07 (d, Pγ), -12.43 (d, Pα).
Synthesis of (Sp)-adenosine-5′-[β-thio, βγ-18O]triphosphate (ATPBβγ18O) (110) via the ephedrine route

![Chemical structure of (Sp)-adenosine-5′-[β-thio, βγ-18O]triphosphate (ATPBβγ18O) (110)](image)

This was prepared in the same way as the unlabelled ATPβS described above, starting with cis 2-chloro-3,4-dimethyl-5-phenyl-1,3,2-oxazaphospholidine-2-thione (88a) (0.2 g, 0.75 mmol) and aqueous Li18OH [0.5 ml of a 3.4 M solution; prepared from lithium metal (11.9 mg, 1.17 mmol) and H218O (0.5 ml of 98% atom excess)]. Subsequent reactions gave ATPBβγ18O (110) (53 µmol, 74% from ADPβγ18O); 31P n.m.r. δ(121.5 MHz, D2O, 0.1 M Tris buffer, EDTA (10 mM), pH 9.3, mixed with authentic ATPβS (ca. 1:1)):

-12.50 p.p.m. [d, Jpαopβ = 27.6 Hz, Pα], -7.02 [d, Jpγopβ = 28.9 Hz, Pγ(βγ18O)], -7.04 [d, 18O isotopomer, 18O isotopic shift = 2.7 Hz, Pγ(βγ18O)], +27.76 [dd, Jpβopγ = 27.6 Hz, JpβopPβ = 28.9 Hz, Pβ(βγ18O)], +27.74 [dd, 18O isotopomer, 18O isotopic shift = 2.7 Hz, Pβ(βγ18O)],

(see Figure 2.21, Chapter 2).

Attempted preparation of adenosine-5′-[γ-thio]triphosphate (ATPγS) (107a) via the ephedrine route

![Chemical structure of ATPγS (107a)](image)

This synthesis was attempted based on the preparation of ATPβS previously described. To a stirred solution of cis 2-oxy-3,4-dimethyl-5-phenyl-1,3,2-oxazaphospholidine-2-thione lithium salt (108) (24.9 mg, 100 µmol) in DMF (2 ml) was added adenosine-5′-diphosphate (ADP free acid) (89.7 mg, 210 µmol). The white heterogeneous mixture was left under the
following conditions:

a) stirred rapidly at 70°C overnight,

b) reaction vessel was placed in an ultrasound bath overnight.

But ion-exchange chromatography of the reaction mixture in each case (50-500 mM TEAB) gave back starting materials, thione (108) and ADP. Changing the solvent to \(N,N'\)-dimethyl-propyleneurea (DMPU) again gave a heterogeneous reaction with no product.

Use of dimethyl sulphoxide (DMSO) as solvent (cf. Blättler and Knowles\textsuperscript{132}) led to complete loss of sulphur in (108).
Kinetic studies on 2,4-dinitrophenyl thiophosphate dianion (141)

![Chemical Structure](image)

(141)

Synthesis of 2,4-dinitrophenyl thiophosphoryl dichloride (142)

![Chemical Structure](image)

(140) (142)

This synthesis was based on a method by Tolkmith.\textsuperscript{172} 2,4-Dinitrophenol (140) (5 g, 27 mmol) (purified by recrystallisation from dry ethanol) was dissolved in dry dioxan (50 ml). Finely powdered lithium hydride (0.22 g, 27 mmol) was added to this clear orange solution over 5 minutes. The solution was then stirred for a further 40 minutes at room temperature. After this time, the dioxan was evaporated under reduced pressure and the resulting orange solid was co-evaporated with dioxan (3 x 20 ml) to leave lithium 2,4-dinitrophenoxide.

The phenoxide was dissolved in acetonitrile (150 ml) and added to a solution of thiophosphoryl chloride (13.7 ml, 135 mmol, 5 eq) in acetonitrile (30 ml) with stirring under nitrogen over a period of 15 minutes. The mixture was stirred overnight at room temperature. After this time, the mixture was filtered and the filtrate evaporated under reduced pressure to leave a light brown oil. Cyclohexane (30 ml) was added and evaporated in the same manner. The residue was extracted twice with a mixture of cyclohexane (30 ml) and light petroleum (30 ml) and then with light petroleum alone (15 ml). The hydrocarbon solution was evaporated under reduced pressure to leave a crude off-white solid.

Recrystallisation of the solid from hexane gave the product (142) as white needles (3.6 g, 42%); m.p. 55-57\(^\circ\)C; \(^1\)H n.m.r. \(\delta\)(90 MHz, CDCl\(_3\)) 7.1 p.p.m. (d, \(J = 9\) Hz), 8.3 (dd, \(J = 9, 3\) Hz).
Hz), 8.9 (d, J = 3 Hz); $^{31}$P n.m.r. $\delta$ (36.3 MHz, CDCl$_3$) +54.2 p.p.m. (s); m/z 320, 318, 316 (M$^+$; 1, 6, 8%), 274, 272, 270 (M$^+$ - NO$_2$; 3, 18, 27), 183 [(NO$_2$)$_2$C$_6$H$_3$O$^+$; 100].

