STEREOCHEMICAL STUDIES IN MECHANISTIC ENZYMOLOGY USING NUCLEOSIDE PHOSPHOROTHIOATES

A Thesis submitted for the degree of
Doctor of Philosophy in the Faculty of Science of
the University of Leicester

by

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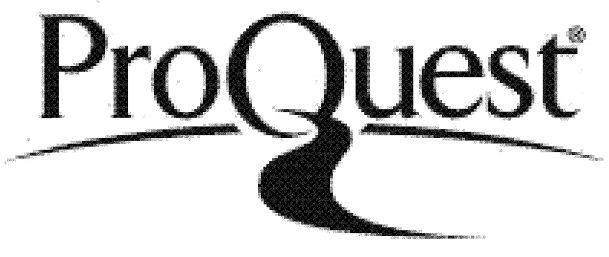
University of Leicester
1990



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Statement

The experimental work in this thesis has been carried out by the author in the Department of Chemistry of the University of Leicester, between October 1984 and October 1987. This work has not been, and is not currently being, presented for any other degree.

J Cummins .

University of Leicester,

January 1990.

To my parents

Acknowledgements

The author would like to thank her supervisor, Dr. B.V.L. Potter, for his constant interest and encouragement, and also members of the Organic Research Laboratory for their friendship.

Thanks also go to M. Robinson, and Dr. D. Turner for assistance with high-field ^{31}P.n.m.r. spectra.

Abstract

'STEREOCHEMICAL STUDIES IN MECHANISTIC ENZYMOLOGY USING NUCLEOSIDE PHOSPHOROTHIOATES'
Jane H.Cummins

A simple method for the configurational analysis of the four major 2'-deoxynucleoside 5'-[^{16}O,^{18}O] phosphorothioates,(1), and adenosine 5'-[^{16}O,^{18}O] phosphorothioate, (1a), is described. The method involves permethylation of (1,1a) with diazomethane or dimethyl sulphate, to generate the corresponding S-methyl-O-methyl phosphorothioate, (2,2a), δP(3:1 DMF:d_4-MeOH) +30ppm. Having assigned the diastereoisomers of (2,2a) to their corresponding ^{31}P.n.m.r. resonances, the ^{18}O-isotope is located by examination of $^{31}P(^{18}O)$ isotope shifts. A larger shift, (0.05ppm), is observed on the diastereoisomer with ^{18}O in the P=O position than in the P-OMe position, (0.02ppm).

Configurations of (2,2a) were assigned by partial hydrolysis of the Sp diastereoisomer of a 1:1 mixture of the diastereoisomers of 2'-deoxyribonucleoside- or adenosine 5'-O-methyl phosphorothioate diester, (3,3a), δP(3:1 DMF:d_4-MeOH) +58ppm, by snake venom phosphodiesterase. Subsequent methylation of (3,3a) yielded correspondingly Sp-deficient samples of (2,2a). In all cases Sp(2,2a) resonates downfield of Rp(2,2a) by ca. 0.08ppm whilst Sp(3,3a) resonates upfield of Rp(3,3a) by ca. 0.12 ppm, and shows shorter retention time on reverse-phase h.p.l.c.

The method is demonstrated by the stereochemical study of the hydrolysis of (3a) by bovine intestinal mucosa 5'-nucleotide phosphodiesterase, (EC 3.1.4.1), shown to proceed with overall retention of configuration at phosphorus, indicating the involvement of a covalent enzyme intermediate.

Mung bean nuclease, (EC.3.1.30.1), is shown to hydrolyse 5'-O-thymidyl 3'-O-(2'-deoxyadenosyl) phosphorothioate with inversion of configuration, consistent with a single step mechanism.

The iodine-mediated desulphurisation of a dinucleoside phosphorothioate, Sp 5'-O-(2'-deoxyadenosyl)-3'-O-thymidyl phosphorothioate, occurs with epimerisation at phosphorus in aqeous pyridine, but with 75% inversion of configuration in aqueous lutidine. These results implicate pyridine as a nucleophilic catalyst in the reaction, several displacements by pyridine causing loss of configuration at the phosphorus centre prior to displacement by water. Less nucleophilic lutidine shows reduced participation, allowing direct displacement of sulphur by water.

CONTENTS

Chapter Two

" Stereochemical Analysis of the Hydrolysis Reaction Catalysed by Bovine Intestinal Mucosa 5'-nucleotide Phosphodiesterase (EC 3.1.4.1.) "

Chapter Three

" Stereochemical Analysis of The Hydrolysis Reaction Catalysed by Mung Bean Nuclease, (EC 3.1.30.1)"

Chapter Four

"Stereochemical Analysis of the Iodine-mediated Desulphurisation of a Dinucleoside Phosphorothioate"

Experimental Details

Notes

In diagrams throughout this thesis the following notation has been used for isotopic labelling:

$O = {}^{16}O$

$\not{O} = {}^{17}O$

$\bullet = {}^{18}O$

Throughout this thesis the Cahn-Ingold-Prelog R,S nomenclature[1] has been used to describe absolute configuration.

In assigning R,S configurational symbols to chiral phosphates exchange of protons, fractional bond orders in anionic forms, and the involvement of double bonds which shift position in the fully protonated species are complicating factors, so that protons, double bonds, and negative charges are ignored when assigning configurational symbols, and are not usually included in structural formulae depicting chiral phosphorus centres. Double bonds are retained in fully alkylated chiral phosphoric esters.

Abbreviations and Symbols

A	Adenine
AMP	Adenosine 5′-phosphate
cAMP	Adenosine 3′-5′-cyclic phosphate
ADP	Adenosine 5′-diphosphate
ATP	Adenosine 5′-triphosphate
AMPS	Adenosine 5′-phosphorothioate
ADPαS	Adenosine 5′-O-(1-thiodiphosphate)
ATPαS	Adenosine 5′-O-(1-thiotriphosphate)
AMPS-OMe	Adenosine 5′-O-methyl phosphorothioate
$AMPS(OMe)_2$	Adenosine 5′-O,O-dimethyl phosphorothioate
AMPSMeOMe	Adenosine 5′-S-methyl-O-methyl phosphorothioate
dAMPS	2′-Deoxyadenosine 5′-phosphorothioate
dAMPS-OMe	2′-Deoxyadenosine 5′-O-methyl phosphorothioate
dAMP-SMe	2′-Deoxyadenosine 5′-S-methyl phosphorothioate
$dAMPS(OMe)_2$	2′-Deoxyadenosine 5′-O,O-dimethyl phosphorothioate
dAMPSMeOMe	2′-Deoxyadenosine 5′-S-methyl-O-methyl phosphorothioate
C	Cytosine
dCMP	2′-Deoxycytidine 5′-phosphate
dCMPS	2′-Deoxycytidine 5′-phosphorothioate
dCMPS-OMe	2′-Deoxycytidine 5′-O-methyl phosphorothioate
dCMP-SMe	2′-Deoxycytidine 5′-S-methyl phosphorothioate
$dCMPS(OMe)_2$	2′-Deoxycytidine 5′-O,O-dimethyl phosphorothioate
dCMPSMeOMe	2′-Deoxycytidine 5′-S-methyl-O-methyl phosphorothioate
G	Guanine
dGMP	2′-Deoxyguanosine 5′-phosphate
dGMPS	2′-Deoxyguanosine 5′-phosphorothioate
dGMPS-OMe	2′-Deoxyguanosine 5′-O-methyl phosphorothioate
dGMP-SMe	2′-Deoxyguanosine 5′-S-methyl phosphorothioate
$dGMPS(OMe)_2$	2′-Deoxyguanosine 5′-O,O-dimethyl phosphorothioate
dGMPSMeOMe	2′-Deoxyguanosine 5′-S-methyl-O-methyl phosphorothioate
T	Thymine
TMP	Thymidine 5′-phosphate
TTP	Thymidine 5′-triphosphate
TMPS	Thymidine 5′-phosphorothioate
TMPS-OMe	Thymidine 5′-O-methyl phosphorothioate

TMP-SMe	Thymidine 5′-S-methyl phosphorothioate
$TMPS(OMe)_2$	Thymidine 5′-O,O-dimethyl phosphorothioate
TMPSMeOMe	Thymidine 5′-S-methyl-O-methyl phosphorothioate
UDP	Uridine 5′-diphosphate
dNTPαS	2′-Deoxynucleoside 5′-O-(1-thiotriphosphate)
d[TpN]	5′-O-Thymidyl 3′-O-nucleoside phosphate
d[ApN]	5′-O-(2′-Deoxyadenosyl) 3′-O-nucleoside phosphate
d[TpA]	5′-O-Thymidyl 3′-O-(2′-deoxyadenosyl) phosphate
d[Tp(S)A]	5′-O-Thymidyl 3′-O-(2′-deoxyadenosyl) phosphorothioate
d[Ap(S)T]	5′-O-(2′-Deoxyadenosyl) 3′-O-thymidyl phosphorothioate
U>pS	Uridine 2′,3′-cyclic phosphorothioate
B	Adenine/guanine/cytosine/thymine
$(MeO)_2PSO^-Na^+$	Sodium O,O-dimethyl phosphorothioate
$(MeO)_2PO_2^-Na^+$	Sodium dimethyl phosphate
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
P_i	Inorganic phosphate
NAD^+	Nicotinamide adenine dinucleotide
FAD	Flavin adenine dinucleotide
CoA	Co-enzyme A
DEAE	Diethylaminoethyl
DMF	Dimethylformamide
DMSO	Dimethyl sulphoxide
d_6-DMSO	Deuteriated dimethyl sulphoxide
MeOH	Methanol
d_4-MeOH	Deuteriated methanol
TEAB	Triethylammonium bicarbonate
TEAA	Triethylammonium acetate
Tris	Tris(hydroxymethyl)methylamine
EDTA	Ethylene diamine-N,N′-tetraacetic acid
h.p.l.c.	High performance liquid chromatography
n.m.r.	Nuclear magnetic resonance
UV	Ultra violet
λmax	Wavelength of maximum absorption
Km	Michaelis constant
Ki	Inhibitor constant
V_o	Initial steady state reaction velocity
Vmax	Maximum possible V_o at fixed enzyme concentration

Results from this thesis have been published as follows:

1. "Stereochemical Evidence for a Phosphorylpyridinium Intermediate in the Iodine-mediated Desulphurisation of a Phosphorothioate Diester." Jane H. Cummins, and Barry V.L. Potter, Journal of the Chemical Society, Chemical Communications, p800-802, (1985).

2. "A Simple Method for the Configurational Analysis of a Deoxynucleoside 5'-[^{16}O,^{18}O,S] Phosphorothioate." Jane H. Cummins, and Barry V.L. Potter, Journal of the Chemical Society, Chemical Communications, p851-853, (1985).

3. "Mung Bean Nuclease Catalyses DNA Cleavage with Inversion of Configuration at Phosphorus." Michael R. Hamblin, Jane H. Cummins, Barry V.L. Potter, Biochemical Society Transactions, 14, p899-900, (1986).

4. "Stereochemical Mechanism of the Cleavage Reaction Catalysed by Bovine Intestinal Mucosa 5'-nucleotide Phosphodiesterase." Jane H. Cummins, Barry V.L. Potter, Biochemical Society Transactions, 14, p1289-1290, (1986).

5. "Mung Bean (*Phaseolus aureus*) Nuclease. A mechanistic investigation of the DNA-cleavage reaction using a dinucleoside phosphorothioate." Michael R. Hamblin, Jane H.Cummins, Barry V.L. Potter, Biochemical Journal, 241, p827-833, (1987).

6. "On the Mechanism of Action of Bovine Intestinal Mucosa 5'-nucleotide Phosphodiesterase. Stereochemical evidence for a nucleotidyl-enzyme intermediate." Jane H. Cummins, Barry V.L. Potter, European Journal of Biochemistry, 162, p123-128, (1987).

7. 'A General Chemical Method for the Sterochemical Analysis of Nucleoside 5'-[^{16}O,^{18}O] Phosphorothioates." Jane H. Cummins, and Barry V.L. Potter, Phosphorus and Sulphur, 30, p589-592, (1987).

INTRODUCTION

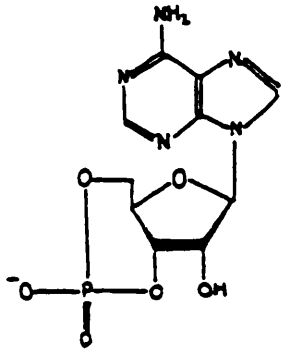
Nucleotides are of central importance in metabolism, and figure largely in the group of molecules that are involved in the metabolic pathways of all forms of life[2]. They participate in nearly all metabolic processes; for example, as the nucleoside 5′-triphosphates they are the activated precursors of the nucleic acids, DNA and its transcript RNA, into which they are incorporated by the nucleotide polymerases. The nucleotide ATP, (adenosine 5′-triphosphate), (Figure I.1), has a central role in energy exchanges in biological systems. It is formed from ADP and P_i using the free energy derived from the oxidation of fuel molecules by chemotrophs, or from light energy trapped by phototrophs, and the free energy released on enzyme-catalysed hydrolysis of its phosphoanhydride bonds is made available to drive thermodynamically unfavourable metabolic processes. The turnover of ATP is very high; during strenuous exercise the rate of consumption by a human may be as high as 0.5 kg per minute.

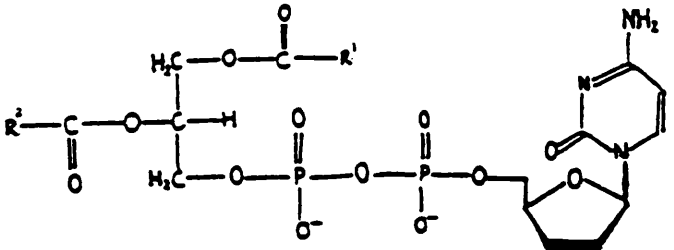
Nucleotide derivatives are activated intermediates in many biosynthetic processes[3], for example, S-adenosyl methionine is the major donor of methyl groups, and cytidine diphosphodiacyl glycerol is the activated intermediate in the synthesis of phosphoglycerides. Adenine nucleotides are components of many coenzymes, including NAD^+ and FAD, which are major electron acceptors in the oxidation of fuel molecules, the reduced form of NAD^+ being the primary electron donor for ATP formation; and Coenzyme A, a carrier of activated acyl groups, in particular bringing activated acyl units into the citric acid cycle for complete oxidation to CO_2. Nucleotide derivatives also act as metabolic regulators[4], cyclic AMP being central to the

Figure I.1

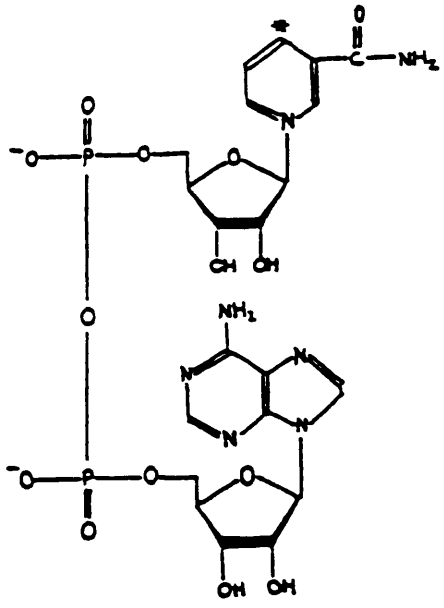
Examples of Nucleotides of Central Importance In Metabolism

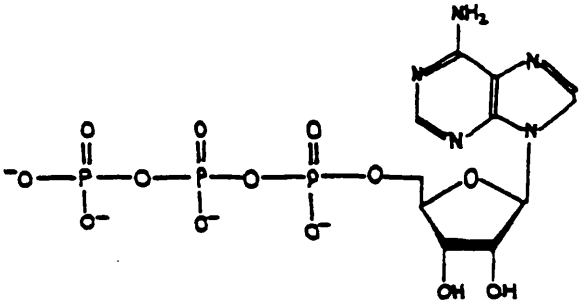
Cyclic adenosine 3',5' monophosphate (cAMP)





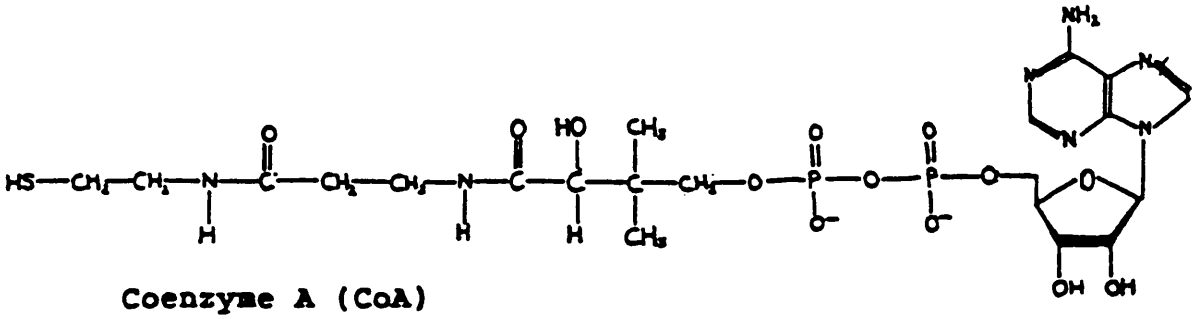
Cytidine diphosphodiacyl-glycerol





Adenosine Triphosphate (ATP)

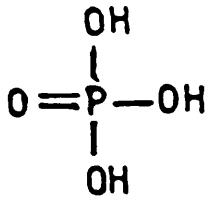
Nicotinamide adenine dinucleotide (NAD)



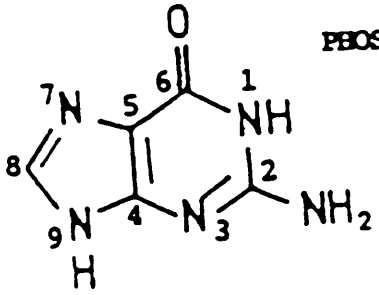
Coenzyme A (CoA)

Figure I.2

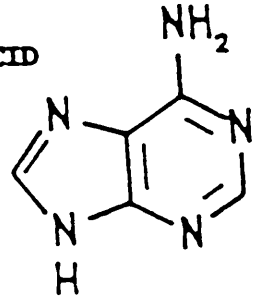
Components of Nucleotides



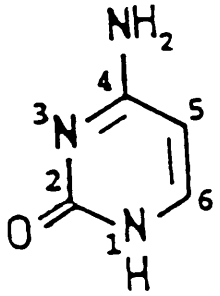
PHOSPHORIC ACID



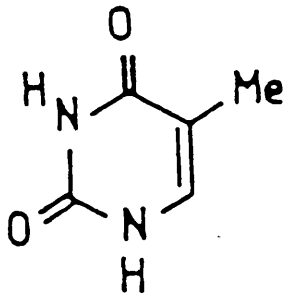
GUANINE



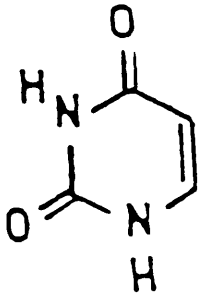
ADENINE



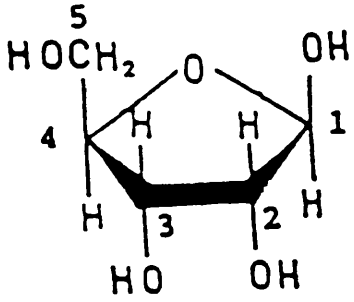
CYTOSINE



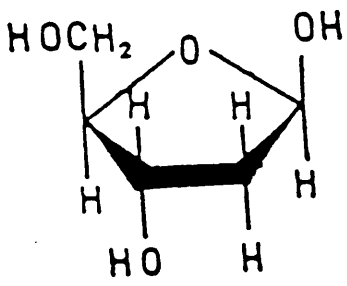
THYMINE



URACIL



β-D-RIBOSE



β-2'-DEOXY-D-RIBOSE

co-ordinated control of glycogen synthesis and degradation, and a mediator of the action of many hormones. Figure I.1 shows examples of some of the most important nucleotide derivatives.