**Hydrolysis of 2,4-dinitrophenyl thiophosphoryl dichloride (142)**

![Diagram of hydrolysis reaction](image)

To a solution of the dichloride (142) (9.5 mg, 30 µmol) in dry dioxan (40 µl) was added 50 mM–NaOH aqueous solution (3.6 ml) at room temperature. $^{31}$P n.m.r. spectroscopy showed the hydrolysis of (142) to be complete after 15 minutes, ($\delta$P +54.2 $\rightarrow$ +50.6 p.p.m.).

After 3 hours, the $^{31}$P n.m.r. spectrum of the reaction mixture showed just one resonance, ($\delta$P +41.1 p.p.m.), which was confirmed as inorganic thiophosphate (identified by spiking the n.m.r. sample with authentic inorganic thiophosphate).

**Kinetic measurements:**

**Atmospheric pressure kinetic runs**

Rate constants at atmospheric pressure were determined from kinetic runs carried out in the thermostatted cell compartment of a Hewlett-Packard HP-8451A Diode Array spectrophotometer as follows:

2,4-Dinitrophenyl thiophosphoryl dichloride (73 mg, 230 µmol) was dissolved in dry dioxan (2 ml). An aliquot of this solution (2.2 µl, 0.25 µmol) was pipetted into a quartz UV cuvette containing 50 mM–NaOH aqueous solution (3 ml), (in all cases, the reactions were carried out with a large excess of NaOH(aq) so that first-order rate law was obeyed). The cuvette was inverted several times and the hydrolysis of 2,4-dinitrophenyl thiophosphate was followed spectrometrically by repeat scan monitoring at regular intervals (4 minutes) between 190-500 nm, (see Figure 5.5, Chapter 5).

The hydrolysis was repeated, but this time the absorbance at 400 nm (maximum...
The absorbance of liberated phenoxide was recorded against time. Time \( t = 0 \) was taken as the time (estimated) when all the dichloride had been hydrolysed. One sample data is shown in Table E.2.

Figure E.1 shows the plot of absorbance against time.

The observed rate constant, \( k_{a(\text{obs})} \), was calculated by computer from the slope of \( \ln \left( \frac{a}{(a-x)} \right) \) against time \( (a = \text{absorbance at time} \ \to, \ x = \text{absorbance at time} \ t) \) for at least two-and-a-half half-lives, shown in Figure E.2 (overleaf).

The observed first-order rate constant, \( k_a \), for the hydrolysis of (141) at atmospheric pressure = \( 6.9 \times 10^{-4} \) s\(^{-1}\).

For a first-order reaction:

\[
\text{Half-life, } \tau = \frac{\ln 2}{k_1}
\]

\[
\therefore \tau = \frac{\ln 2}{6.9 \times 10^{-4}} = 16.7 \text{ minutes}
\]
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High pressure kinetic runs

Rate constants at high pressures were determined by the use of the apparatus shown in Figure E.3, which works on the simple principle of thermostatting a relatively large volume of reaction mixture under pressure.

The high pressure apparatus and solutions of the reactants were thermostatted for about 15 minutes; then a run was started by mixing a dioxan solution of 0.12 M-2,4-dinitrophenyl thiophosphoryl dichloride (100 μl) with an aqueous solution of 50 mM-NaOH (130 ml) to give a final concentration of 0.08 mM in dichloride. Most of this solution was transferred to the cell of the high pressure apparatus, which was inserted into the bomb, pressurised, and then left for several minutes to re-equilibrate to bath temperature (25°C). Meanwhile, an aliquot of about 3 ml was run into a 1 cm quartz cuvette in the thermostatted cell compartment of the spectrophotometer. The rate constant for reaction at atmospheric pressure was obtained for this sample by recording the absorbance changes (due to the release of 2,4-dinitrophenoxide) as a function of time at 400 nm.
FiguRe E.3 The apparatus used for high pressure kinetic runs.
Aliquots of the pressurised reaction solution were released at intervals through the valve indicated in Figure E.3; careful operation of the high pressure valve permitted the ejection of about 3 ml of solution directly into the 1 cm quartz cuvette. The absorbance of each ejected aliquot was read off the spectrophotometer as quickly as possible. The Madan Air Hydro Power unit automatically restored the pressure to the value set at the start of the run within 2 or 3 seconds of ejecting the aliquot. Some 12 to 15 samples were taken during each run; times of sampling were chosen so that run was monitored over between 2 and 3 half-lives at approximately equal time intervals. Rate constants were calculated graphically for atmospheric pressure and high pressure runs from the slope of \( \ln \left[ a/(a-x) \right] \) against time.