STRUCTURE OF NUCLEOSIDES AND NUCLEOTIDES

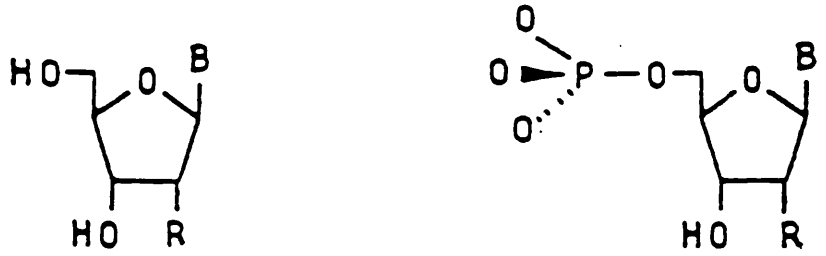
A nucleoside consists of a nitrogenous base; either a purine or a pyrimidine derivative, attached to a pentose sugar unit, either a D-ribose or 2'-deoxy-D-ribose, via a β-glycosidic linkage to C-1'. The major purine derivatives are Adenine and Guanine, and the major pyrimidines are Cytosine, Thymine, and Uracil. (Figure I.2). The pyrimidine bases are attatched to the sugar unit via N-9, and the pyrimidines by N-1. There are two series of nucleosides corresponding to D-ribose and 2'-deoxy D-ribose. In the deoxy- series Uracil is replaced by its C-5' methylated analogue Thymine, whilst Thymine does not occur in the ribo-series, where it is replaced by Uracil.

A nucleotide is a phosphate ester of a nucleoside in which at least one of the hydroxyl groups of the pentose moiety is esterified, the most common site being C-5'. As for nucleosides, there is a ribo- and a 2'- deoxy-ribo series, depending on the nature of the sugar component. Figure I.3 and its accompanying key demonstrates the structure and nomenclature of some of the common nucleosides and nucleotides.

The nucleic acids, DNA and RNA, are covalently linked chains of 2'-deoxy-ribo- and ribo- nucleotides respectively, in which phosphodiester bridges link the 3'-hydroxyl of one nucleotide with the 5'-hydroxyl of the next. (Figure I.4). Duplex DNA consists of two antiparallel polynucleotide chains arranged in a

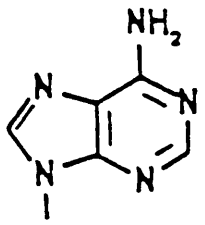
Figure I.3

General Structure of Nucleosides and Nucleotides

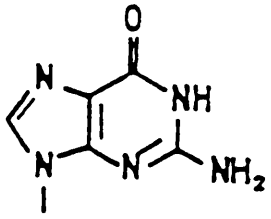


R = H, OH

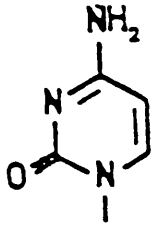
B =



(a)



(b)



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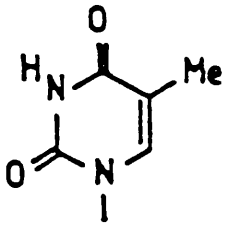
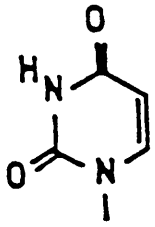


(d)



(e)

Key to Figure I.3

R =OH, RIBO SERIES

B	BASE	NUCLEOSIDE(1)	NUCLEOTIDE(2)
a	ADENINE	ADENOSINE	ADENOSINE 5'-MONOPHOSPHATE (adenylic acid, AMP)
b	GUANINE	GUANOSINE	GUANOSINE 5'-MONOPHOSPHATE (guanylic acid, GMP)
c	CYTOSINE	CYTIDINE	CYTIDINE 5'-MONOPHOSPHATE (cytidylic acid, CMP)
e	URACIL	URIDINE	URIDINE 5'-MONOPHOSPHATE (uridylic acid, UMP)

R = H, 2'-DEOXY RIBO SERIES

B	BASE	NUCLEOSIDE(1)	NUCLEOTIDE(2)
a	ADENINE	2'-DEOXYADENOSINE	2'-DEOXY ADENOSINE 5'-MONOPHOSPHATE (2'-deoxyadenylic acid, dAMP)
b	GUANINE	2'-DEOXYGANOSINE	2'-DEOXYGUANOSINE 5'-MONOPHOSPHATE (2'-deoxyguanylic acid, dGMP)
c	CYTOSINE	2'-LEOXYCYTIDINE	2'-DEOXYCYTIDINE 5'-MONOPHOSPHATE (2'-deoxycytidylic acid, dCMP)
d	THYMINE	2'-DEOXYTHYMIDINE	2'-DEOXYTHYMIDINE 5'-MONOPHOSPHATE (2'-deoxythymidiylic, acid dTMP)

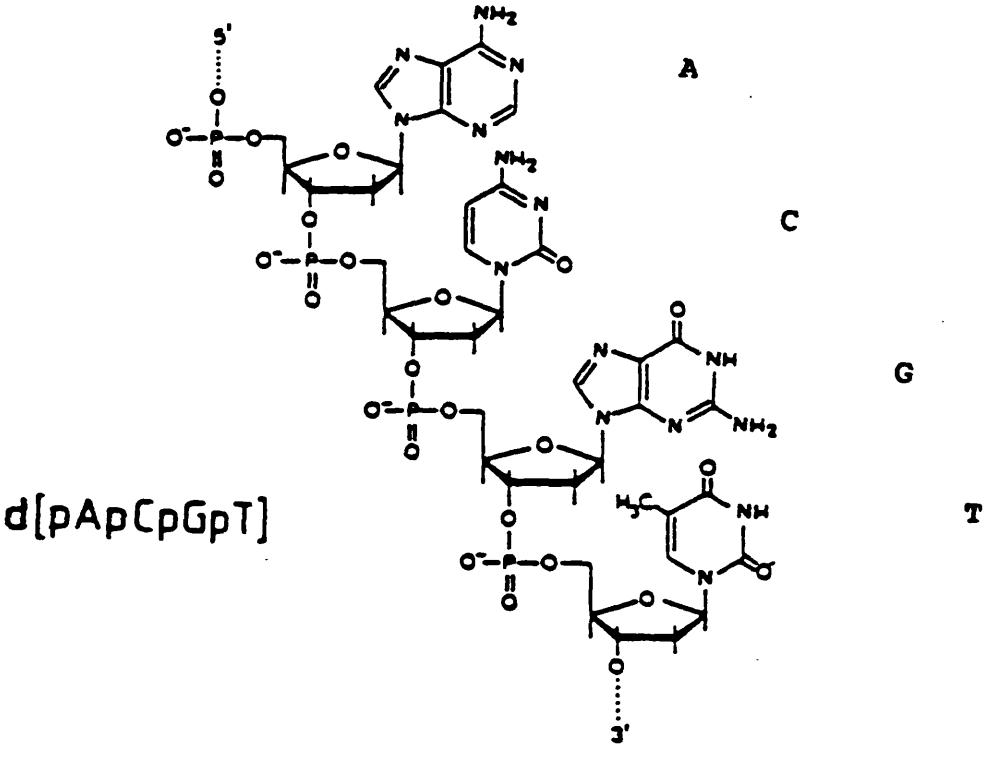
double helix about a common axis; the hydrophilic sugar phosphate backbones form the outer surface of the molecule, and the hydrophobic bases are directed towards the inside. The adenine bases in each chain are always paired with the thymine bases in the other chain, and guanine bases are always paired with cytosine bases, each purine-pyrimidine pair being linked by hydrogen bonds, as shown in Figure I.4.

Because of the central role of nucleotides in metabolism considerable interest has been focussed on the way in which they interact with the metabolic enzymes that handle them.

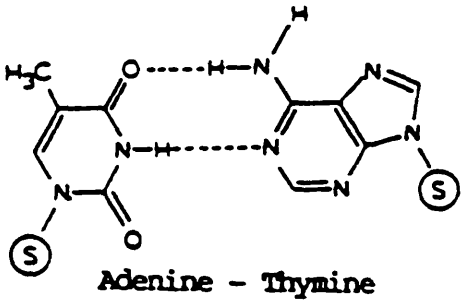
A wide range of enzymes are known which catalyse displacement at phosphorus centres, with transfer of the phosphoryl, or nucleotidyl group to various acceptor molecules, including water, carboxylic acids, alchohols, nitrogenous compounds, and phosphoryl compounds, (figure I.5). Although in most cases the overall reaction has been elucidated, the mechanistic details of the steps involved are often not understood.

Figure I.4

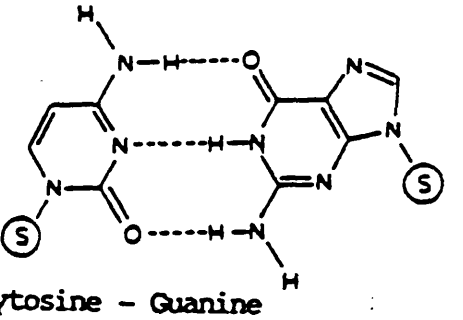
Watson-Crick Structure For DNA



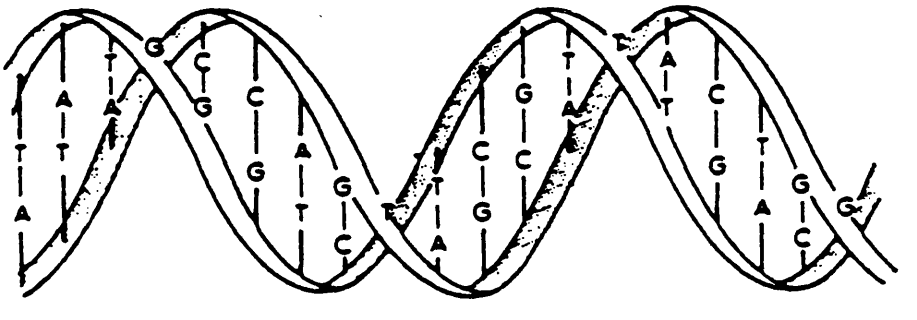
Hydrogen bonding between purine-pyrimidine base pairs in duplex DNA



Adenine - Thymine



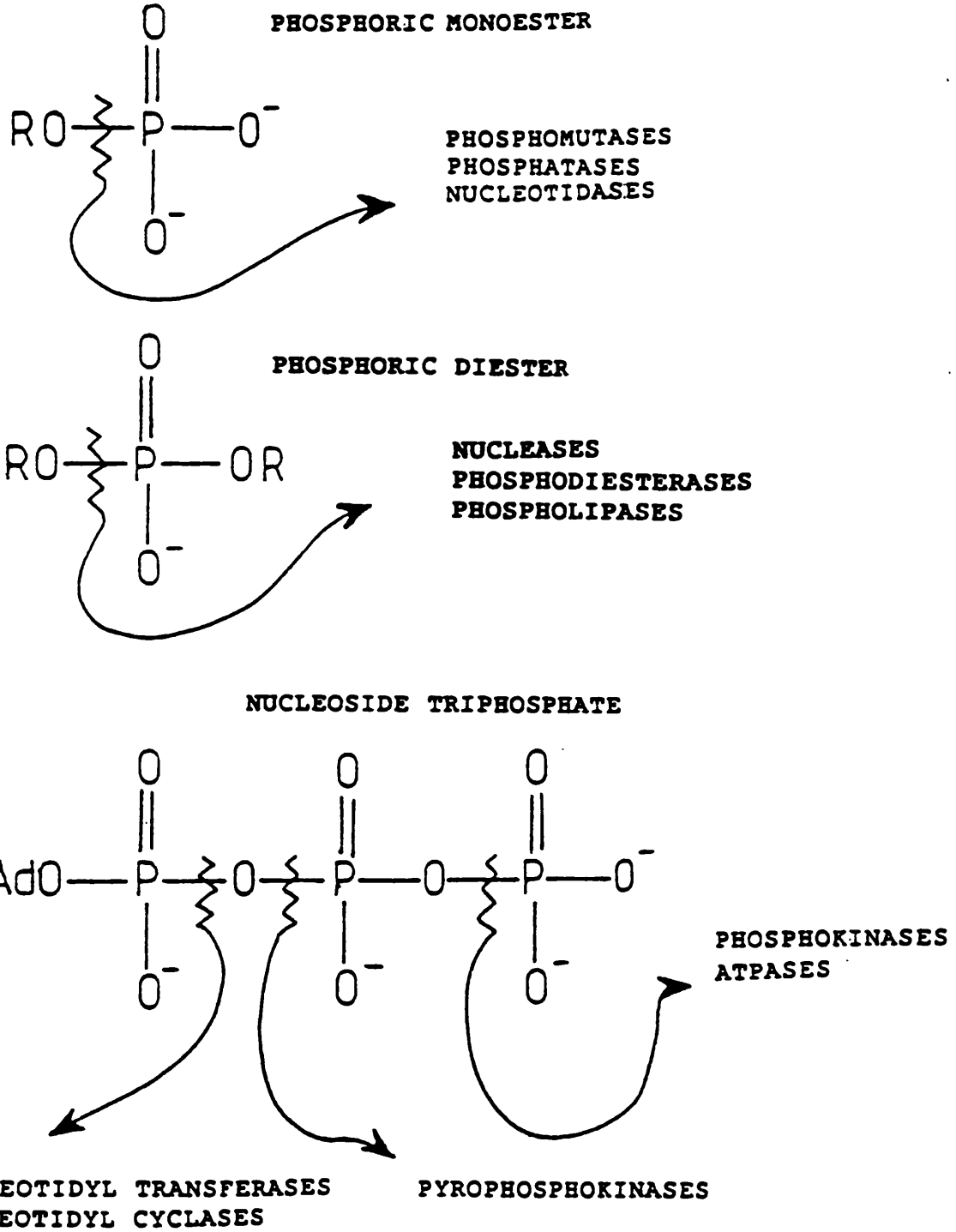
Cytosine - Guanine



Double helix Structure of DNA

Figure I.5

Summary of The Classes of Enzymes That Catalyse Displacement at Phosphorus



Nucleoside Phosphorothioates as Substrate Analogues;

Analogues of naturally occurring substrates have wide application in the elucidation of the nature and mechanisms of enzyme reactions, and have been synthesized as inhibitors, suicide substrates, transition state analogues, and spectroscopic probes. They differ from the natural substrate in one or more properties that make them suitable for use in investigation of various aspects of enzyme action.

Nucleoside phosphorothioates are analogues of nucleotides in which the non-bridging oxygen atom of a phosphate group is replaced by sulphur[5-7]. This modification can be introduced into almost all nucleoside phosphates, the sulphur atom being incorporated into nucleoside monophosphate, and at the α, β, or γ-phosphate of a nucleoside di-, or triphosphate, or at an internucleotidic phosphate linkage. In many cases these substrate analogues are more stable against enzyme-catalysed hydrolysis than the natural substrates,[8-10] facilitating the determination of the importance of bond cleavage for a particular nucleotide-mediated effect, the degree of reduction of hydrolysis rates relative to normal substrates varying between enzymes. In contrast phosphorothioates are often good substrates for enzyme-catalysed reactions where the phosphorothioate or nucleotide group is transferred to an acceptor other than water. Thus the enzymatic incorporation of phosphorothioate groups into DNA by the action of DNA polymerases on dNTPαS proceeds quite efficiently,[9,11] leading to the formation of internucleotide linkages which are generally hydrolysed more slowly than the normal phosphate linkages, often to the extent that hydrolysis is

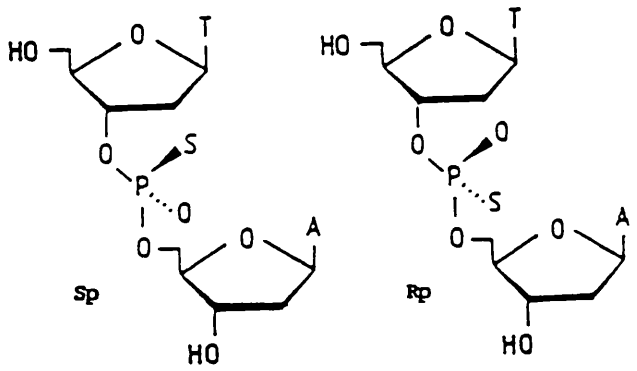
not detectable. Substitution of sulphur for oxygen occurs with retention of the essential features of the normal phosphate group, such as structure and negative charge, so that the overall DNA structure is not seriously disrupted. However charge distribution is likely to differ considerably, charge being localised on the non-bridging sulphur atom of the phosphorothioate linkage[11a], rather than being shared between two non-bridging oxygens in a normal phosphate linkage.

A particularly useful feature of nucleoside phosphorothioates arises from the fact that substitution of oxygen by sulphur confers chirality on the phosphorothioate centre when two or more non-equivalent residues are linked to it. Since nucleosides themselves are chiral this leads to the existence of diastereoisomers of nucleoside phosphorothioates, the number, (2^n), of which depends on the number, n, of phosphorothioate centres. This opens the way for use of these compounds in investigations of the stereochemical course of enzyme-catalysed reactions, and the stereoselectivity of an enzyme for a particular diastereoisomer of a nucleoside phosphorothioate. Figure I.6 shows the diastereoisomers of a dinucleoside phosphorothioate arising from the substitution of sulphur for a non-bridging oxygen atom in the corresponding dinucleoside monophosphate.

Stereochemical Studies Using Nucleotide Phosphorothioates

The stereochemical course of any chemical reaction is dictated by the structure of the transition state of that reaction, or in multistep reactions, by the structures of the transition states and intermediates, so that reaction mechanisms and

Figure I.6
Diastereoisomers of d[Tp(S)A]



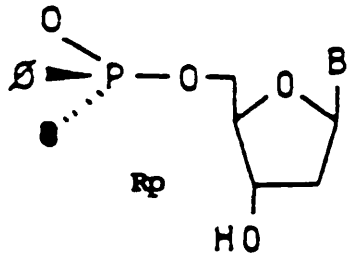
stereochemistry are closely related. A knowledge of the stereochemical course of a reaction is generally held to be one of the most useful kinds of information for distinguishing between possible reaction mechanisms, and this applies equally to enzyme-catalysed reactions.

In order to follow the stereochemical course of a given reaction it is necessary to have chirality in both substrate and product, and suitable methods for their configurational analysis. Stereochemical studies of enzyme-catalysed phosphoryl and nucleotidyl transfer reactions have relied on two major approaches, these being the use of isotopic substitution with the heavy isotopes of oxygen[12,13], to give isotopically chiral phosphates, and the incorporation of sulphur and one or more of the isotopes of oxygen to generate chiral phosphorothioates[5-7], (figure I.7).