A graph of \( \log k_p/k_s \) against pressure was plotted (see Figure 5.6, Chapter 5) from which the slope gave the volume of activation (\( \Delta V^\ddagger \)) for the hydrolysis of (141),

\[
\Delta V^\ddagger = +11 \text{ cm}^3 \text{ mol}^{-1}
\]
Preparation of 2-hydroxy-5-nitrobenzyl alcohol (148)

To a stirred solution of 2-hydroxy-5-nitrobenzaldehyde (147) (7.5 g, 44.9 mmol) in methanol (30 ml) and dioxan (30 ml) was added a solution of sodium borohydride (0.56 g, 14.8 mmol) in water (10 ml) and 2M-NaOH (1 ml) at a rate of 0.5 ml per minute with occasional cooling (cold water) to keep the reaction at 15-20°C. The bright yellow mixture was stirred for a further 2 hours after which time most of the solvent was removed under reduced pressure. The residue was diluted with 0.2 M sulphuric acid solution (60 ml) and the mixture extracted with dry ether (3 x 30 ml). The organic extracts were washed with water (80 ml), dried (MgSO₄), filtered and the solvent was removed under reduced pressure to give a yellow solid which was recrystallised from water. Hard yellow crystals of (148) were obtained (6.2 g, 82%); m.p. 126-127°C (Lit., 127-128°C); Rf = 0.28 (light petroleum/ethyl acetate, 1:1); \( \nu_{max} \) (Nujol) 3400br cm⁻¹ (OH); \( ^1H \) n.m.r. \( \delta \) (300 MHz, CD₃OD) 4.65 p.p.m. (2H, s, ArCHO), 4.90 (br s, OH), 6.85 (1H, d, J = 8.9 Hz, Ar), 8.00 (1H, dd, J = 2.9, 8.9 Hz, Ar), 8.25 (1H, d, J = 2.9 Hz, Ar); m/z 169 (M⁺; 35%), 151 (M⁺ - H₂O; 50), 65 (C₅H₅⁺; 100).

Synthesis of 2-acetoxy-5-nitrobenzyl acetate (149)
To a stirred solution of the diol (148) (3.5 g, 20.7 mmol) in dry pyridine (13 ml) was added dry acetic anhydride (12.6 ml, 133.5 mmol) at room temperature. After 3 hours, the reaction mixture was saturated with water (100 ml) and then extracted with ethyl acetate (3 x 30 ml). The combined organic extracts were washed successively with 0.2 M HCl (40 ml) and water (40 ml), dried (Na$_2$SO$_4$), and then filtered. The solvent was evaporated under reduced pressure to leave a pale brown oil which when dried in vacuo at room temperature for 2 hours gave an off-white solid. This solid was recrystallised from di-isopropyl ether to give (149) as white crystals, (4.8 g, 92%); m.p. 56-58°C (Lit. 57-58°C); R$_f$ = 0.29 (light petroleum/ethyl acetate, 3:1); $\nu_{\text{max}}$ (CH$_2$Cl$_2$) 1740st, 1770st cm$^{-1}$ (C=O); $^1$H n.m.r. 6(300 MHz, CD$_3$OD) 2.10 p.p.m. (3H, s, OH$_2$OAc), 2.40 (3H, s, ArOAc), 5.15 (2H, s, CH$_2$OAc), 7.25, (1H, d, J = 8.9 Hz, Ar), 8.15 (1H, dd, J = 3, 8.9 Hz, Ar), 8.25 (1H, d, J = 3 Hz, Ar); m/z 253 (M$^+$, 5%), 211 (M$^+$ - C$_2$H$_2$O; 100).

**Synthesis of 2-hydroxy-5-nitrobenzyl acetate (146a)**

![Chemical structure of 149 and 146a](image)

The diacetylated compound (149) (2.4 g, 9.5 mmol) was dissolved in acetone (40 ml) and to this solution was added 1 M Na$_2$CO$_3$ (28.5 ml, 28.5 mmol). To make the mixture homogeneous, water (30 ml) was added and the solution stirred at 20°C for 2 hours. After this time, most of the acetone was evaporated under reduced pressure and the resulting mixture was diluted with water (100 ml) and acidified with 0.5 M HCl (50 ml). The mixture was extracted with ethyl acetate (3 x 60 ml) and the organic layer was washed with water (30 ml), dried (MgSO$_4$), filtered and then evaporated to give a green oil. This was chromatographed on silica with light petroleum - ethyl acetate (2:1) (R$_f$ = 0.20) as eluant to give the monoacetylated compound (146a) as a white solid (1.2 g, 61%); m.p. 99-102°C.
(recrystallised from H$_2$O); $\nu_{\text{max}}$ (Nujol) 3200br (OH), 1710st cm$^{-1}$ (C=O); $^1$H n.m.r. $\delta$(300 MHz, CD$_3$OD) 2.10 p.p.m. (3H, s, CH$_2$OAc), 4.8 (br s, OH, HOD), 5.15 (2H, s, CH$_2$OAc), 6.95 (1H, d, J = 8.9 Hz, Ar), 8.00 (1H, dd, J = 3, 8.9 Hz, Ar), 8.15 (1H, d, J = 3 Hz, Ar); m/z (E.I.) 211 (M$^+$; 80%), 194 (M$^+$ - OH; 15), 169 (M$^+$ - C$_2$H$_2$O; 60), 151 (M$^+$ - CH$_3$CO$_2$H; 100), 65 (C$_5$H$_5^+$; 100), (Found: M$^+$ 211.0481, C$_9$H$_9$NO$_5$ requires M 211.0481).