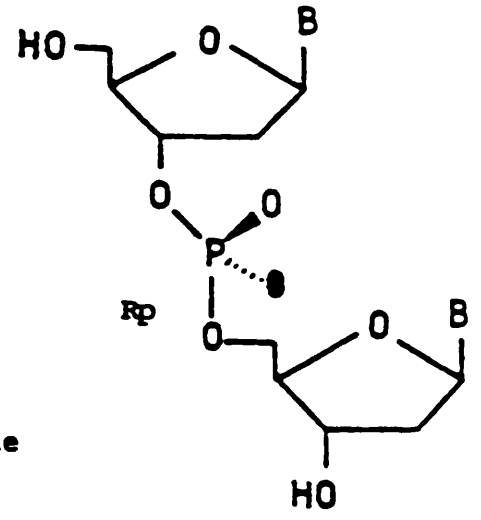
The phosphorothioate approach to the stereochemical analysis of nucleotidyl and phosphoryl transfer reactions was the earliest, and synthetically most straightforward. Phosphorothioate diastereoisomers can be separated by conventional chromatographic

Figure I.7

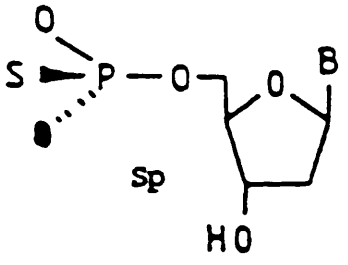
Isotopically Chiral Phosphates and Thiophosphates



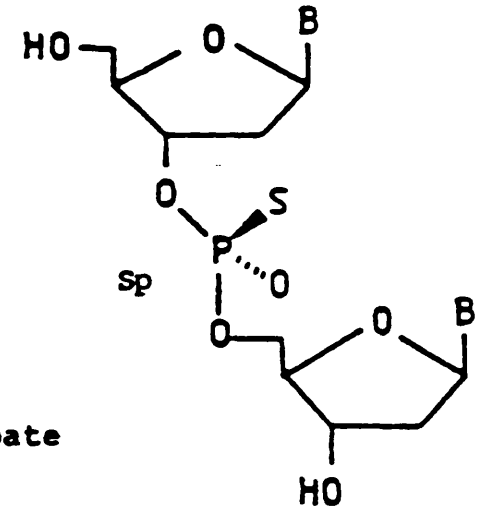
Nucleoside 5′-phosphate



Dinucleoside 3′-5′-phosphate



Nucleoside 5′-phosphorothioate



Dinucleoside 3′-5′-phosphorothioate

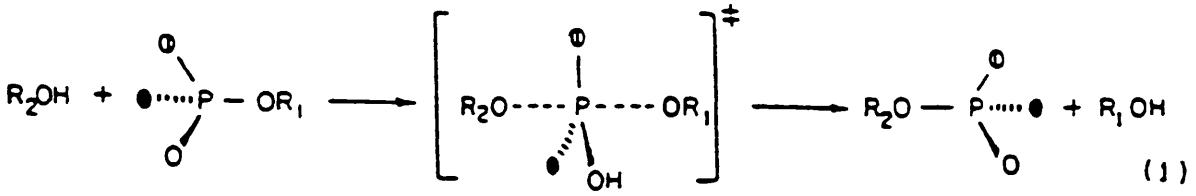
techniques, and the ability of a number of enzymes to distinguish between the pro-chiral oxygen atoms of a thiophosphoryl group can be exploited in their synthesis and configurational analysis. The isotopically chiral phosphate approach was developed as a result of fears that phosphorothioates might be handled differently by enzymes than the natural substrate, so that studies based on their use might give misleading results. Syntheses of oxygen chiral phosphate esters must be stereospecific as the isotopomers cannot be separated by physical methods.

It is with the phosphorothioate substrate analogue approach that the work in this thesis is primarily concerned.

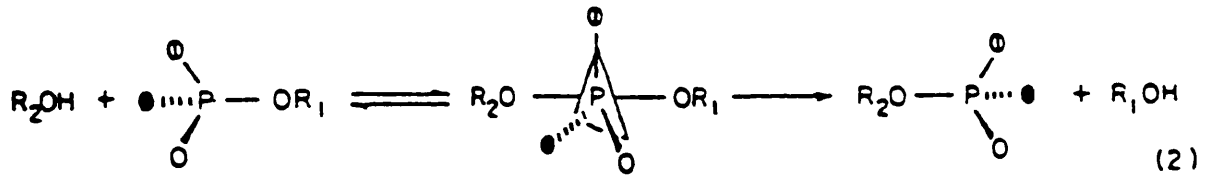
Stereochemistry of Substitution in Phosphates

On the basis of detailed studies of non-enzymatic reactions, mechanisms of nucleophilic substitution at phosphorus in phosphates have been proposed[14,15], and these are the chemical mechanisms that may be considered for enzymatic reactions[5,16]. As in carbon chemistry, stereochemistry is a useful means of distinguishing between them. The two major types of mechanism can be described as ASSOCIATIVE and DISSOCIATIVE, these representing the extremes between which the mechanism of a given reaction may fall.

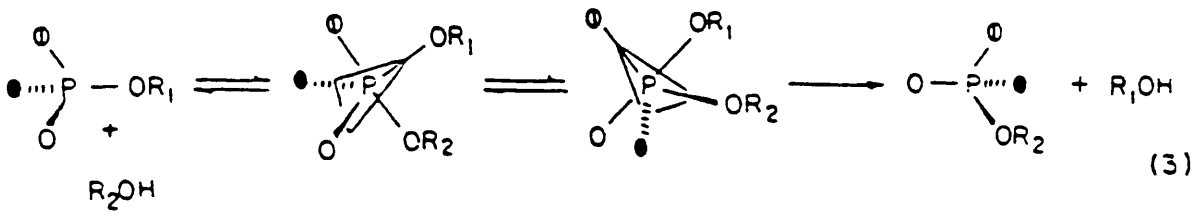
In an associative mechanism the tetra co-ordinated phosphorus atom expands its co-ordination number to five to generate a penta co-ordinate trigonal bipyramidal species.



Equation 1 represents such a mechanism for an oxygen chiral phosphate ester; the displacing nucleophile attacks from the side opposite the leaving group, displacing it in a single step via a penta co-ordinate trigonal bipyramidal transition state. The stereochemical consequence of this mechanism of nucleophilic substitution at phosphorus would be inversion of configuration at the chiral phosphorus centre.



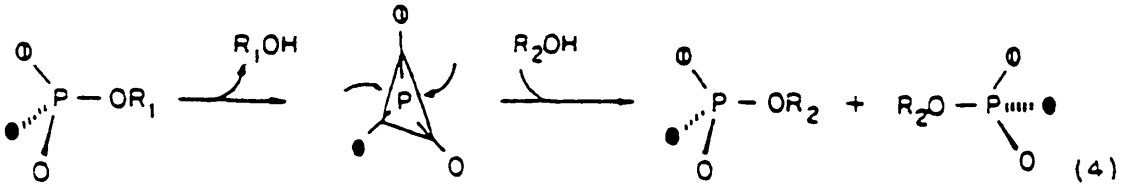
Equation 2 represents an alternative associative mechanism, differing from equation 1 in the involvement of an intermediate; the attacking nucleophile approaches from the side opposite to that from which the leaving group will depart, and forms a bond to the phosphorus atom. The resulting pentacovalent trigonal bipyramidal intermediate shown has both nucleophile and leaving group in apical positions, the other three substituents being in the equatorial plane. The intermediate decomposes by departure of the apical leaving group, with formation of products, the stereochemical consequence of this mechanism being inversion of configuration at the phosphorus centre.



(3)

Equation 3 represents a further associative-type mechanism differing from that of equation 2 in that the leaving group is in the equatorial plane of the first trigonal bipyramidal intermediate, known as ADJACENT ATTACK. Since, by the rules governing the formation and breakdown of such intermediates[15], the leaving group cannot depart from this position, a pseudorotatory rearrangement of the first intermediate must occur so as to bring the leaving group into an apical position, from which it can depart.[17] Pseudorotation can occur in two ways; either ^{16}O or ^{18}O of the oxygen chiral phosphate in Equation 3 may serve as the pivot, but both routes occur with an overall stereochemical consequence of retention of configuration at the phosphorus centre[18].

In the dissociative type pathway of substitution the co-ordination number of phosphorus decreases from four to three, to produce a monomeric "metaphosphate" intermediate which adds to a nucleophile to yield product.



(4)

Equation 4 represents a dissociative SN_1P-type of mechanism in which the leaving group is expelled in the rate-limiting step by the phosphate undergoing substitution, generating a planar electrophilic intermediate, analagous to the carbonium ion of carbon chemistry. Since metaphosphate is planar, and can capture the nucleophile at either face, this mechanism might be expected to result in loss of configuration at chirally substituted phosphates.

Studies of reactions expected to proceed via a metaphosphate intermediate indicate that only in aprotic solvents, such as acetonitrile, does phosphoryl transfer occur with extensive racemisation at the chiral centre[19,20], whilst in protic solvents, such as alchohol/water mixtures, inversion of configuration is observed[21-23]. The observation of inversion of configuration in protic solvents for a dissociative mechanism, such as the hydrolysis of monoesters of phosphoric acid, can be explained in terms of a PREASSOCIATIVE reaction pathway[24,25]. In protic solvent the lifetime of the metaphosphate monoanion generated by the dissociation is so short that it does not escape the solvent cage in which it is formed before reaction with a nucleophilic solvent molecule; hence the stereochemistry of the substitution is controlled. It might be expected that a similar

preassociative condition would apply to reaction at an enzyme active site since a substrate molecule bound at an enzyme active site is held in position with respect to nucleophile, be it an enzyme functional group or a bound water molecule, so that inversion is likely to be the stereochemical result of what is strictly a dissociative mechanism. Hence, whilst the observation of racemisation in an enzyme catalysed transfer would provide positive evidence for a dissociative mechanism involving a free metaphosphate intermediate, observation of inversion is ambiguous[14] as to the nature of the mechanism of the transfer reaction.

Results of stereochemical studies to date[5-7,16,26] of enzyme-catalysed substitution in biological phosphates, using either oxygen-isotope labelled substrates or chiral [^{16}O,^{18}O] phosphorothioate analogues of substrates indicate that substitution proceeds by an "in-line" mechanism which inverts the configuration at the chiral phosphorus centre, consistent with equations 1 and 2, but not with the adjacent attack of equation 3, which has not been observed in enzyme reactions. The metaphosphate mechanism of equation 4 is also a possibility in view of the likelihood that interactions of such an intermediate with the active site of an enzyme, protecting it from capture by water, would also control the stereochemical course, and prevent loss of configuration. To explain the data in terms of equation 4 it must be assumed that these interactions lead to in-line attack, and inversion of configuration at phosphorus. **Thus a single enzyme catalysed substitution at phosphorus is accompanied by inversion of configuration at the phosphorus centre.**

Since each enzymatic substitution proceeds with inversion of

configuration at phosphorus, the overall stereochemistry observed for a reaction provides information as to the NUMBER of substitution steps in the catalytic pathway. This information is important in determining whether catalysis involves a bringing together of reactants at the active site with direct transfer of the phosphoryl group in a single displacement, (5), or whether covalent catalysis by an enzymic nucleophile occurs with bond formation between substrate and active site in a double displacement pathway leading to the formation of a phosphorylated enzyme intermediate E-XP, (6).

$$A\text{-}P + BH \rightleftharpoons AH + B\text{-}P \qquad (5)$$

$$E\text{-}XH + A\text{-}P \rightleftharpoons E\text{-}XP + AH \qquad (6)$$
$$E\text{-}XP + BH \rightleftharpoons E\text{-}XH + BP$$

The observation of inversion of configuration indicates that the enzyme catalysed reaction proceeds via a single displacement at phosphorus, without formation of a covalent substrate-enzyme intermediate, whilst retention of configuration indicates the formation and breakdown of a covalently-bound phosphoryl or nucleotidyl enzyme intermediate in the reaction pathway, requiring two displacements at phosphorus. Prior to the stereochemical test the majority of mechanistic information was obtained from kinetic studies, allowing the order in which substrates bind and dissociate from the enzyme to be determined. The only basis on which to assign a single-displacement pathway was failure to isolate and characterise a phosphorylated enzyme intermediate E-XP, the usual non-stereochemical evidence being unable to provide a positive indication of a single displacement.

Stereochemical analysis is now recognised as being the best way of obtaining information concerning the participation or not of covalent enzyme-bound intermediates in enzyme-catalysed phosphoryl transfer reactions.

Although the observation of retention of configuration might be explained in terms of the mechanism of equation 3, involving pseudorotation of a penta-cordinate intermediate, for most enzymes for which retention has been observed[7] there has been strong evidence for the existence of a covalent enzyme intermediate, (either it has been isolated, or kinetic data indicates its involvement). Thus, to date, there is no evidence compelling consideration of more complex reaction mechanisms, invoking pseudorotation, more than two displacements at phosphorus, or the ambiguous breakdown of a potential enzyme acyl-phosphate intermediate[16].

In some cases the stereochemical course of an enzyme-catalysed reaction has been determined using a chiral phosphorothioate analogue, and an isotopically chiral analogue, and in all cases the stereochemical outcome has been the same[7,26]. This result dispels doubts as to the relevance of the stereochemical conclusions drawn from studies using chiral phosphorothioate analogues of the natural substrates.

Table I.1 shows a summary of enzymes investigated to date, with references[7].

TABLE I.1

RESULTS OF STEROCHEMICAL INVESTIGATIONS OF ENZYMES

Enzyme	Stereochemical Course	Method: Phosphoro-thioate	Method: Oxygen Isotope
Nucleotide Transferases			
Ribonuclease A			
(transesterification)	inversion	27,28	
Ribonuclease T_1	inversion	29	
Ribonuclease T_2	inversion	10	
Enterobacter aerogenes phosphohydrolyase	inversion	30	
E.coli-dependent RNA polymerase;			
initiation	inversion	31	
elongation	inversion	32	
DNA-dependant DNA polymerase			
E.coli I	inversion	9,33	
Phage T4	inversion	34	
Phage T7	inversion	35	
Micrococcus luteus	inversion	36	
Reverse transcriptase	inversion	8	
Polynucleotide phosphorylase			
exchange	retention	37	
elongation	inversion	38	
t-RNA Nucleotidyl transferase	inversion	39	
RNA-ligase			
ligation step	inversion	40	
UDP Glucose pyrophosphorylase	inversion	41	
Galactose-1-phosphate-uridyl transferase	retention	41	
Adenylate cyclase			
bacterial	inversion	42	43
mammalian	inversion	44	
Guanylate cyclase	inversion	45	45
Acetyl CoA-synthetase			
activation step	inversion	46,47	
Tyr-tRNA synthetase			
activation step	inversion	48	49

Enzyme	Stereochemical Course	Method	
		Phosphoro-thioate	Oxygen Isotope
Met-tRNA synthetase activation step	inversion	48	50
Phe-tRNA synthetase activation step	inversion	51	
Ileu-tRNA synthetase activation step	inversion	52	
ATP Sulphurylase	inversion		53
Phospholipase D	retention		54
NAD Phosphorylase	inversion		55
Calf spleen phosphodiesterase (nucleotidyl transferase activity)	retention	102	
Nucleases			
Ribonuclease A hydrolytic step	inversion	56	
Cyclic nucleotide phosphodiesterase (bovine)	inversion	57,58	59,60
Cyclic AMP Phosphodiesterase (yeast)	inversion	58	
Snake venom phosphodiesterase	retention	61,62	63,64
Nuclease S1	inversion	65	
Restriction endonuclease EcoRI	inversion	66	
3'—>5' exonuclease of T4 DNA polymerase	inversion	67	
Nuclease P1	inversion		68
DNAse 1 (bovine pancreas)	inversion		69
Exonuclease 1 (E.coli)	inversion	70	
Mung Bean nuclease	inversion	101	
Bovine Intestinal 5'-nucleotide phosphodiesterase	retention	100	
Bovine Spleen Phosphodiesterase	retention		178
Kinases			
Acetate kinase	inversion		78
Adenosine kinase	inversion	72	
Adenylate kinase	inversion	41,73	

Enzyme	Stereochemical COURSE	Method PHOSPHORO-THIOATE	Method OXYGEN ISOTOPE
Creatine Kinase	inversion		74
Glucokinase	inversion		75
Glycerol Kinase	inversion	76,77	77,78
Hexokinase	inversion	76	78,163
Nucleoside diphosphate kinase	retention	41	
Nucleoside phosphotransferase	retention	80	
Phosphofructokinase	inversion		80
Phosphoglycerate kinase	inversion	86,87	
Polynucleotide kinase	inversion	77,81	82
Pyruvate kinase	inversion	76	78,83
Ribulose phosphate kinase	inversion	84	
Thymidine kinase (herpes simplex 1)	inversion		85
Phosphatase and NTPase			
Acid phosphatase	retention		88
Alkaline phosphatase	retention		89
Mitochondrial ATPase	inversion	87	
Myosin ATPase	inversion	90	
Sarcoplasmic reticulum ATPase	retention	91	
Elongation factor G GTPase	inversion	92	
Elongation factor T GTPase	inversion	93	
Thermophilic bacterium PS3 ATPase	inversion	94	
Pyrophosphatase	inversion	95	
Glucose-6-phosphatase	retention	79a	
Snake venom 5'-nucleotidase	inversion	86	
Phosphoenol pyruvate carboxylase	inversion	96	
Adenylosuccinate Synthetase	inversion	97	
Phosphoenol Pyruvate carboxykinase	inversion	98	
Mutases			
Phosphoglucomutase	retention		79
Phosphoglycerate mutase (muscle)	retention		99
Phosphoglycerate mutase (Wheatgerm)	retention		99

CHAPTER ONE

A GENERAL METHOD FOR THE CONFIGURATIONAL ANALYSIS OF NUCLEOSIDE 5'-[^{16}O,^{18}O] PHOSPHOROTHIOATES.

Requirements for Stereochemical Studies:

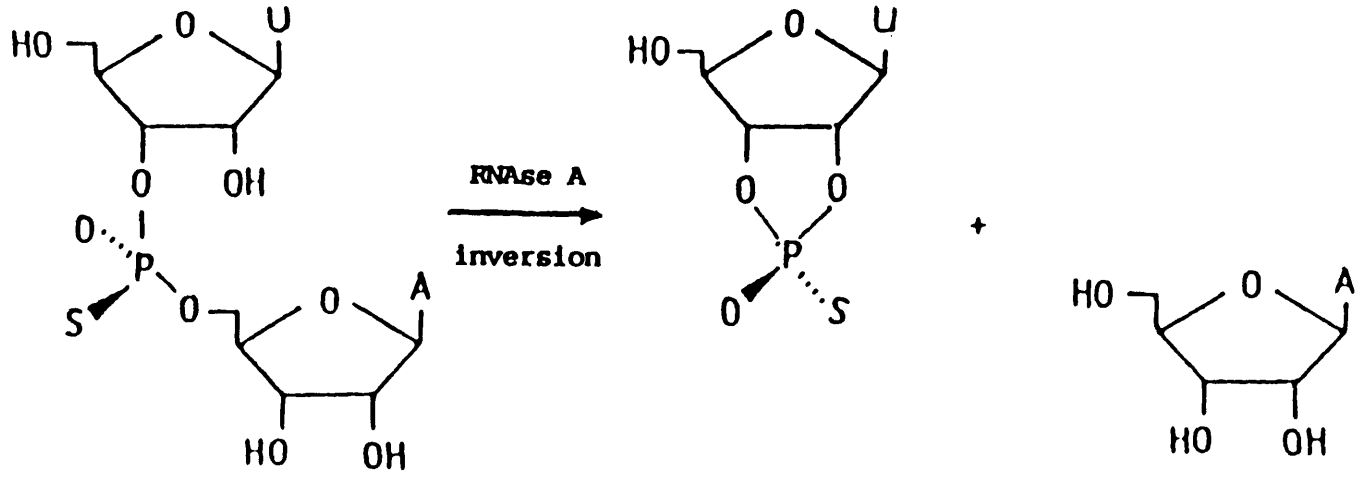
Stereochemical analysis of many enzyme-catalysed phosphoryl transfer reactions has provided insight into the probable mechanisms of such reactions. These investigations require chirality at the phosphorus centre in both substrate and product, and methods for the configurational analysis of the latter. The two major approaches to this type of study have utilized substrate analogues with chirality at the active phosphorus by virtue of substitution with the stable isotopes of oxygen[12,16,17], or by use of one heavy oxygen isotope and sulphur in a chiral phosphorothioate analogue[5-7]. It is with the first-developed, and experimentally easier phosphorothioate approach that the work reported in this thesis is primarily concerned.