**Synthesis of 2-(hydroxymethyl)-4-nitrophenyl [^{16}O]thiophosphate (145) via the ephedrine route**

![Chemical Structure](image)

**Preparation of cis (4S,5R)-2-(2-acetoxymethyl-4-nitrophenox)-3,4-dimethyl-5-phenyl-1,3,2-oxazaphospholidine-2-thione (150)**

![Chemical Structures](image)

To a solution of 2-hydroxy-5-nitrobenzyl acetate (146a) (1.1 g, 5.2 mmol) in dry dioxan (20 ml) was added triethylamine (0.72 ml, 5.2 mmol) followed by cis 2-chloro-3,4-dimethyl-5-phenyl-1,3,2-oxazaphospholidine-2-thione (88a) (1.3 g, 5.1 mmol) in dioxan (20 ml) over 5 minutes at room temperature. The mixture was then heated at 60°C in an oil bath for 50 hours. After this time, the reaction was cooled, filtered and the solvent evaporated under reduced pressure to give an orange oil. The oil was chromatographed on silica using hexane–dichloromethane (1:3) ($R_f = 0.41$) as eluant to give a pale yellow oil (1.47 g, 66%). The oil was recrystallised from di-isopropyl ether to give the title compound (150) as pale
yellow crystals, m.p. 70-71°C; (Found: C, 52.39%; H, 4.95; N, 6.24. C₁₉H₂₁N₂O₆PS requires C, 52.29%; H, 4.85; N, 6.42). \( \nu_{\text{max}} \) (Nujol) 1740 cm\(^{-1}\) (C=O); \(^1\)H n.m.r. \( \delta \) (300 MHz, CDCl\(_3\)) 0.90 p.p.m. (3H, d, J = 7 Hz, CH\(_3\)), 2.15 (3H, s, OAc), 2.95 (3H, d, \( ^3 \)J\(_{PH} = 12.4\) Hz, N-CH\(_3\)), 3.80 (1H, dqm, J = 7 Hz, \( ^3 \)J\(_{PH} = 18.5\) Hz, H-4), 5.25 (2H, AB q, J = 13.5 Hz, ArCH\(_2\)), 5.80 (1H, dd, J = 7 Hz, \( ^3 \)J\(_{PH} = 2.7\) Hz, H-5), 7.40 (6H, m, Ph, Ar), 8.25 (1H, dd, J = 2.8, 8.9 Hz, Ar). 8.35 (1H, d, J = 2.8 Hz, Ar); \(^31\)P n.m.r. \( \delta \) (36.3 MHz, CDCl\(_3\)) -57.2 p.p.m. (s); m/z 436 (M\(^+\); 70%), 376 (M\(^+\) - CH\(_3\)CO\(_2\)H; 80).

\( P-N \) bond cleavage of diester amidate (150)

\[ \text{Ph} \quad \text{OAc} \quad \text{O} \quad \text{S} \quad \text{Me} \quad \text{Me} \quad \text{Me} \]

The diester amidate (150) (0.52 g, 1.2 mmol) was dissolved in dry dioxan (0.8 ml) and added to a solution of trifluoroacetic acid (0.32 ml, 4.2 mmol) and water (0.7 ml). After 40 minutes, \(^31\)P n.m.r. showed that no starting material remained (\( \delta \)p +77.2 \( \rightarrow \) +52.4 p.p.m.). Solvent removal under reduced pressure gave a white solid which was co-evaporated with dry dioxan (4 x 4 ml) to leave the compound (151) as its zwitterion. \(^1\)H n.m.r. \( \delta \) (90 MHz, CDCl\(_3\)) 1.2 p.p.m. (3H, d, J = 7 Hz, CH\(_3\)), 2.1 (3H, s, OAc), 3.0 (3H, s, N-CH\(_3\)), 4.2 (1H, dq, J = 7 Hz, H-2), 5.1 (2H, br s, NH\(_2\)), 5.3 (2H, s, CH\(_2\)OAc), 6.2 (1H, dd, J = 7 Hz, \( ^3 \)J\(_{PH} = 3\) Hz, H-1), 7.2 (5H, m, Ph), 7.5 (1H, d, J = 9 Hz, Ar), 8.1 (1H, dd, J = 3, 9 Hz, Ar), 8.2 (1H, d, J = 3 Hz, Ar); \(^31\)P n.m.r. \( \delta \) (36.3 MHz, CDCl\(_3\)) +52.3 p.p.m. (s).
The diester (151) (0.45 g, 0.99 mmol) as its zwitterion was dissolved in a mixture of chloroform (3 ml) and DMF (5 ml). Methyl iodide (1.23 ml, 19.8 mmol, 20 eq) was added and the mixture stirred at room temperature. The reaction was monitored by $^{31}$P n.m.r. spectroscopy. After 1 hour, no starting material remained ($\delta$ 52.2 → 26.9 p.p.m.). Solvent and excess methyl iodide were evaporated to leave a yellow oil which was co-evaporated with chloroform (4 x 4 ml) to give the S-methylated derivative (152) as a yellow foam. $^1$H n.m.r. (partial) $\delta$(90 MHz, CDCl$_3$) 2.4 p.p.m. (3H, d, $\delta$J$_{PH}$ = 30 Hz, SCH$_3$); $^{31}$P n.m.r. $\delta$(36.3 MHz, CDCl$_3$) +26.7 p.p.m. (s).