The level of isotopic substitution necessary for an investigation of an enzyme-catalysed substitution at phosphorus using a phosphorothioate substrate analogue depends upon the nature of the reaction catalysed by the enzyme under investigation. Thus, nucleotidyl transfer reactions which occur by way of a transesterification, in which both substrate and product are diastereoisomeric nucleoside phosphorothioates, require no isotopic substitution, and stereochemical analysis is relatively straightforward. Configurations can be determined by h.p.l.c. or ^{31}P n.m.r. spectroscopy by comparison with diastereoisomers of known configuration obtained by chemical synthesis, or by further degradation using a nuclease with established specificity for one diastereoisomer. The polymerisation reactions catalysed by the nucleoside triphosphate

Figure 1.1

Investigation of Intramolecular Esterification

Catalysed By RNAse A



Rp Up(S)A

endo U>pS

adenosine

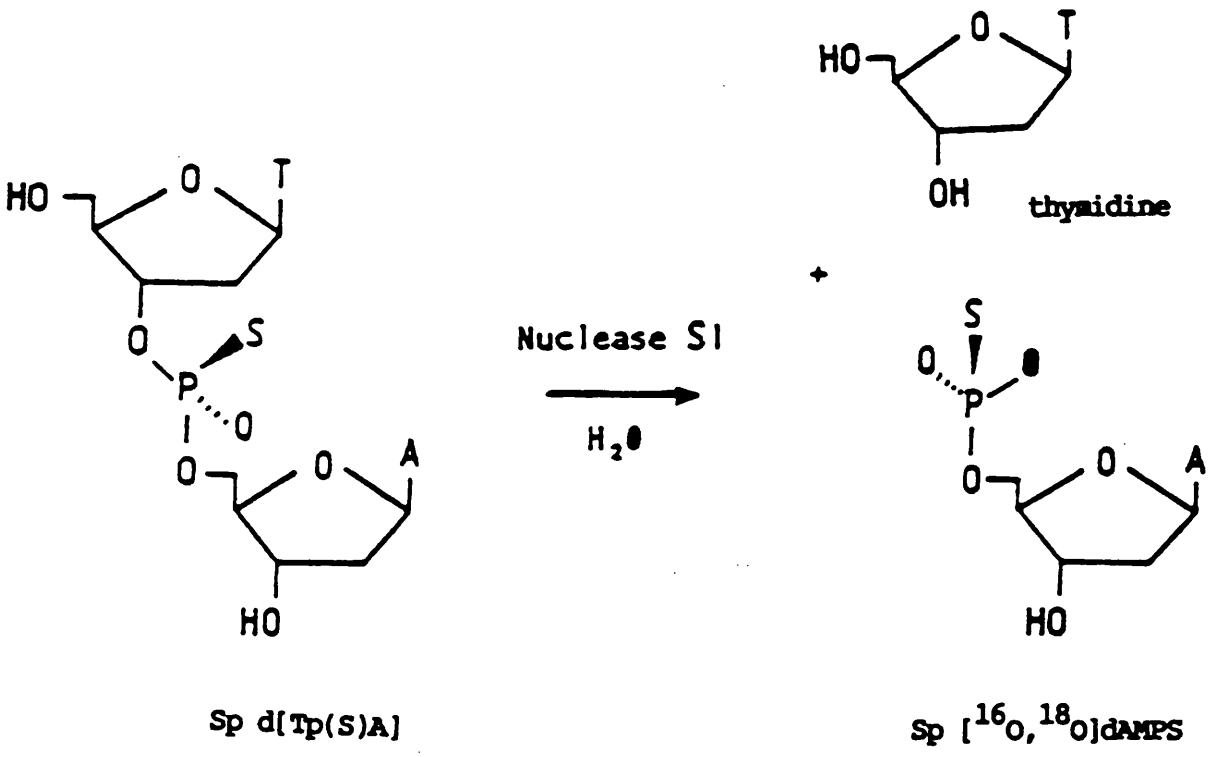
polymerases fall into this category, as do the cyclisation of nucleoside triphosphates to 3′-5′-cyclic phosphorothioates, catalysed by the adenyl and guanylyl cyclases, and intramolecular transesterification of internucleotidic phosphorothioate linkages by RNAse A, T1, and T2, to generate nucleoside 2′-3′-cyclic phosphorothioates. An example of this type of reaction is shown in Figure 1.1. The first step in ribonuclease action, i.e. the cleavage of a phosphodiester bridge to form a 2′,3′- cyclic nucleotide intermediate, was investigated by Usher et. al.[28] and was shown to proceed with inversion of configuration at the phosphorus centre. The dinucleoside phosphorothioate 5′-O-uridyl-3′-O-adenosyl phosphorothioate, Up(S)A, was synthesised with the Rp configuration at the phosphorus centre, and shown to be cyclised by RNAse A to endo uridine 2′,3′-cyclic phosphorothioate, the configuration of which had already been assigned by X-ray crystallography[103].

Investigations of enzymes that catalyse nucleotidyl transfer to water, such as the nucleases, require an internucleotidic phosphorothioate linkage in the substrate, the simplest unit to meet this requirement being a dinucleoside phosphorothioate. In order for chirality to be maintained in the product of hydrolysis, transfer must be to ^{18}O-labelled water so that the product is a chiral [^{16}O,^{18}O] nucleoside phosphorothioate, and a method of configurational analysis of the product is required. For example the stereochemical course of DNA hydrolysis by Nuclease S1 was investigated[65] by hydrolysing a sample of Sp 5′-O-(2′-deoxyadenosyl)-3′-O-thymidyl phosphorothioate, d[Tp(S)A], in ^{18}O-labelled water to generate chiral [^{16}O,^{18}O]2′-deoxyadenosine 5′-phosphorothioate, Figure 1.2.

When the enzyme under investigation catalyses phosphoryl

Figure 1.2

Investigation of the Hydrolysis Catalysed By Nuclease S1



transfer, and the phosphoryl acceptor is not water, as for the kinases, it is necessary for the substrate to contain an ^{18}O label at the phosphoryl centre in order for the stereochemistry of the transfer to be monitored. An example of this kind of reaction is the thiophosphoryl transfer catalysed by adenylate kinase;[73]

$$ATP\gamma S + AMP \rightleftharpoons ADP + ADP\beta S$$

The stereochemistry of the reaction was investigated by thiophosphorylation of AMP using Rp $ATP\beta\gamma^{18}O\gamma S\gamma^{18}O$ as a thiophosphoryl donor substrate to produce chiral $ADP\beta S\beta^{18}O$, Figure 1.3.

When phosphoryl transfer is to water, as catalysed by the ATPases and GTPases, the water in the reaction must be ^{17}O-labelled, the reaction product being $[^{16}O,^{17}O,^{18}O]$ phosphorothioate, in order for chirality to be maintained in the product. Alternatively the ^{17}O-label may be incorporated into the substrate, and the reaction conducted in ^{18}O-labelled water. An example of this type of investigation is that of the hydrolysis of AMP to adenosine and inorganic phosphate catalysed by snake venom 5'-nucleotidase[86]. Chiral $[^{16}O,^{18}O]$AMPS was hydrolysed in ^{17}O-labelled water to generate chiral $[^{16}O,^{17}O,^{18}O]$ thiophosphate, Figure 1.4.

Synthesis of Nucleoside Phosphorothioates;

Methods for the synthesis of sulphur substrate analogues are fairly well developed, routes being available to substrate analogues suitable for the investigation of a wide range of enzyme types[3]. Thus nucleoside 5'- mono-phosphorothioates are generally prepared by thiophosphorylation of unprotected

Figure 1.3

Investigation of Thiophosphoryl Transfer Catalysed By Adenylate Kinase

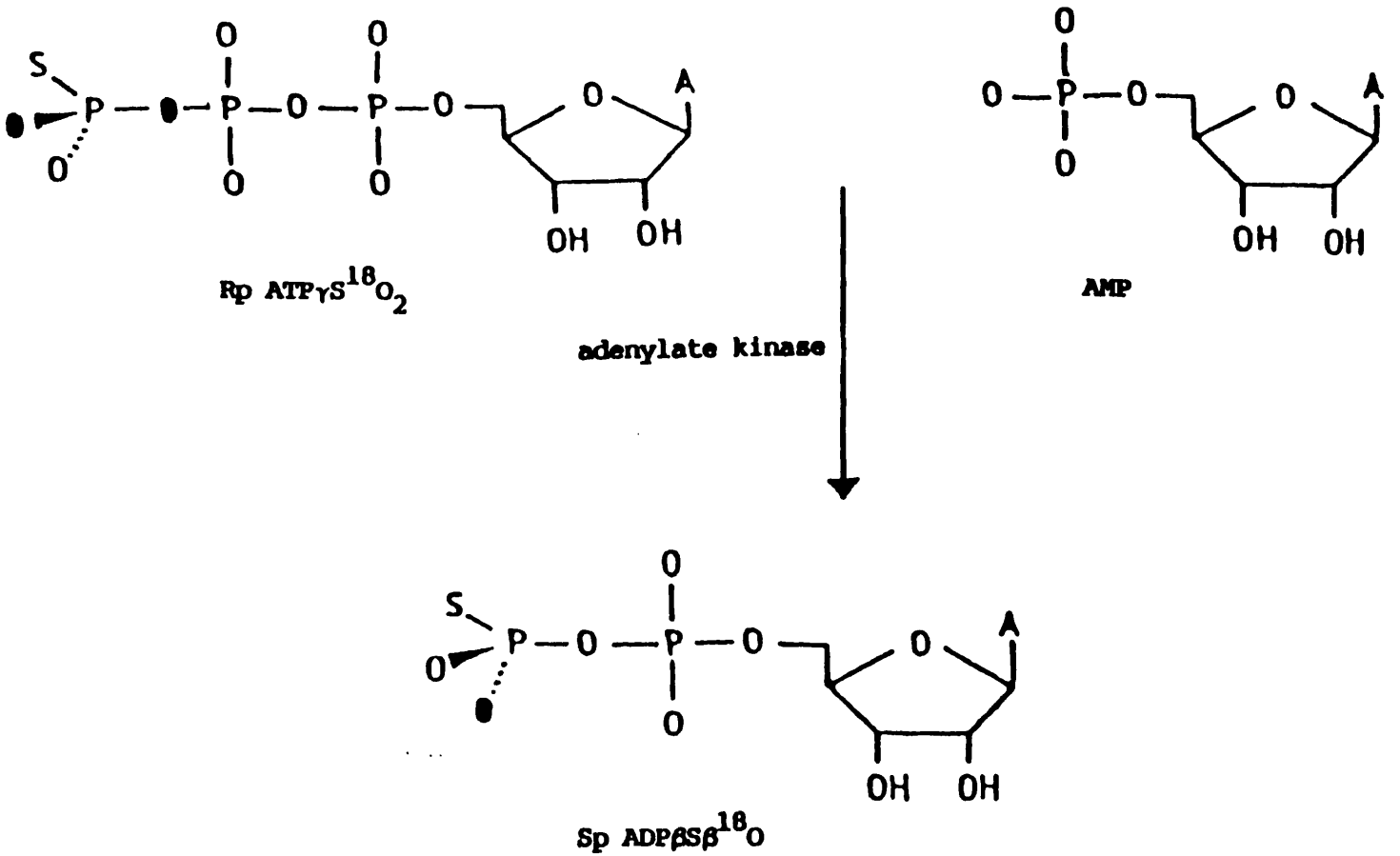
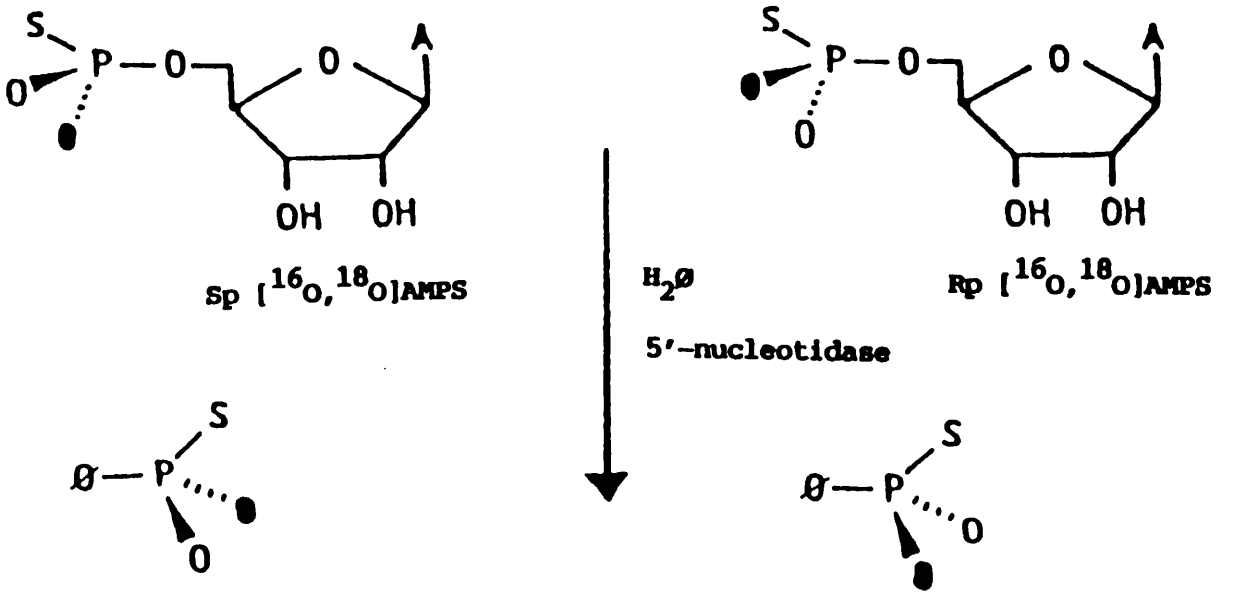


Figure 1.4

Investigation of the Hydrolysis of AMPS by Snake Venom 5′-Nucleotidase



ribonucleotides, and deoxyribonucleotides, with thiophosphoryl chloride, using either triethyl or trimethyl phosphate as a solvent. Nucleoside 5'-O-(1-thiodiphosphates) and (1-thiotriphosphates) are generally prepared from the corresponding nucleoside 5'-monophosphorothioate by activation of the phosphorothioate with diphenyl phosphorochloridate, and subsequent reaction of the resulting anhydride with phosphate or pyrophosphate. These chemical methods of synthesis yield mixtures of diastereoisomers which can be distinguished by ^{31}P n.m.r. spectroscopy, or reverse-phase h.p.l.c., and partially separated by ion-exchange chromatography on DEAE-Sephadex. Small scale separations are possible by h.p.l.c. Pure diastereoisomers can be obtained by various methods based on stereoselective enzymatic phosphorylation. For example, Sp ATPαS can be obtained by stereoselective phosphorylation of adenosine 5'-phosphorothioate, AMPS, by adenylate kinase.[104,116] Other combinations of enzymatic reactions have been worked out to permit stereospecific synthesis of a variety of nucleoside 5'-O-phosphorothioates.[7]

Phosphorothioate-containing dinucleotides and oligonucleotides can be prepared by adaptations of the methods for the preparation of dinucleotides and oligonucleotides[7]. Thus the phosphite approach for synthesis of dinucleoside phosphate linkages has been adapted to dinucleoside phosphorothioate synthesis, by the replacement of the iodine/water oxidation step by the addition of sulphur to the phosphite triester[7].

Configurational Analysis of Nucleoside Phosphorothioates;

Enzymatic methods have been applied to the assignment of configuration of nucleoside phosphorothioates by comparison of

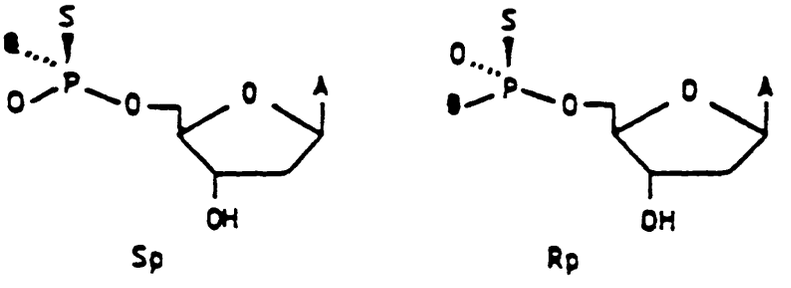
the relative rates at which each diastereoisomer is handled by an enzyme with the relative rates for substrate analogues, the absolute configurations of which are known. Since it is expected that, due to the nature of the active site, a given enzyme will only utilise substrates with a particular configuration at phosphorus, the configuration of the diastereoisomer that is handled best by the enzyme is assignable by analogy. This was first demonstrated by an investigation of snake venom phosphodiesterase, which was observed to hydrolyse the Sp enantiomer of 4-nitrophenyl phenyl-phosphonothioate more than 50 times faster than the Rp enantiomer,[105] and to hydrolyse the Rp diastereoisomer of 5′-O-adenosyl 5′-O-uridyl phosphorothioate, Up(S)A,[32] but not the Sp diasteroisomer. By analogy it was expected that the diastereoisomer of ATPαS that is hydrolysed would have the Rp configuration,[61] and this assignment has since been supported by the assignment of the Sp configuration to the 4-nitrophenyl ester of AMPS that is NOT hydrolysed, determined by X-ray crystallography,[106] and also by the stereospecific synthesis of ^{18}O ADPαS.[107]

Methodology for configurational analysis of phosphorothioates containing heavy oxygen isotopes is not so well developed as the methods for their preparation, and currently limits their application.