To a solution of the above S-methylated compound (152) (0.55 g, 0.92 mmol) in chloroform (4 ml) under a nitrogen hood was added trimethylsilyl iodide (0.52 ml, 3.68 mmol) via a graduated glass pipette. The resultant dark brown mixture was stirred for 2.5 hours at room temperature, by which time $^{31}$P n.m.r. showed the reaction to be complete ($\delta$ 26.0 → +36.4 p.p.m.). The solvent and excess trimethylsilyl iodide were removed under reduced pressure and the dark red oil obtained was dissolved in a solution of sodium bicarbonate (0.46 g, 5.52 mmol, 6 eq) in water (5 ml) and mercaptoethanol (0.4 ml). A bright yellow solution was obtained which was diluted with water (100 ml) and the product
purified by ion-exchange chromatography using DEAE-Sephadex A-25 (140 ml). The column was eluted with TEAB buffer (50-250 mM, vol. 1000 ml, pH 7.6) at 60 ml per hour. The eluate was continuously monitored by a UV spectrophotometer at 315 nm. Two peaks were recorded on the UV chromatogram. The combined product-containing fractions (eluted at 210 mM buffer concentration) were evaporated under a high vacuum, and residual TEAB removed by repeated evaporation (under reduced pressure) of aliquots of methanol (4 x 5 ml), to give the product (154) as the bis-triethylammonium salt (0.44 g, 72%); \( \lambda_{\text{max}} \) (H\( _2 \)O) 315 nm; \( ^1 \)H n.m.r. \( \delta \)(90 MHz, CD\( _3 \)OD) 1.3 p.p.m. (18H, t, \( J = 7 \) Hz, NCH\( _2 \)CH\( _3 \)), 2.2 (3H, s, OAc), 3.3 (12H, q, \( J = 7 \) Hz, NCH\( _2 \)CH\( _3 \)), 5.3 (2H, s, CH\( _2 \)OAc), 7.5-8.3 (3H, br m, Ar); \( ^{31} \)P n.m.r. \( \delta \)(36.3 MHz, CD\( _3 \)OD) +44.2 p.p.m. (s).

**Removal of acetate group from thiophosphate (154)**

![Diagram](image)

The thiophosphate (154) (0.36 g, 0.71 mmol) was dissolved in water (4 ml) and to this was added a solution of 3M–NaOH (0.95 ml, 2.8 mmol, 4 eq). The resulting bright yellow solution was stirred at room temperature for 2 hours. After this time, the mixture was diluted with more water (100 ml) and applied to an ion-exchange column. A gradient of 50-250 mM-TEAB (vol. 1000 ml, pH 7.7) was applied at a rate of 60 ml per hour. The eluate was monitored at \( \lambda \) 315 nm. The deprotected product eluted at 220 mM buffer concentration; the appropriate fractions were collected together, concentrated and the residual TEAB removed as before to give the product (145), a yellow oil as the bis-triethylammonium salt (0.28 g, 86%); \( \lambda_{\text{max}} \) (H\( _2 \)O) 315 nm; \( ^1 \)H n.m.r. \( \delta \)(90 MHz, CD\( _3 \)OD) 1.3 p.p.m. (19H, t, \( J = 7 \) Hz, NCH\( _2 \)CH\( _3 \)), 3.2 (12H, q, \( J = 7 \) Hz, NCH\( _2 \)CH\( _3 \)), 4.7 (2H, s, CH\( _2 \)OH), 5.1 (br s, CH\( _2 \)OH, HOD), 7.6-8.3 (3H, br m, Ar); \( ^{31} \)P n.m.r. \( \delta \)(36.3 MHz, CD\( _3 \)OD) +44.1 p.p.m. (s).
Solvolysis of 2-(hydroxymethyl)-4-nitrophenyl thiophosphate (145) in tert-butanol

To a solution of the bis-triethylammonium salt of (145) (0.12 g, 260 µmol) in water (5 ml) was added a 40% aqueous solution of tetra-n-butylammonium hydroxide (337 µl, 520 µmol, 2 eq). [At this stage, no decomposition of (145) had occurred as shown by $^{31}$P n.m.r. spectroscopy, δp 40.8 p.p.m.; $\lambda_{\text{max}}$ 315 nm.] The pH of the resulting solution was 10.6. The water was evaporated (but not to dryness) under a high vacuum; further portions of water were added and successively removed (5 x 3 ml) until there was a little water remaining (~150 µl). To this aqueous compound was added dry tert-butanol (100 ml) with rapid stirring. After 3 minutes of vigorous stirring, tert-butanol was removed under reduced pressure. The $^{31}$P n.m.r. spectrum of the resulting bright yellow oil showed three main peaks, $^{31}$P n.m.r. δ(36.3 MHz, tert-butanol/H$_2$O) +45.5 p.p.m. (s), +37.8 (s), 36.2 (br s), see Figure 6.13, Chapter 6; $\lambda_{\text{max}}$ (tert-butanol/H$_2$O) 415 nm].

The bright yellow oil was dissolved in water (100 ml) and applied to an ion-exchange column (140 ml). A linear gradient of 50-250 mM-TEAB (vol. 1200 ml, pH 7.6) was applied at a rate of 60 ml per hour. The eluate was monitored at λ 210 nm; two main peaks were recorded on the UV chromatogram. Each fraction was collected and concentrated, and the residual TEAB was removed as before.

*First fraction:* This eluted at 180 mM buffer concentration and was isolated as a white gum. This fraction was identified as tert-butyl thiophosphate (bis-triethylammonium salt)
(160) (37.8 mg, 102 μmol; \( \lambda_{\text{max}} \) (H₂O) 210 nm; \(^1\)H n.m.r. δ(90 MHz, CD₃OD) 1.3 p.p.m. (18H, t, J = 7 Hz, NCH₂CH₃), 1.4 (9H, s, Bu'), 3.2 (12H, q, J = 7 Hz, NCH₂CH₃); \(^{31}\)P n.m.r. δ(36.3 MHz, CD₃OD) +44.7 p.p.m. (s); m/z (FAB) Found: \([M + H]^+ 373.2654, \text{C}_{16}\text{H}_{41}\text{O}_3\text{N}_2\text{PS} + \text{H}^+ \text{requires } 373.5480.

**Second fraction:** This eluted at 210 mM buffer concentration and was isolated as a bright yellow gum. This fraction contained a mixture of inorganic thiophosphate (77) and 2-(hydroxymethyl)-4-nitrophenolate (148). \( \lambda_{\text{max}} \) (H₂O) 400 nm; \(^1\)H n.m.r. δ(90 MHz, CD₃OD) 1.3 p.p.m. (11H, t, J = 7 Hz, NCH₂CH₃), 3.2 (8H, q, J = 7 Hz, NCH₂CH₃), 4.6 (2H, s, ArCH₂), 6.8 (1H, d, J = 9 Hz, Ar), 8.0 (1H, dd, J = 3, 9 Hz, Ar), 8.3 (1H, d, J = 3 Hz, Ar); \(^{31}\)P n.m.r. δ(36.3 MHz, CD₃OD) +36.8 p.p.m. (s).

Further elution of the column with 500-700 mM-TEAB (same conditions as before) gave another product which eluted at 650 mM buffer concentration. Isolation and concentration of this fraction gave a bright yellow gum. This was suspected to be the rearranged product, 2-hydroxy-5-nitrobenzyl thiophosphate (161) (~41 mg, ~71 μmol; \( \lambda_{\text{max}} \) (H₂O) 400 nm; \(^1\)H n.m.r. δ(90 MHz, CD₃OD) 1.2 p.p.m. (30H, t, J = 7 Hz, NCH₂CH₃), 3.0 (17H, q, J = 7 Hz, NCH₂CH₃), 4.8 (13H, m, ArOH, HOD, ArCH₂OP), 6.8 (1H, d, J = 9 Hz, Ar), 8.0 (1H, dd, J = 3, 9 Hz, Ar), 8.2 (1H, d, J = 3 Hz, Ar); \(^{31}\)P n.m.r. δ(36.3 MHz, CD₃OD) +47.3 p.p.m. (s), [\(^1\)H-coupled (t, J_{PH} = 9 Hz)].

**Synthesis of (Rp)-2-(hydroxymethyl)-4-nitrophenyl [\(^{16}\)O,\(^{18}\)O]thiophosphate (145a)**

![Chemical structure of (150) and (145a)]

This was prepared in the same manner as the unlabelled material using (150) (0.55 g, 1.27 mmol) as the starting material with H₂\(^{16}\)O (0.24 ml)/H₂\(^{18}\)O (0.56 ml) and trifluoroacetic acid (0.34 ml, 4.45 mmol). This gave the title compound (145a) (0.42 g, 71%).
Solvolysis of (Rp)-2-(hydroxymethyl)-4-nitrophenyl [\(^{16}\text{O},{^{18}}\text{O}\)] thiophosphate (145a) in tert-butanol

![Chemical structure of (Rp)-2-(hydroxymethyl)-4-nitrophenyl thiophosphate (145a)]

Solvolysis of \(^{18}\text{O}\)-labelled thiophosphate (145a) (0.12 g, 260 \(\mu\)mol) and the isolation of the products was conducted in the same manner as the unlabelled material. The following compounds were isolated: tert-butyl [\(^{16}\text{O},{^{18}}\text{O}\)] thiophosphate (160), inorganic [\(^{16}\text{O},{^{18}}\text{O}\)] thiophosphate (77), 2-(hydroxymethyl)-4-nitrophenolate (148) and 2-hydroxy-5-nitrobenzyl [\(^{16}\text{O},{^{18}}\text{O}\)] thiophosphate (161).