An enzymatic method for the configurational analysis of 2′-deoxyadenosine 5′-[^{16}O,^{18}O] phosphorothioate exists, based on the enzyme-catalysed phosphorylation of [^{16}O,^{18}O]dAMPS by adenylate kinase coupled to pyruvate kinase, to generate the Sp diasteroisomer of [α-^{18}O]dATPαS,[33,104] (scheme 1.1). The ^{18}O-isotope is either in a bridging position between the α and β phosphorus or is in a non-bridging position on the α phosphorus

Scheme 1.1

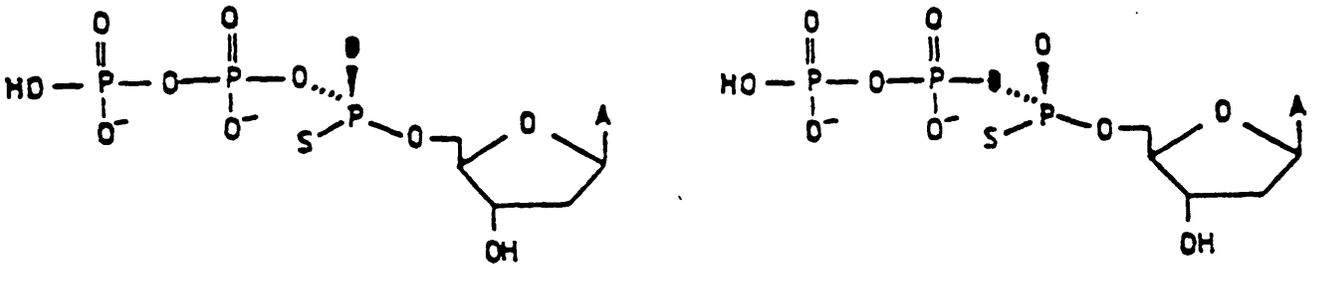
Stereospecific Enzymatic Phosphorylation of dAMPS to Sp ATPαS



MYOKINASE | PYRUVATE KINASE

ATP | PEP

Sp ATPαS



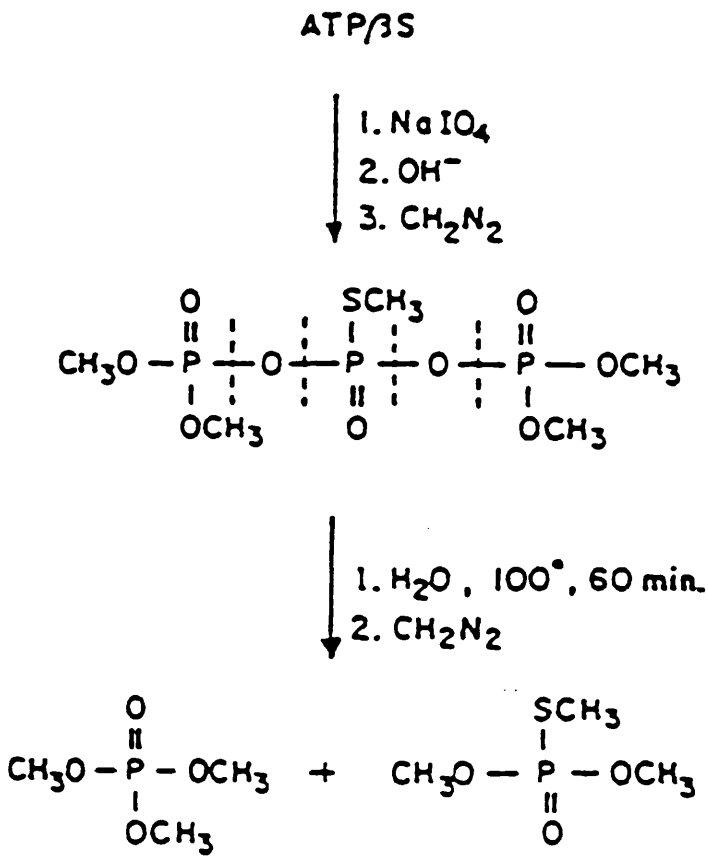
centre, depending upon the configuration of the [^{18}O] phosphorothioate starting material. Methods for configurational analysis of such ^{18}O-labelled phosphorothioates rely on either mass spectrometry[51,73,113] or ^{31}P n.m.r. spectroscopy[13,109] for the location of ^{18}O-isotopes. In 1978 it was observed that ^{18}O directly bonded to phosphorus causes a small, but measurable shift in the ^{31}P n.m.r. resonance,[110,111] the magnitude of which is a function of the order of the ^{31}P(^{18}O) bond, being ca. 0.02ppm per bond in phosphate.[112] This was followed in 1979 by the observation that ^{17}O bonded directly to phosphorus causes the ^{31}P n.m.r. resonances to be greatly broadened by the quadrupolar effect of ^{17}O, I=5/2, so that they effectively disappear.[112] These two facts have been central to the application of ^{31}P n.m.r. spectroscopy to the configurational analysis of oxygen chiral phosphorothioate, and phosphates. Thus examination of the high-field ^{31}P n.m.r. spectrum of the nucleoside α-thio triphosphate permits the ^{18}O isotope to be located by examination of ^{18}O-isotope shifts on the signals due to the α and β phosphorus atom; if the ^{18}O is non-bridging a relatively large shift is expected on the α phosphorus signal, whilst if it is bridging smaller shifts are expected on both α and β signals. Having located the ^{18}O isotope position in the Sp [α^{18}O] dATPαS, the position of the isotope in the original [^{16}O,^{18}O]dAMPS is known.

Mass spectrometric methods have the virtue of greater sensitivity than ^{31}P n.m.r. spectroscopic methods but early approaches required extensive enzymatic and chemical manipulations in order to separate the sulphur and oxygen isotope labels, and to produce sufficiently volatile derivatives for analysis[73,113,114]. A procedure for location of ^{18}O-isotopes in

nucleoside triphosphorothioates was first developed by Richard and Frey[114] for location of ^{18}O in a sample of ATPβSβ^{18}O. The analysis route is shown in Scheme 1.2, and requires degradation of the nucleoside triphosphorothioate to the corresponding triphosphorothioate, permethylation with diazomethane, and hydrolysis to dimethyl phosphate and methyl phosphorothioate. Further methylation with diazomethane yields a mixture of trimethyl phosphate and trimethyl phosphorothioate, which can be analysed by mass spectrometry. Since hydrolysis of the permethylated triphosphorothioate occurs with nearly equal partitioning of bridging oxygens into both the phosphate and the phosphorothioate, if ^{18}O had been bridging in ATPβSβ^{18}O both trimethyl phosphate and trimethyl phosphorothioate products would be enriched in ^{18}O; if non-bridging only the phosphorothioate product would be enriched. Similarly for the analysis of [α^{18}O]dATPαS; detection of ^{18}O in both trimethyl phosphate and trimethyl phosphorothioate indicates that ^{18}O is bridging, whilst its detection in trimethyl phosphorothioate alone indicates ^{18}O-is non-bridging in [α^{18}O]dATPαS.

The recent development of Fast Atom Bombardment mass spectrometry,[128] suitable for use with underivatised nucleotides,[51] provides an estimated 50-200 fold increase in sensitivity over ^{31}P n.m.r. techniques, yet is still chemically simple. The ATPαS molecule can be subjected to negative ion Fast Atom Bombardment spectroscopy, in which the diphosphate sequence ion, arising from cleavage of the P_α–$O_{\alpha\beta}$ bond, is of most interest.[109a] This method of analysis was originally developed for the configurational analysis of [^{16}O,^{18}O]AMPS,[5-7,116] but is applicable to phosphorylation of dAMPS using larger amounts of enzyme and longer reaction times[33], and the position of the

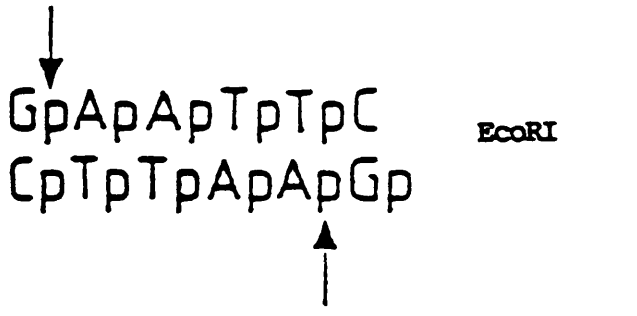
Scheme 1.2
Method for Location of ^{18}O-isotopes in ^{18}O-labelled Triphosphorothioates



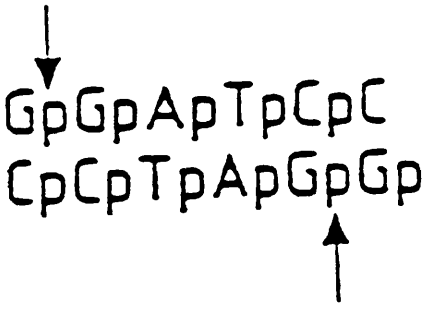
^{18}O-isotope can be determined using either ^{31}P n.m.r. spectroscopy, or F.A.B mass spectroscopy.[107-109a]

The site specific DNA restriction endonucleases[117,118] are a large and useful group of enzymes that recognise a specific base sequence in duplex DNA, typically 4-6 base pairs long, and cleave both strands of the DNA at fixed locations relative to the recognition site. Figure 1.5 shows some examples of restriction endonucleases, their recognition sequences, and the points at which they cleave DNA. These enzymes have wide application in genetic manipulation as tools for gene cloning and for mapping studies, but although well characterised in terms of their recognition sites and overall reactions, little is known of the mechanism by which the cleavage sites are recognised and the way the substrates are bound to the active sites. DNA fragments containing the recognition sequences of various restriction endonucleases have been synthesized with sulphur incorporated at the cleavage site, and have been shown to be hydrolysed at a reduced rate by the restriction endonucleases.[119,120] Thus the stereochemical analysis of the reactions catalysed by the restriction endonucleases is possible by synthesis of the substrate analogue incorporating S at the cleavage site, followed by enzymatic hydrolysis in ^{18}O-labelled water to generate an oligonucleotide with a chiral ^{18}O-containing phosphorothioate group at the 5′-terminus. Further hydrolysis of this product allows the chiral nucleoside 5′-[^{16}O,^{18}O]phosphorothioate to be isolated. Configurational analysis of this chiral mononucleoside 5′-phosphorothioate with respect to starting material thus indicates the stereochemical course of the reaction.[66] The stereochemical course of the hydrolysis reaction

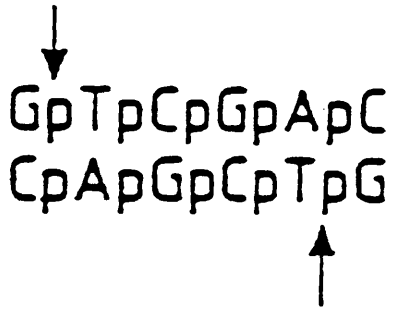
Figure 1.5
Examples of the Recognition Sites of Some
Restriction Endonucleases



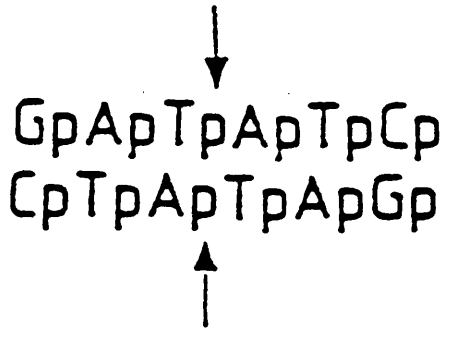
EcoRI



BamHI



SalI



EcoRV

catalysed by the restriction endonuclease EcoRI has been investigated using this approach.[66] The Rp diastereoisomer of d(pGGsAATTCC), which has the chiral phosphorothioate linkage between the deoxyadenosine and deoxyguanosine residues, was used as the substrate, and the hydrolysis conducted in ^{18}O-labelled water. The products of the cleavage reaction were d(pGG) and d([^{16}O,^{18}O,S]pAATTCC), further hydrolysis of the latter by Nuclease P1 yielding 2'-deoxyadenosine 5'-[^{16}O,^{18}O] phosphorothioate of unknown configuration. Stereospecific phosphorylation with adenylate kinase and pyruvate kinase gave Sp [$\alpha^{18}O$]deoxyadenosine 5'-O-(1-thiotriphosphate), ^{31}P n.m.r. analysis of which allowed the ^{18}O to be located in a bridging position between the α and β phosphorus atoms. Thus the EcoRI was shown to catalyse hydrolysis with INVERSION of configuration at the phosphorus centre, consistent with a mechanism involving direct nucleophilic attack of H_2O at phosphorus without involvement of a covalent enzyme intermediate.

Since restriction endonucleases are known that cleave next to all four nucleoside residues in DNA, a general method is required for the configurational analysis of the 2'-deoxynucleoside 5'-[^{16}O,^{18}O] phosphorothioates before a thorough stereochemical investigation of this type of enzyme can be undertaken. The sulphur substrate analogue approach was favoured here, since current methods for the configurational analysis of oxygen chiral phosphates require relatively large amounts of material, whilst site-modified oligonucleotides can only be synthesized on a small scale, (usually no more than 10μmol, and often considerably less).

Overview

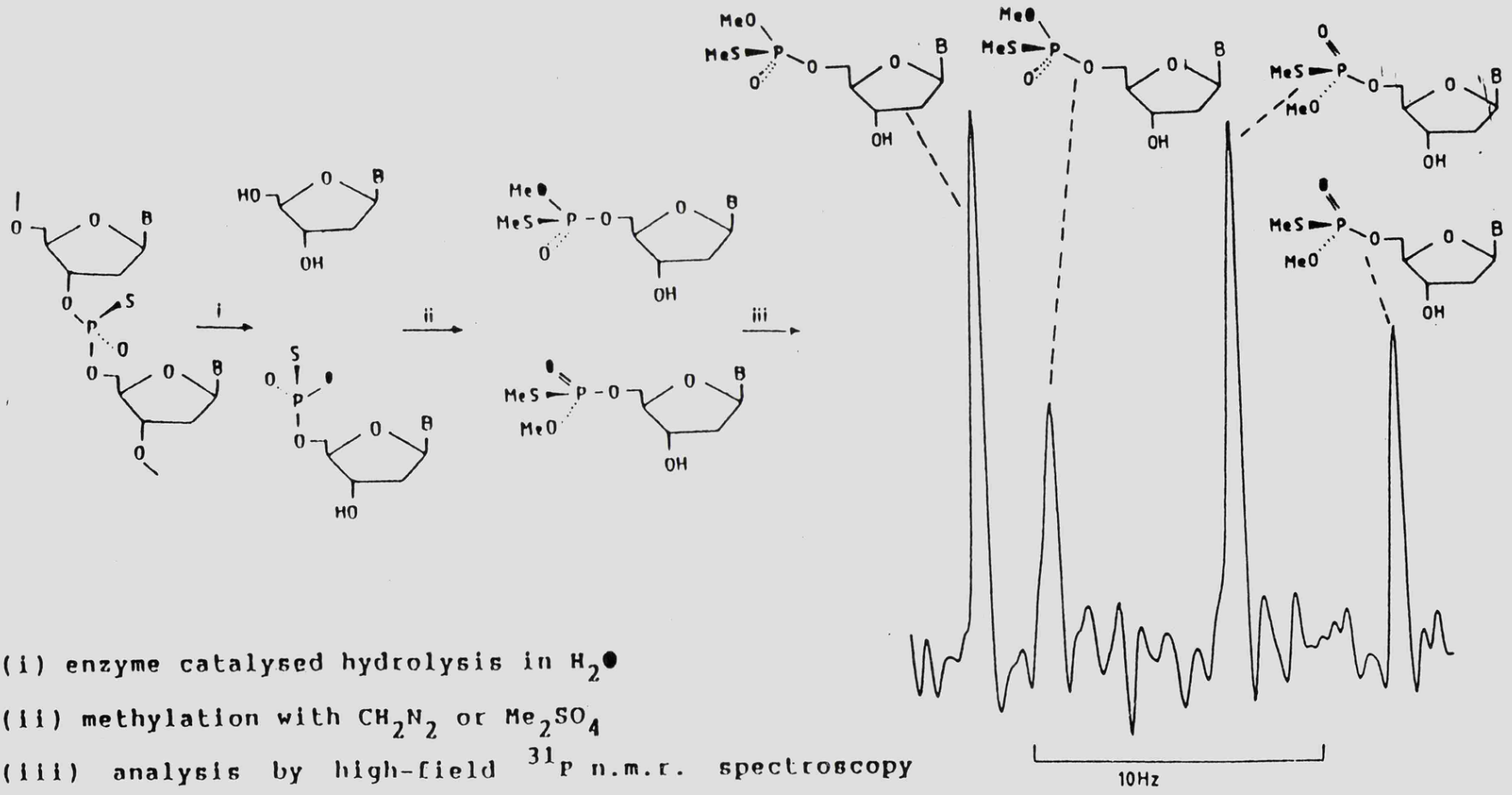
Nucleoside 5′-[^{16}O,^{18}O] phosphorothioates are the product of enzyme-catalysed hydrolysis of phosphorothioate diesters in ^{18}O-labelled water, and a general method for their configurational analysis is applicable to investigations of the stereochemistry of reactions catalysed by phosphodiesterases and nucleases, including the site-specific restriction endonucleases.

^{31}P n.m.r. spectroscopy was chosen as the analytical tool for location of ^{18}O-isotopes, ^{31}P n.m.r. spectra being relatively easy to record and interpret, whilst still offering adequate sensitivity.

In order to distinguish between the oxygen isotopes in a nucleoside [^{16}O,^{18}O] phosphorothioate it is necessary to make them chemically nonequivalent. This can be achieved by simple methylation on oxygen, which occurs without disturbing the stereochemistry of the phosphorus centre, and results in a pair of diastereoisomers with non-equivalent ^{31}P n.m.r. resonances. Provided that the ^{31}P n.m.r. resonances are assignable to the corresponding diastereoisomer, the position of the ^{18}O-isotope in the oxygen-methylated material can be determined by comparison of the magnitudes of $^{31}P(^{18}O)$ isotope shifts[110,111] on the resonances of the unlabelled diastereoisomers. Since the magnitude of these shifts is a function of the order of the phosphorus-oxygen bond[112] the diastereoisomer in which the ^{18}O-isotope is in the P-OMe position is expected to show a smaller $^{31}P(^{18}O)$ isotope shift relative to the unlabelled material than the diastereoisomer in which the ^{18}O-isotope is in the P=O position. The method is summarised in Scheme 1.3.

Scheme 1.3

Summary of a Simple Method For Configurational Analysis of [^{16}O,^{18}O] nucleoside 5'-O-Phosphorothioates



(i) enzyme catalysed hydrolysis in H_2O

(ii) methylation with CH_2N_2 or Me_2SO_4

(iii) analysis by high-field ^{31}P n.m.r. spectroscopy

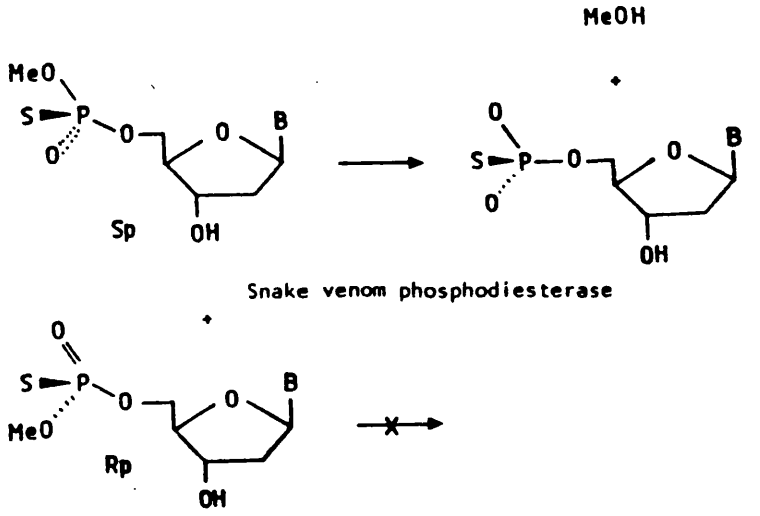
The ^{31}P n.m.r. resonances of the diastereoisomers of the nucleoside 5′-S-methyl-O-methyl phosphorothioate triesters derived from all four major deoxyribonucleoside phosphorothioates, and for adenosine phosphorothioate in the ribo-series, have been assigned here so that the way is open for use of this method in stereochemical analysis of a wide range of enzyme-catalysed substitutions at phosphorus.