**Configurational analysis of 2-(hydroxymethyl)-4-nitrophenyl [\(^{16}\text{O},{^{18}}\text{O}\)] thiophosphate (145a)**

\[ \begin{align*}
\text{O}_2\text{N} &- O - \text{Ph} - \text{S} \\
\text{OH} &\quad \text{O} \\
\text{O} &\quad 2\text{Bu}_4\text{N} \\
\end{align*} \]

(145a)

2-(Hydroxymethyl)-4-nitrophenyl [\(^{16}\text{O},{^{18}}\text{O}\)] thiophosphate (145a) (20 \(\mu\)mol) was dissolved in methanol (0.2 ml) and triethylamine (2.8 \(\mu\)l, 20 \(\mu\)mol). To this solution was added myrtenyl bromide (3.6 \(\mu\)l, 20 \(\mu\)mol) and the reaction mixture was then stirred for 5 minutes at room temperature. After this time, the reaction was shown to be complete by \(^{31}\text{P}\) n.m.r. spectroscopy (\(\delta p +44.1 \rightarrow +15.2\) p.p.m.). Filtration and evaporation of solvent gave the \(S\)-alkylated derivative of (145a).

The \(S\)-alkylated derivative of (145a) was then dissolved in chloroform (0.2 ml) and triethylamine (5.6 \(\mu\)l, 40 \(\mu\)mol) and benzoyl chloride (5.8 \(\mu\)l, 50 \(\mu\)mol) was added. The resulting reaction mixture was stirred for 10 minutes, by which time \(O\)-benzoylation was
shown to be complete by $^{31}$P n.m.r. spectroscopy ($\delta p +15.2 \rightarrow +21.6$ p.p.m.). Unfortunately, the product was later found to have cyclised.

$^{31}$P n.m.r. $\delta$(121.5 MHz, CDCl$_3$): +21.59 p.p.m. (s), +21.52 (s), +21.47 (s, $^{18}$O isotopic shift = 6.3 Hz), (see Figure 6.12, Chapter 6).

**Configurational analysis of tert-butyl [16O,18O]thiophosphate (160) (isolated from the solvolysis reaction)**

**S-Alkylation:**

\[ \text{Bu'O-PO}_3\text{S} \quad \text{Br} \quad \text{V} \quad \quad \text{Bu'O-PO}_3\text{S} \quad \text{O} \quad \text{V} \quad \text{CH} \]

(160)  (163)

** tert-Butyl [16O,18O]thiophosphate (160) (mono-triethylammonium salt) (13.5 mg, 50 µmol) was dissolved in dry methanol (0.4 ml) and triethylamine (7 µl, 50 µmol), and myrtenyl bromide (9 µl, 50 µmol) was added. The reaction mixture was stirred for 5 minutes at room temperature, by which time the reaction was shown to be complete by $^{31}$P n.m.r. spectroscopy, ($\delta p +44.4 \rightarrow 14.5$ p.p.m.).** Solvent evaporation under reduced pressure gave the S-alkylated thiophosphate (163).

**O-Benzoylation:**

\[ \text{Bu'O-PO}_3\text{S} \quad \quad \text{PhCOCl} \quad \quad \text{Bu'O-PO}_3\text{S} \quad \quad \text{O} \quad \text{OCOPh} \]

(163)  (164)

The S-alkylated compound (163) was dissolved in chloroform (0.3 ml) and triethylamine (7 µl, 50 µmol) and benzoyl chloride (17 µl, 150 µmol) was added. The reaction mixture was stirred for 10 minutes, by which time there was no starting material remaining as judged by $^{31}$P n.m.r. spectroscopy, ($\delta p +14.5 \rightarrow +18.6$ p.p.m.). The solvent was removed by evaporation under reduced pressure and the white solid recovered was washed with dry
ether (3 x 3 ml). The ether washings were then filtered and solvent evaporation gave a clear oil which was dissolved in CDCl₃ for immediate high-field ³¹P n.m.r. This showed the tert-butyl [¹⁶O,¹⁸O]thiophosphate, isolated from the solvolysis reaction to be totally racemic.

³¹P n.m.r. δ(121.5 MHz, CDCl₃):

 tert-Butyl [¹⁶O,¹⁸O]thiophosphate, +18.54 p.p.m. (s), +18.52 (s, ¹⁸O isotopic shift = 2.2 Hz), +18.49 (s, ¹⁸O isotopic shift = 5.7 Hz), +18.47 (s), +18.45 (s, ¹⁸O isotopic shift = 2.2 Hz), +18.42 (s, ¹⁸O isotopic shift = 5.7 Hz), (see Figure 6.17, Chapter 6).

Configurational analysis of 2-hydroxy-5-nitrobenzyl [¹⁶O,¹⁸O]thiophosphate (161) (isolated from the solvolysis reaction)

(161) was S-alkylated with myrtenyl bromide as before. This S-alkylated compound (~20 μmol) was dissolved in chloroform (150 μl) and triethylamine (5.6 μl, 40 μmol, 2 eq) and benzoyl chloride (8.1 μl, 70 μmol, 3.5 eq) was added. The reaction mixture was stirred for 10 minutes by which time the reaction was shown to be complete by ³¹P n.m.r. (δp +14.2 → +24.9 p.p.m.). The solvent was removed and the white solid recovered was washed with ether (3 x 3 ml). Filtration and evaporation of the ether washings gave a light yellow oil which was dissolved in CDCl₃ for high-field ³¹P n.m.r. This showed the 2-hydroxy-5-nitrobenzyl [¹⁶O,¹⁸O]thiophosphate (161) isolated from the solvolysis reaction to be 65% racemic, with 35% excess retention.