Approach

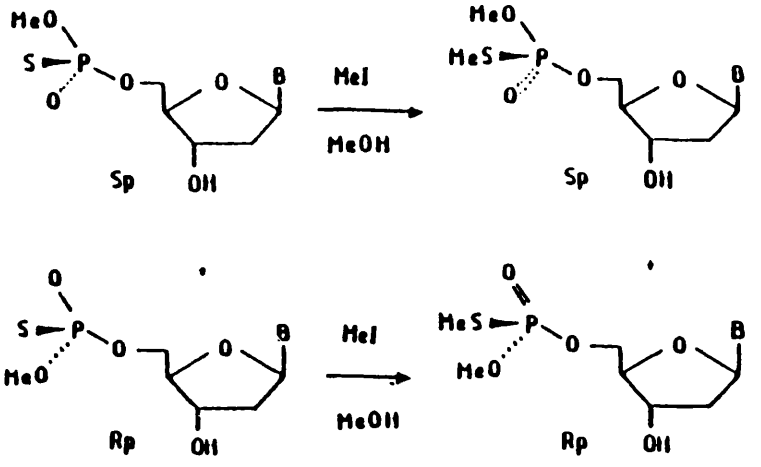
In order to establish the position of oxygen isotopes within a given nucleoside 5′-S-methyl-O-methyl phosphorothioate triester by ^{31}P.n.m.r. spectroscopic analysis it was first necessary to assign the absolute configurations of the diastereoisomers of the unlabelled triester to their corresponding ^{31}P n.m.r. resonances. This was achieved by examination of the relative intensities of the ^{31}P n.m.r. resonances of an asymmetric mixture of the diastereoisomeric triesters of known composition. Samples of asymmetric mixtures of the triesters of known composition were obtained by partial selective enzymatic hydrolysis of a 1:1 synthetic mixture of diastereoisomers of the corresponding nucleoside 5′-O-methyl phosphorothioate diester, using snake venom phosphodiesterase. A knowledge of the previously documented[61,62,105,106,139] selectivity of snake venom phosphodiesterase allowed the configurations of the O-methyl diester to be assigned from the relative intensities of their ^{31}P n.m.r. resonances. Simple S-methylation with methyl iodide generated the corresponding mixture of S-methyl-O-methyl triesters; since methylation occurs without affecting the configuration at the phosphorus centre, the composition of the mixture of diastereoisomers was known, allowing configurational

Scheme 1.4

Summary of the Route to Configurational Assignments of Nucleoside 5′-S-methyl-O-methyl Phosphorothioate Triesters



(a) configurational assignment of the diastereoisomers of the nucleoside 5′-O-methyl phosphorothioate diester by partial selective hydrolysis of the Sp diastereoisomer



(b) configurational assignment of the diastereoisomers of the nucleoside 5′-S-methyl-O-methyl phosphorothioate triesters by methylation of Sp deficient samples of the corresponding nucleoside 5′-O-methyl phosphorothioate diesters

assignments to be made in the resulting ^{31}P n.m.r. spectrum by examination of relative peak heights. The route to these configurational assignments is summarised in Scheme 1.4.

1. Configurational Assignment of Nucleoside 5'-S-methyl-O-methyl Phosphorothioate Triesters

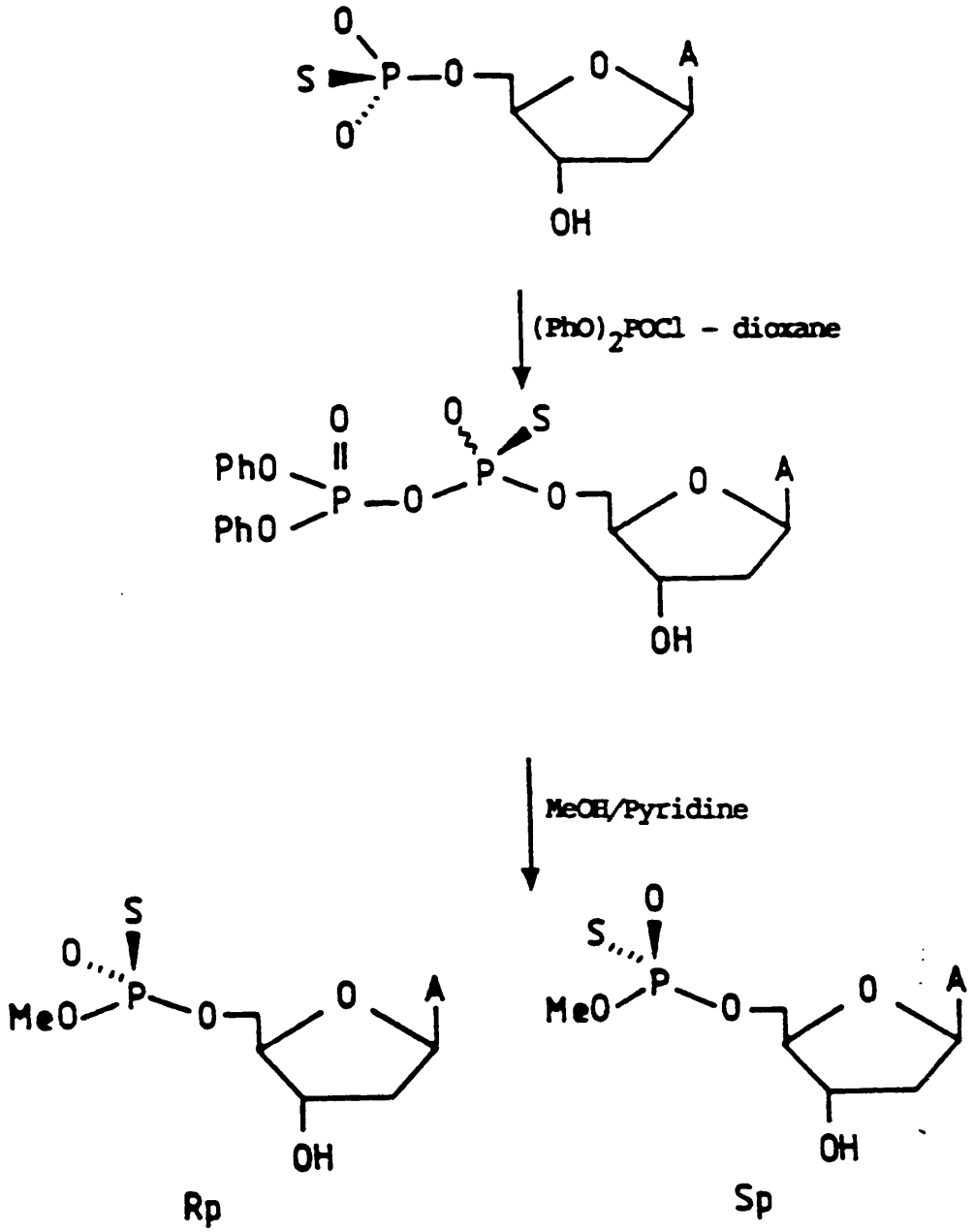
1.1. Preparation of Nucleoside 5'-O-methyl Phosphorothioate Esters.

Two methods were applied to the synthesis of the nucleoside 5'-O-methyl phosphorothioates; the first, illustrated in Scheme 1.5, is based on that of Michelson et al.,[121a] and was applied to the preparation of 2'-deoxyadenosine 5'-O-methyl phosphorothioate. It requires the initial preparation of the 2'-deoxyadenosine 5'-phosphorothioate, which is activated with diphenyl phosphorochloridate to generate an anhydride intermediate. Treatment with excess methanol results in displacement of the diphenyl phosphate, with formation of an approximately 1:1 mixture of the diastereoisomers of 2'-deoxyadenosine 5'-O-methyl phosphorothioate.

The second method involves the synthesis of nucleoside 5'-O-methyl phosphorothioate esters from the free nucleosides[121b] by the procedure illustrated in Scheme 1.6. The free nucleoside is dissolved in either triethyl or trimethyl phosphate and thiophosphorylated with thiophosphoryl chloride to yield the corresponding nucleoside 5'-O-phosphorothiodichloridate. Treatment with excess methanol in pyridine generates the corresponding O,O-dimethyl phosphorothioate triester, resonating at δ 71ppm. (The bulk of the phosphorus-containing solvent is removed at this stage by extraction into petrol or toluene, allowing the reaction to be monitored by ^{31}P n.m.r.

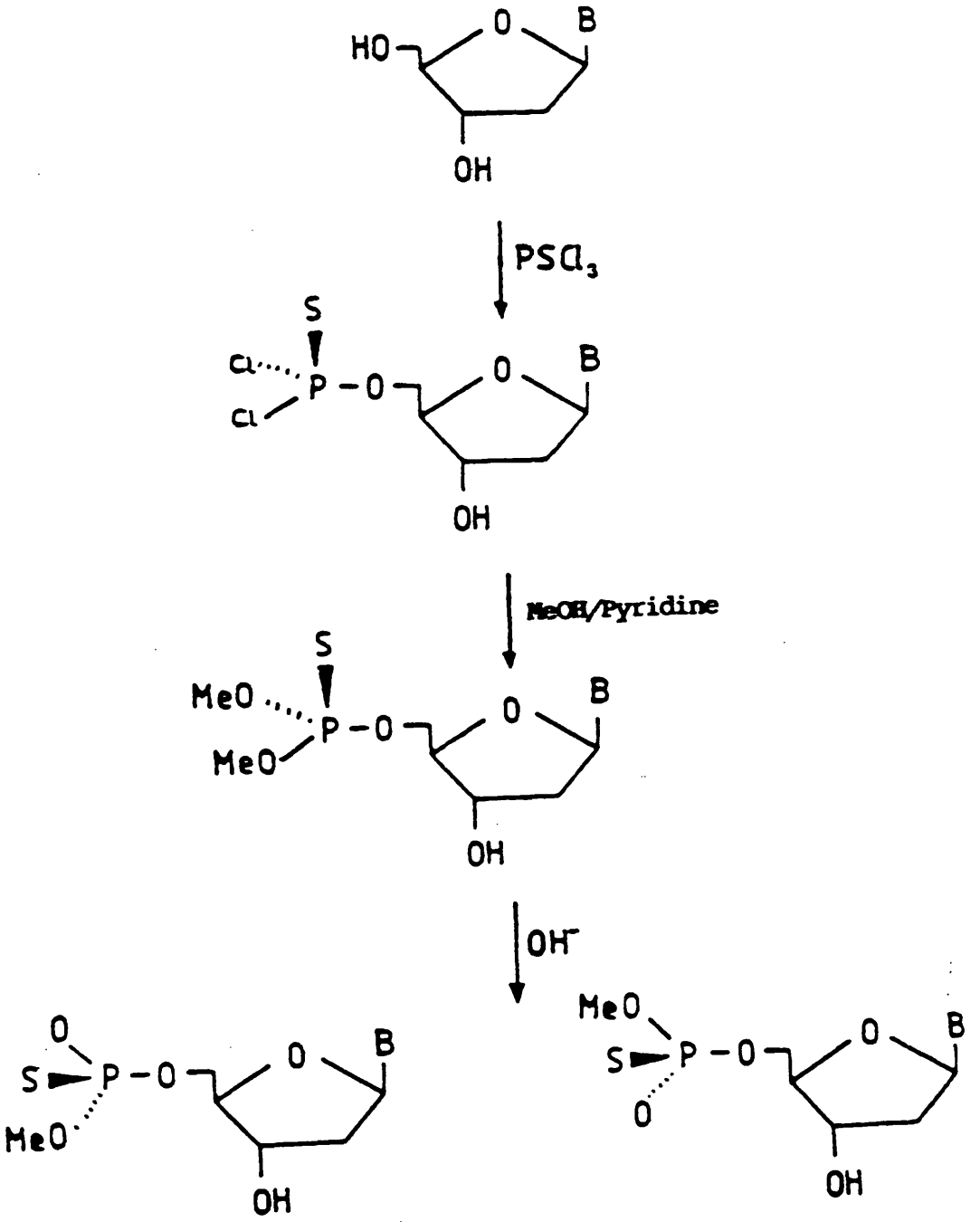
Scheme 1.5

Summary of the Synthesis of Nucleoside 5'-O-methyl Phosphorothioates from the Corresponding Nucleoside 5'-O-phosphorothioate



Scheme 1.6

Summary of the Synthesis of Nucleoside 5'-O-methyl Phosphorothioates from the Corresponding Nucleosides



spectroscopy.) Treatment of the O,O-dimethyl triester, with aqueous sodium hydroxide solution for 1.5h at 50°C results in mono-demethylation to the nucleoside 5'-O-methyl phosphorothioate diester, the diastereoisomers being formed in approximately equal amounts, as judged by h.p.l.c. and ^{31}P n.m.r. integration. After purification by ion-exchange chromatography the diastereoisomers could be readily distinguished by reverse phase h.p.l.c., and high-field ^{31}P n.m.r. spectroscopy.

Figure 1.6 shows a representative h.p.l.c. trace and high-field ^{31}P n.m.r. spectrum of the diastereoisomers of the nucleoside 5'-O-methyl phosphorothioate diesters.

1.2. Partial Hydrolysis With Snake Venom Phosphodiesterase

The absolute configurations of the diastereoisomers of the O-methyl diesters of the nucleoside 5'-phosphorothioates could be assigned by partial selective digestion with snake venom phosphodiesterase, (EC 3.1.4.1), an enzyme reported to cleave methyl esters of 5'-nucleotides, including adenosine 5'-O-phosphate methyl ester.[128a] Snake Venom phosphodiesterase is known to preferentially hydrolyse one diastereoisomer of phosphorothioate esters or anhydrides, usually the Rp,[61,62,106] and the Sp enantiomer of 4-nitrophenyl phenyl phosphonothioate.[105] Figure 1.7 shows some examples of phosphorothioates that are hydrolysed by snake venom phosphodiesterase, represented in such a way as to permit comparison of the positions of the groups which are cleaved. Due to the very large difference in reaction rate, of the order of 10^3, observed between the two diastereoisomers of phosphorothioate analogues,[106] it is assumed that all phosphorothioate analogues that are substrates for snake venom

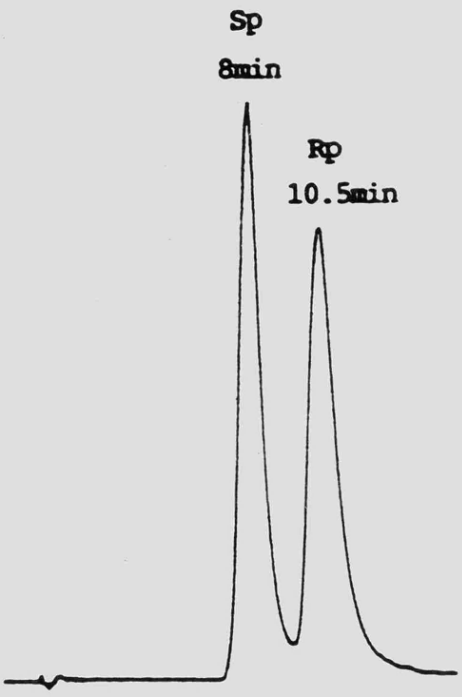
phosphodiesterase have the same configuration at phosphorus. Thus the absolute configurations of nucleoside phosphorothioates can be indirectly assigned by comparison of the relative rates at which the diastereoisomers are handled by the enzyme with those for compounds for which the configuration has been established directly by X-ray structural analysis. This indirect method of configurational assignment was used to assign the Rp configuration to the diastereoisomer of ATPαS which is the preferred substrate for snake venom phosphodiesterase,[61] and can similarily be applied to predict the structure of the diastereoisomer of a nucleoside 5'-O-methyl phosphorothioate diester which will be the favoured substrate for snake venom phosphodiesterase. It should be remembered that this method of indirect assignment presumes that the hydrolysis of the different substrates occur at the same active site, and with mechanisms with identical stereochemical consequences.

Thus it can be seen that the diastereoisomer of a nucleoside 5'-O-methyl phosphorothioate diester that is expected to be a substrate for the snake venom phosphodiesterase enzyme has the Sp configuration at phosphorus, as a result of the MeO- group having a lower priority than the 3'-O nucleotidyl group of the dinucleoside phosphorothioates, according to the sequence rules governing R and S notation[1].

Samples of ca. 1:1 mixtures of diastereoisomers of the 5'-O-methyl phosphorothioates were incubated with snake venom phosphodiesterase and the progress of the reaction monitored by examination of aliquots of the reaction mixture by h.p.l.c. After a 24h incubation of dAMPS-OMe with snake venom phosphodiesterase, at pH 9.6, and 36°C, h.p.l.c. analysis

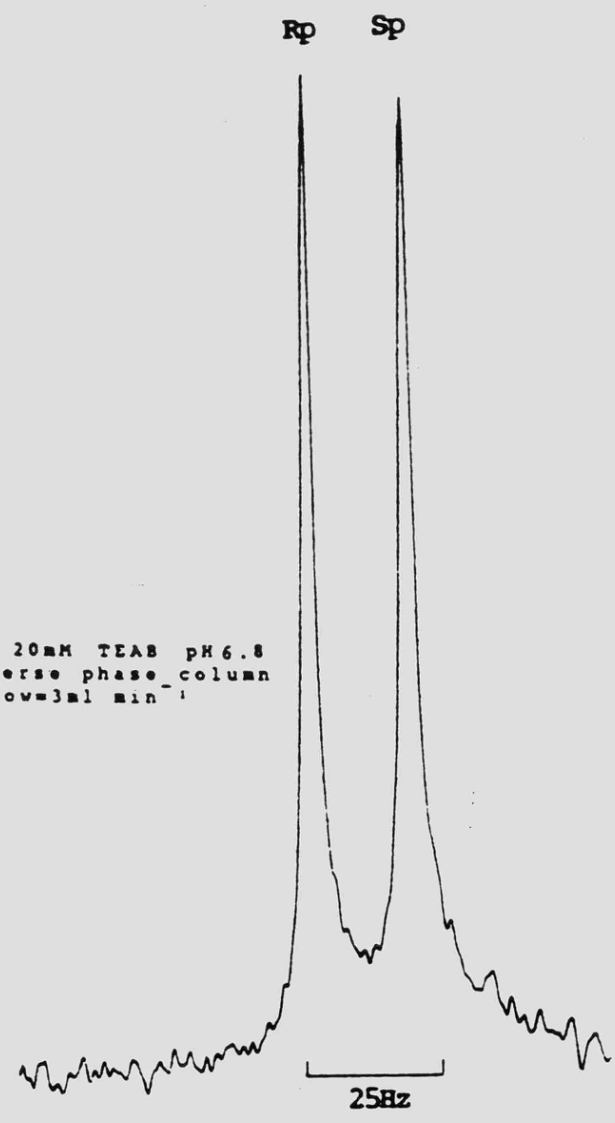
Figure 1.6

Representative H.p.l.c. Trace and High Field ^{31}P n.m.r. Spectrum of the Diastereoisomers of Nucleoside 5'-O-methyl Phosphorothioates



(a) H.p.l.c. Trace

Isocratic elution with 3% MeCN in 20mM TEAB pH 6.8 using an Partisil PXS10/ 25μ ODS reverse phase column UV detector at l=259nm, temp=20°C, flow=3ml min^{-1}

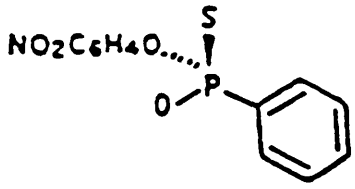


(b) High Field ^{31}P n.m.r. Spectrum

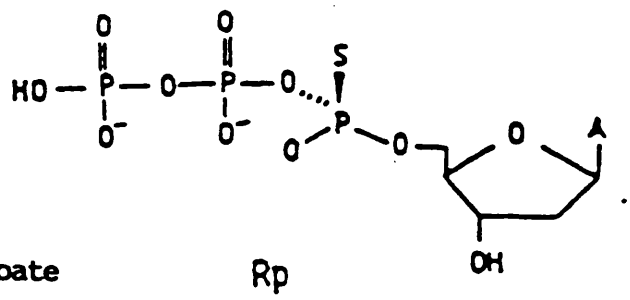
Conc. ca. 100mM AMPS-Ome in 3:1 v/v DMF:D$_4$MeOH ^{31}P n.m.r. parameters were: field strength 121.5MHz, sweep width 1213Hz, pulse width 5.0μs, aquisition time 2.2s, block size 16K, zero-filled to 32K, no. transients 508.