³¹P n.m.r. δ(121.5 MHz, CDCl₃); 2-hydroxy-5-nitrobenzyl [¹⁶O,¹⁸O]thiophosphate, +24.93 (s), +24.91 (s, ¹⁸O isotopic shift = 2.2 Hz), +24.89 (s, ¹⁸O isotopic shift = 5.8 Hz), +24.92 (s), +24.90 (s, 2.2 Hz), +24.88 (s, ¹⁸O isotopic shift = 5.7 Hz), (see Figure 6.18, Chapter 6).
The thiophosphate (161) (triethylammonium salt) (~30 μmol) was dissolved in methanol (0.25 ml) and triethylamine (4.2 μl, 30 μmol, 1 eq) and myrtenyl bromide (5.4 μl, 30 μmol, 1 eq) was added. The reaction mixture was stirred for 5 minutes at room temperature by which time the S-alkylation was shown to be complete by $^{31}$P n.m.r. ($\delta p +47.3 \rightarrow +15.5$ p.p.m.). Solvent evaporation gave the S-alkylated derivative as a yellow oil.

The above S-alkylated compound was dissolved in DMF (0.2 ml) and triethylamine (8.4 μl, 60 μmol, 2 eq) and dimethyl sulphate (56.8 μl, 0.6 mmol, 20 eq) was added. After 4 hours stirring at room temperature, the reaction was complete by $^{31}$P n.m.r. analysis ($\delta p +15.5 \rightarrow +27.7$ p.p.m.). The reaction mixture was diluted into water (20 ml) and extracted with ether (3 x 12 ml). The combined organic extracts were dried (MgSO$_4$) and then evaporated under reduced pressure. The resulting yellow oil was purified by flash silica chromatography (chloroform - 2% methanol, R$_f$ = 0.22) to give the derivatised compound, O-methyl-S-myrtenyl-(2-methoxy-5-nitrobenzyl) thiophosphate (165), as a light yellow oil (7 mg, ~55%) along with some O-methoxy-S-myrtenyl-(2-hydroxy-5-nitrobenzyl) thiophosphate. $^1$H n.m.r. (partial) $\delta$(300 MHz, CDCl$_3$) 3.50 p.p.m. (2H, m, SCH$_2$), 3.80 (3H, d, $J_{PH} = 12.8$ Hz, POCH$_3$), 3.95 (s, ArOCH$_3$), 5.20 (2H, br d, $J_{PH} = 9$ Hz, ArCH$_2$OP).
X-RAY CRYSTAL STRUCTURE DATA FOR THE THIONE (92)

Crystal data C₁₅H₁₇O₄N₂PS, M = 364.36, Tetragonal, space group = P4₁2₂, a = 8.933(18), b = 8.933(18), c = 46.257(38) Å, U = 3691.54 Å³, Z = 8, μ = 2.35 cm⁻¹, λ(Mo-Kα) = 0.7107 Å, F(000) = 1512 D, D₀ = 1.307 g cm⁻³.

The unit cell parameters were determined by least squares refinement of omega measurements for different layers. The intensities of 1787 unique reflections with 2θ < 45° and (+h, +k, +l) were measured on a Stoe STADI-2 Weissenberg diffractometer, with graphite monochromated Mo-Kα radiation using an omega-scan technique. The data were corrected for Lorentz and polarisation effects to yield 1219 reflections with I > 3σ(I).

The structure was solved using the TREF option of SHELXS. All subsequent calculations were carried out using the computer program SHELX-76. The hydrogen atoms H1 and H2 were located on the difference Fourier map, and refined as normal atoms. All other hydrogen atoms were included in calculated positions (C-H = 1.08 Å). The isotropic thermal parameters of the phenyl hydrogens were refined as a group. All other atoms were refined with anisotropic thermal parameters.

Final cycles of refinement employed a weighting parameter g(0.00022) {w = 1/[σ²(F) + g(F)²]} and gave the final residual indices R{=Σ(|Fo|−|Fc|)/Σ|Fo|} 0.0538 and RW{=ΣW(|Fo|−|Fc|)²/ΣW|Fo|²}¹/₂ 0.0464. The final difference Fourier was featureless and an analysis of the weighting scheme over |Fo| and sinθ/λ was satisfactory.

The geometry of the molecule is shown in Figure A.1.

Acknowledgements - Leicester University Computer Centre who provided support and facilities for X-ray single crystal calculations and G. M. Sheldrick for the use of SHELXS.

FIGURE A.1  X-ray crystal structure of cis (4S,5R)-2-(4-nitrophenoxy)-3,4-dimethyl-5-phenyl-1,3,2-oxazaphospholidine-2-thione (92).
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**TABLE A.3**

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### TABLE A.5

Non-bonded contacts (Å) for thione (92) \( \text{C}_{16}\text{H}_{17}\text{O}_{4}\text{N}_{2}\text{PS} \)

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