Figure 1.7

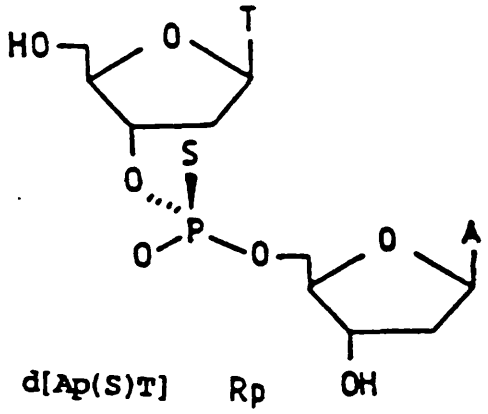
Examples of Substrates of Snake Venom 5'-nucleotide Phosphodiesterase

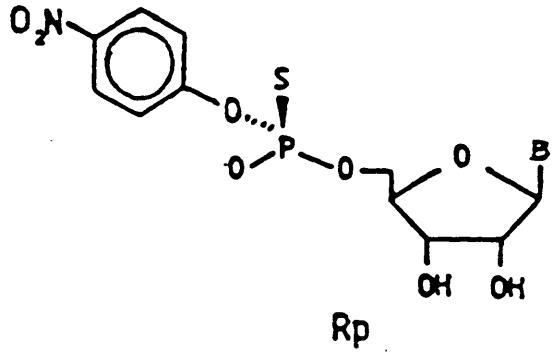


4-nitrophenyl phenyl-phosphonothioate

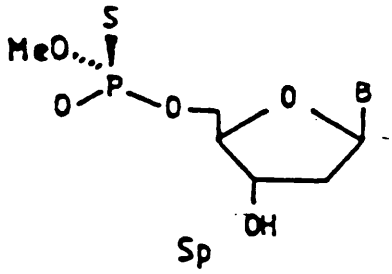


ATPαS





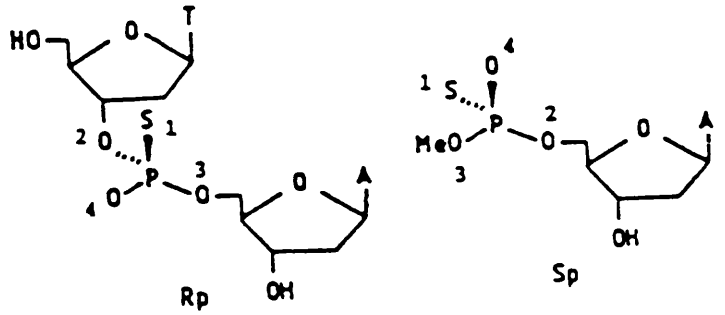
adenosine 5'-O-(4-nitrophenyl)phosphorothioate



Nucleoside 5'-O-methyl Phosphorothioate

indicated a reduction in the amount of 'fast' diastereoisomer, (i.e. the diastereoisomer with the shorter retention time), relative to the 'slow' diastereoisomer, (that with the longer

Figure 1.8



retention time), with concomitant appearance of a peak at shorter retention time, which was identified as dAMPS by co-injection with authentic sample. Comparison of the relative peak heights by h.p.l.c. integration indicated that ca. 28% of the Sp diastereoisomer had been hydrolysed, representing a turnover of ca. 0.2μmol of substrate by 0.5 units of enzyme over a period of 24h under the conditions employed. Thus the 'fast' peak of reduced intensity could be assigned to the Sp diastereoisomer of dAMPS-OMe. In this way incubation with snake venom phosphodiesterase could be used to generate samples of the diastereoisomeric O-methyl diester mixture deficient in the Sp diastereoisomer, the latter having been hydrolysed to the corresponding nucleoside phosphorothioate and methanol.

The nucleoside 5'-phosphorothioate released as the initial product of hydrolysis was observed to undergo slow desulphurisation to the corresponding phosphate which, in turn, became a substrate for phosphatases associated with the enzyme preparation, leading to the release of free nucleoside. This desulphurisation and subsequent dephosphorylation has

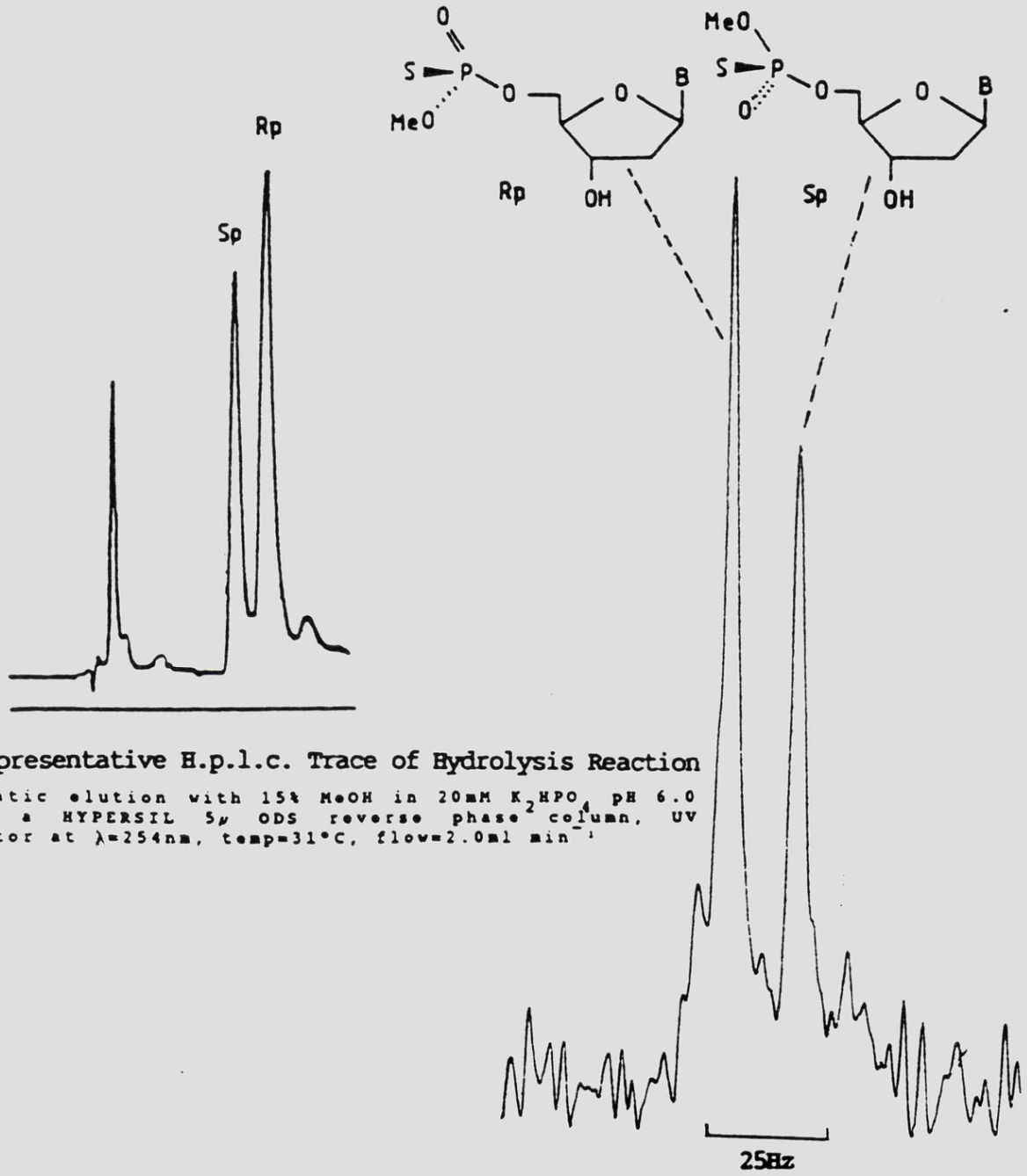
frequently been observed during studies of snake venom phosphodiesterase using phosphorothioate substrates.[61,62,139]

Nuclease P1, a random endonuclease, known to hydrolyse the Sp diastereoismer of dinucleoside phosphorothioates,[68] was tested as an alternative reagent to snake venom phosphodiesterase for the partial selective hydrolysis of nucleoside 5'-O-methyl phosphorothioates. This enzyme shows the opposite stereoselectivity to the snake venom phosphodiesterase, and hence would be expected to hydrolyse the Rp diastereoisomer of this substrate. It is easier to handle, being commercially available as a solid, and relatively free from contaminating activities, and is reported to hydrolyse phosphorothioates without any of the partial desulphurisation seen with the snake venom enzyme. However the result obtained from h.p.l.c. analysis of an incubation of AMPS-OMe with Nuclease P1 after 24h indicated little, if any, hydrolysis of the Rp diastereoisomer, the enzyme probably requiring another nucleotide residue to function efficiently. Hence Nuclease P1 was not a suitable alternative reagent to snake venom phosphodiesterase.

The Sp deficient samples of nucleoside 5'-O-methyl phosphorothioate obtained by partial hydrolysis with snake venom phosphodiesterase were separated from the other components of the incubation mixture by ion-exchange chromatography, and examined by high-field ^{31}P n.m.r. spectroscopy. Figure 1.9 shows a representative h.p.l.c. trace of the hydrolysis reaction, and high field ^{31}P n.m.r. spectrum of a resulting sample of Sp-deficient nucleoside 5'-O-methyl phosphorothioate.

Figure 1.9

Hydrolysis of Nucleoside 5′-O-methyl Phosphorothioate by Snake Venom Phosphodiesterase



(a) Representative H.p.l.c. Trace of Hydrolysis Reaction

Isocratic elution with 15% MeOH in 20mM K_2HPO_4 pH 6.0 using a HYPERSIL 5μ ODS reverse phase column, UV detector at λ=254nm, temp=31°C, flow=2.0ml min^{-1}

(b) Representative High Field ^{31}P n.m.r. Spectrum of Resulting Sp- Deficient Nucleoside 5′-O-methyl Phosphorothioate

Conc. ca. 8mM dGMPS-OMe in 3:1 v/v DMF:D_4-MeOH ^{31}P n.m.r. parameters were: field strength 121.5MHz, sweep width 1824Hz, pulse width 5.0μs, aquisition time 2.2s, block size 16K, no. transients 19,718, Line broadening -1.0Hz.

1.3. Configurational Assignment of Diastereoisomers by ^{31}P n.m.r. Spectroscopy.

For the O-methyl diesters of all four deoxyribonucleoside 5'-phosphorothioates the signal of reduced intensity, assigned to the Sp diastereoisomer of the nucleoside 5'-O-methyl phosphorothioate, resonated UPFIELD of that due to the Rp diastereoisomer in solvents of DMF and methanol. Similarly for all cases examined, the Sp diastereoisomer showed a shorter retention time on reverse-phase h.p.l.c. than the Rp diastereoisomer. This can be compared with the case for dinucleoside phosphorothioates[6,7], where the diastereoisomer which is a substrate for snake venom phosphodiesterase resonates downfield in the high-field ^{31}P n.m.r. spectrum, although it still shows a shorter retention time on reverse-phase h.p.l.c. Thus it appears that, whilst replacement of a 3'-nucleoside group by a simple methyl group does not affect relative chromatographic mobility, the ^{31}P n.m.r. resonances are reversed. This is not simply a solvent effect as the ^{31}P n.m.r. spectra were determined in both DMF/methanol and in water, and the spectra of the dinucleoside phosphorothioates determined in aqueous media. The ^{31}P n.m.r. diastereoisomeric separation was much smaller in water, (0.009 ppm), compared with DMF/methanol, (0.16 ppm). Thus, the lack of a 3'-nucleoside moiety in the O-methyl diester changes the ^{31}P n.m.r. properties relative to the dinucleoside phosphorothioates.

1.4. Conversion of O-Methyl Diesters to Corresponding S-Methyl-O-Methyl Triesters:

The nucleoside 5'-O-methyl phosphorothioate diesters could be cleanly and quantitatively converted to the corresponding phosphorothioate-S-methyl-O-methyl triesters by methylation on

sulphur using excess methyl iodide in methanol for 1-3h at room temperature. Methylation is accompanied by an upfield shift in the ^{31}P n.m.r. resonances from δ +58ppm to δ +32ppm (methanol), and the diastereoisomers of the S-methyl-O-methyl triester could easily be distinguished by high-field ^{31}P n.m.r. spectroscopy in DMF/methanol. Examination of the ^{1}H n.m.r. spectra of the diastereoisomeric S-methyl-O-methyl triesters obtained in this way showed signals attributable to the S-methyl and O-methyl protons, δ 3.8 J_{PH} 12.7 Hz, and δ 2.2, J_{PH} 15.4 Hz, respectively. Methylation of samples of nucleoside 5'-O-methyl phosphorothioate which had been subjected to partial selective digestion of the Sp diastereoisomer gave rise to samples of the S-methyl-O-methyl triester correspondingly deficient in the Sp diastereoisomer, methylation occurring without a change in configurational assignment.

Treatment of the nucleoside 5'-O-methyl phosphorothioates with methyl iodide was not expected to result in methylation of the heterocyclic base when methanol was used as a solvent for the reaction, in spite of reports of base-methylation of nucleosides by methyl iodide in solvents such as DMSO and N,N-dimethyl acetamide. Treatment of ATPαS with a 50-fold excess of methyl iodide in methanol for 2h at room temperature has been shown by ^{1}H n.m.r. spectroscopy not to result in base-methylation,[68] whilst treatment of adenosine 3',5'-cyclic phosphate for 20h at room temperature with excess methyl iodide in DMSO has been shown to result in base-methylation.[122,177] Similarly, treatment of adenosine with methyl iodide using N,N-dimethyl acetamide as a solvent is reported to yield N^1-methyl adenosine quantitatively.[123]

The UV spectra of the S-methyl-O-methyl phosphorothioates

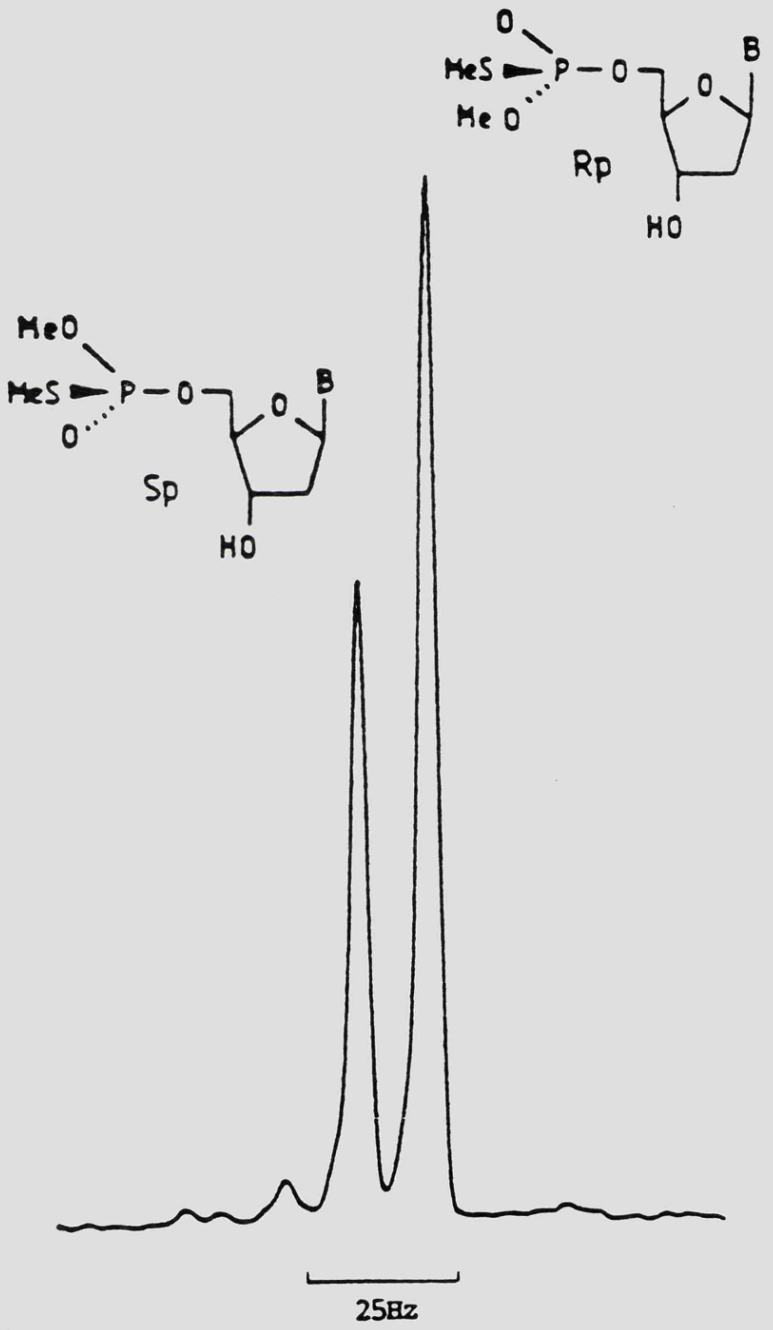
provided a useful indication as to whether or not base-methylation had occurred; the UV spectra of nucleosides and nucleotide derivatives are characteristic of the hetereocyclic base, and changes to the base are usually accompanied by easily observable changes in the UV spectrum, such as shifts in λmax of several nm.

1.5. Configurational Assignment of the Nucleoside S-methyl-O-methyl Phosphorothioate triesters by ^{31}P n.m.r. Spectroscopy

Examination of these samples by high-field ^{31}P n.m.r. spectroscopy in DMF/methanol allowed the diastereoisomers to be assigned to the corresponding ^{31}P n.m.r. resonance, the signal of reduced intensity being assigned to the Sp diastereoisomer. In all cases it was found that the Sp diastereoisomers of the nucleoside 5'-phosphorothioate S-methyl-O-methyl triesters resonated downfield of the Rp diastereoisomer, the diastereoisomeric separation being of the order of 0.08ppm. Hence, the ^{31}P n.m.r. resonances of the diastereoisomers of nucleoside 5'-phosphorothioates S-methyl-O-methyl triesters were assigned to their absolute configurations at phosphorus. Figure 1.10 shows a representative high-field ^{31}P n.m.r. spectrum of a sample of the Sp deficient triesters, generated as described. The very small peaks observed are probably due to traces of base-methylated material.

Figure 1.10

Representative High Field ^{31}P n.m.r. Spectrum of the Sp- Deficient Mixture of Diastereoisomers of Nucleoside 5'-S-methyl-O-methyl Phosphorothioate Triesters



Conc. ca. 14mM dCMPS-OMe in 3:1 v/v DMF: D_4-MeOH ^{31}P n.m.r. parameters were: field strength 121.5MHz, sweep -width 10000Hz, pulse width 15.0μs, aquisition time 1.6s, block size 16K, no. transients 33,625 Gaussian broadening 0.1Hz.

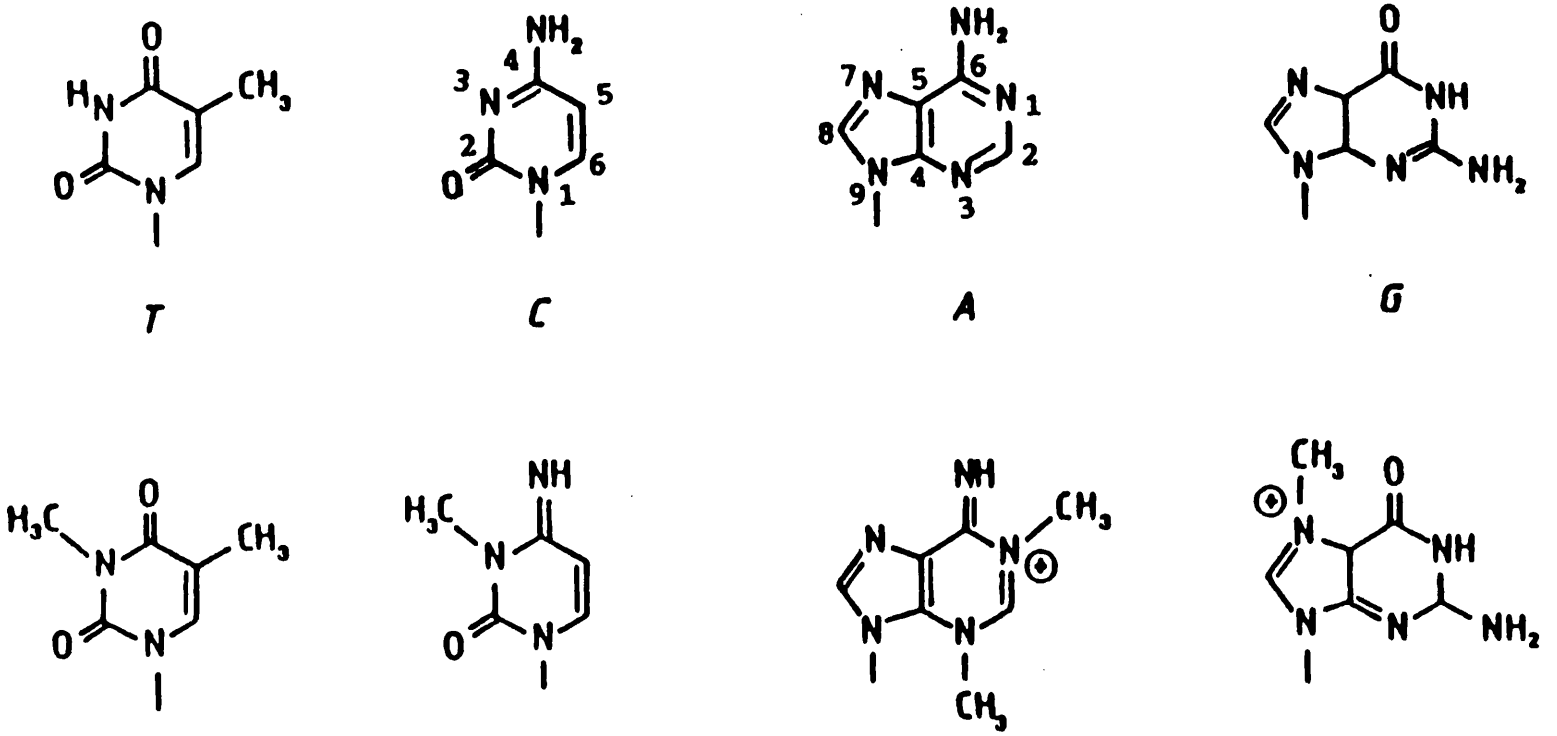
2.DIRECT METHYLATION OF NUCLEOSIDE 5'-O-PHOSPHOROTHIOATES;

Methylation of nucleoside 5'-O-phosphorothioates with either diazomethane or dimethyl sulphate leads to direct formation of the corresponding nucleoside 5'-phosphorothioate -S-methyl-O-methyl triesters. Since the ^{31}P n.m.r. resonances of the diastereoisomers of these triesters have been assigned as described for all four deoxy-ribonucleoside phosphorothioate -S-methyl-O-methyl triesters, and for adenosine 5'-S-methyl-O-methyl phosphorothioate triester, the location of ^{18}O-isotope in nucleoside 5'-[^{16}O,^{18}O] phosphorothioates of unknown configuration at phosphorus is possible for all four of the major deoxyribonucleoside phosphorothioates, and for adenosine 5'-phosphorothioate, using ^{31}P n.m.r. spectroscopy.

Apart from methylation at the phosphorothioate moiety, some methylation of the nucleoside base was expected[122], the extent and site of methylation depending on the methylating agent employed, the nucleoside phosphorothioate involved, and on the reaction conditions. Figure 1.11 shows some of the relevant methylated analogues of the heterocyclic bases. Base methylation of the phosphorothioate triesters was accompanied by small shifts in the ^{31}P n.m.r. resonances of the order of ca. 0.02ppm. Apart from reducing the quality of the spectrum obtainable from small samples in terms of signal:noise ratios, (by splitting of signals), the occurrence of signals only slightly shifted from the main triester resonances by base-methylation might lead to problems in distinguishing base-methylated material from isotope-shifted material. Thus, in order to obtain a clean pair of diastereoisomeric resonances suitable for the observation of

Figure 1.11

Some Methylated Analogues of the Nucleoside Bases



isotope shifts, it was necessary to establish conditions under which the yields of triester are optimal, and for which base-methylation, if it occurs, is either negligible or complete.

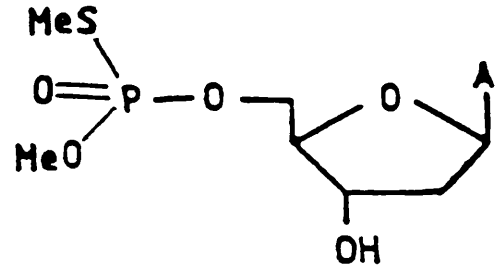
2.1. Diazomethane as a methylating agent:

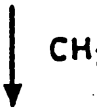
Using diazomethane as a methylating agent rapid methylation on sulphur of the phosphorothioate was observed, accompanied by an upfield shift in the ^{31}P n.m.r. resonance from δ_P(MeOH) +58ppm to δ_P(MeOH) +21ppm. Further methylation on oxygen to yield the triester then occurred more slowly with a downfield shift to +32ppm, (methanol). Thus, exposure of a sample of the triethylammonium salt of dAMPS dissolved in methanol to a slight excess of diazomethane for a few seconds resulted in complete methylation on sulphur, accompanied by ca. 40% O-methylation, as judged by the relative intensities of the ^{31}P n.m.r. resonances. A 10 min exposure resulted in ca 50% O-methylation, whilst 1.5h yielded ca. 70% O-methylation. Initially methanol was used as the solvent for these methylation reactions, however significant amounts of desulphurisation were observed at reaction times above ca. 3-4h, whilst methylation on oxygen was still incomplete. Thus a 4h exposure of dAMPS to excess ethereal diazomethane in methanol led to a yield of the S-methyl-O-methyl triester of ca. 60% with ca. 20% desulphurisation. Loss of sulphur was accompanied by a characteristic large upfield shift in the ^{31}P n.m.r. resonance to ca. δ 0ppm. A possible mechanism for desulphurisation is shown in Scheme I.7. This shows desulphuristion occurring as a result of nucleophilic attack by methanol on a species generated by permethylation of the S atom.

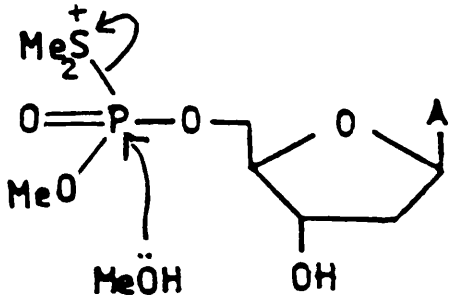
Scheme 1.7

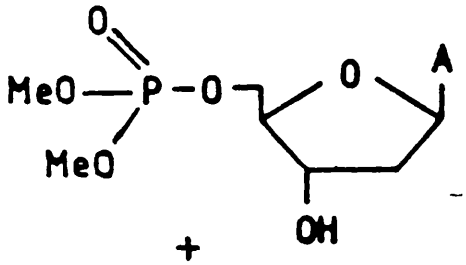
Desulphurisation of -S-Methyl-O-Methyl Triester

By Diazomethane in Methanol









+

Me_2S

When DMF was used as a solvent instead of methanol desulphurisation was not observed, and reactions could be left for 1-2 days with excess diazomethane to achieve optimal yields of triester. Thus DMF became the favoured solvent for methylation reactions using diazomethane.

Yields of triester obtainable from methylation with ethereal diazomethane in DMF for 1-2 days at room temperature were of the order of 80-100%, in the presence of the corresponding S-methyl diester, as judged by relative intensities of ^{31}P n.m.r. resonances at low field. Complications arose in some cases as a result of methylation of the nucleoside base. It was not expected that methylation of the nucleoside base would reverse the configurational assignments of the ^{31}P n.m.r. resonances of the diastereoisomeric triesters in view of the fact that all four deoxyribo-nucleoside 5'-phosphorothioates and adenosine 5'-phosphorothioate showed the same configurational assignments in their diesters and triesters, and that base-methylation was accompanied by a relatively small shift in the ^{31}P n.m.r. resonances. It was nevertheless necessary to correlate the direction of resonance of the diastereoisomers of the triester obtained from different routes in order to prove that the configurational assignments were unaffected by base-methylation.

2.1.1. Adenosine- and 2'-deoxyadenosine 5'-O-phosphorothioate:

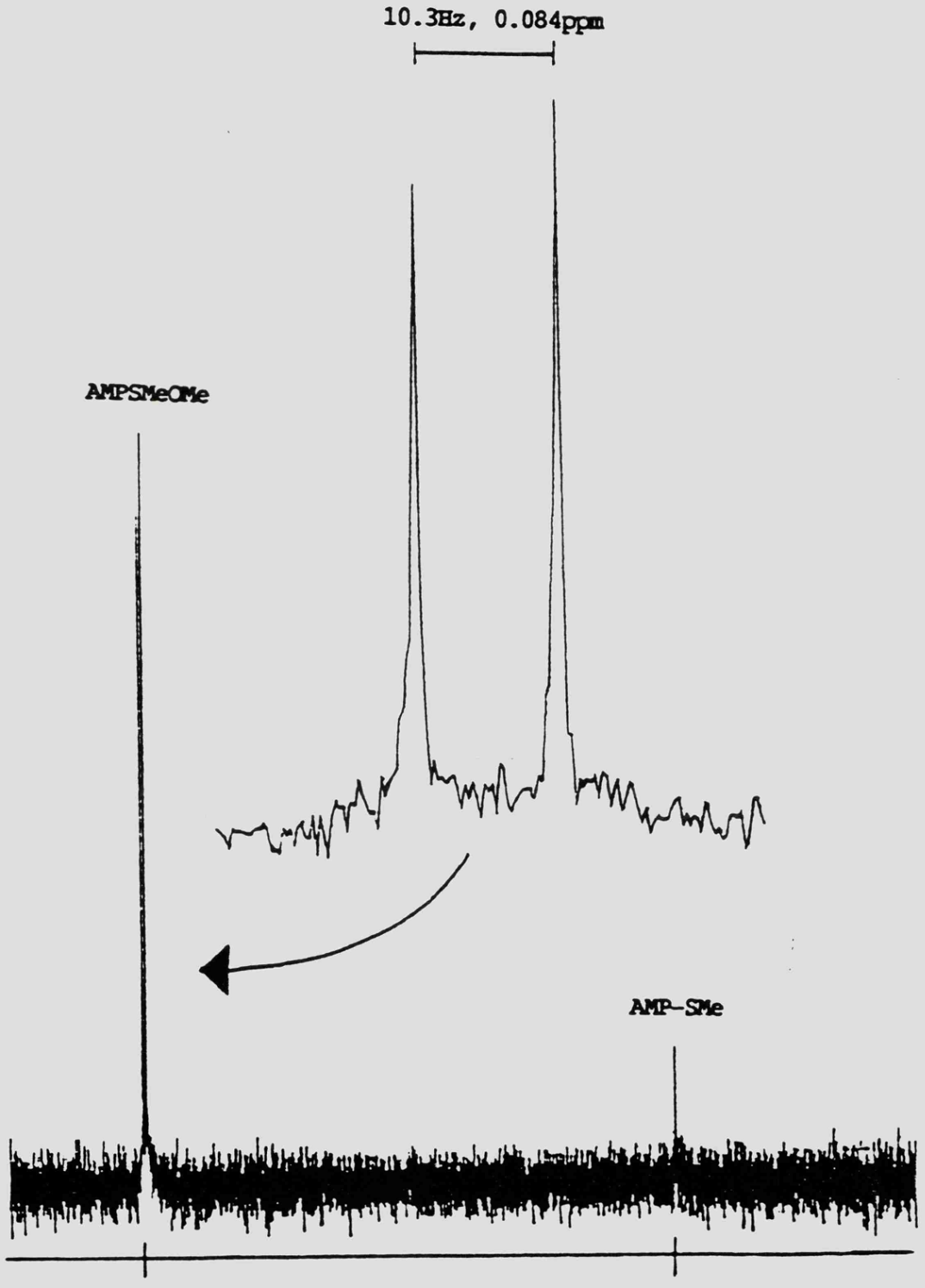
The reported relative susceptibilities of the nucleosides to base methylation by diazomethane are;[122]

guanosine > thymidine > cytidine > adenosine

and it was expected that the corresponding nucleoside phosphorothioates would show similar relative susceptibilities. Treatment of AMPS and dAMPS with excess diazomethane in DMF for 1-2 days at room temperature resulted in 80-90% yield of the diastereoisomers of the S-methyl-O-methyl triester, which appeared as a clean pair of resonances in the high-field ^{31}P n.m.r. spectrum, (Figure 1.12, Figure 1.13). In the case of dAMPS traces of low intensity resonances, attributable to base-methylated material, were apparent, (ca 5-10%). That the diastereoisomeric resonances were due to triester that was not methylated on the purine base was indicated by examination of the UV spectrum of the methylated material, which showed a λmax of 259nm, characteristic of unmodified base. Had extensive base-methylation occurred a shift in λmax of the UV absorption to 261nm,[122] characteristic of N^1-methylation of the purine base would be expected. When a sample of the triester obtained by methylation of the nucleoside 5'-phosphorothioate with diazomethane was combined with material obtained by methylation of the O-methyl diester with methyl iodide, and the ^{31}P n.m.r. spectrum of the mixture examined, a single pair of resonances was observed, suggesting that the material obtained by the two routes was the same. That this material was not methylated on the purine base was further supported by the fact that treatment of

Figure 1.12

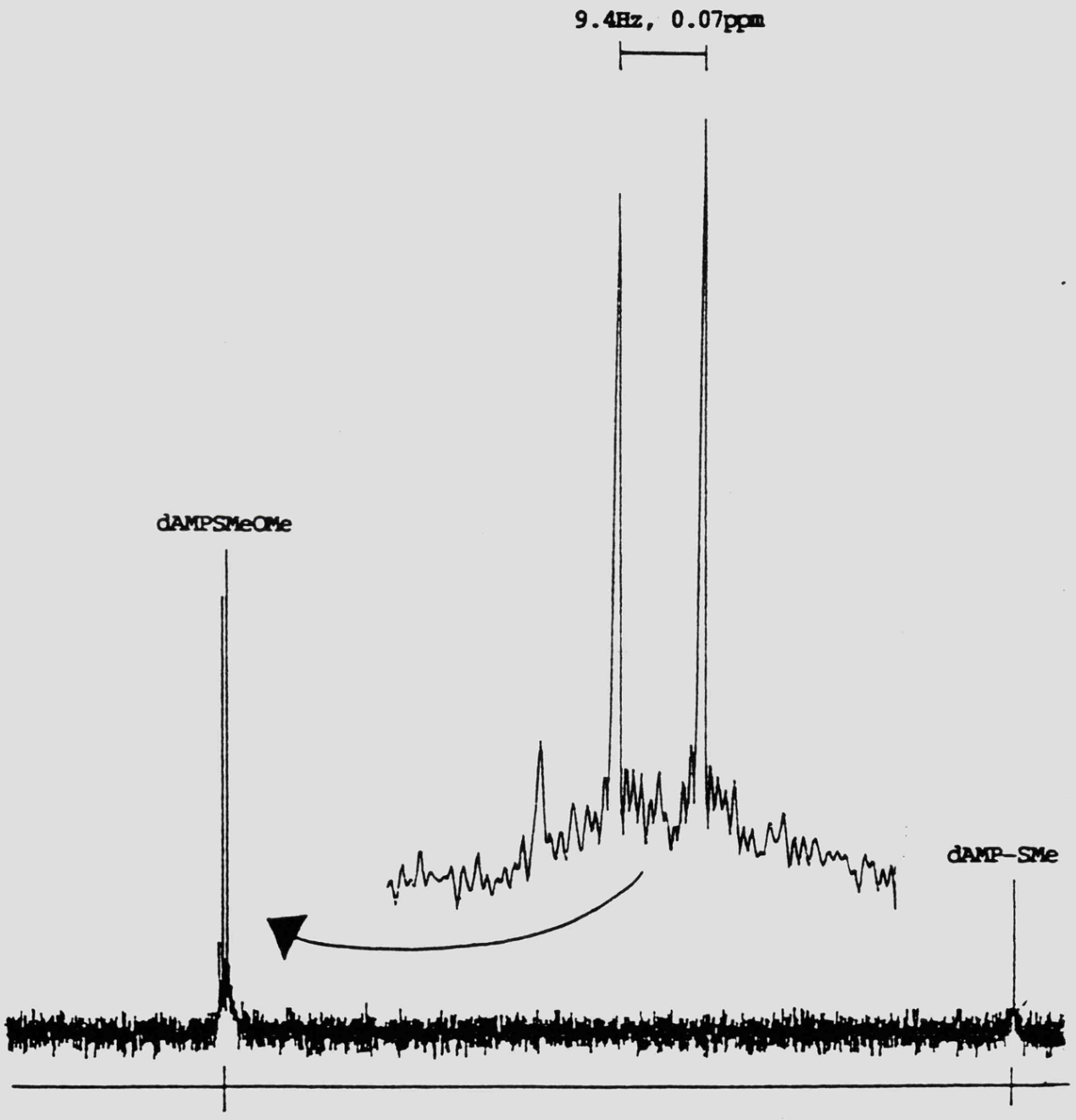
High Field ^{31}P n.m.r. Spectrum of the Diastereoisomers of AMPSMeOMe Obtained by Treatment of AMPS with Diazomethane



Conc. ca. 50mM AMPSMeOMe in 3:1 v/v DMF:D_4-MeOH ^{31}P n.m.r. parameters were: field strength 121.5MHz, sweep width 4504Hz, pulse width 5.0μs, aquisition time 1.8s, block size 16K, no. transients 577.

Figure 1.13

High Field ^{31}P n.m.r. Spectrum of the Diastereoisomers of dAMPSMeOMe Obtained by Treatment of dAMPS with Diazomethane



Conc. ca. 50mM dAMPSMeOMe in 3:1 v/v DMF:D_4-MeOH
^{31}P n.m.r. parameters were: field strength 121.5MHz, sweep width 3650Hz, pulse width 5.0μs, aquisition time 1.2s, block size 8K, no. transients 1340.

ATPαS with a 50-fold excess of methyl iodide in methanol for 2h at room temperature has already been shown by ^1H n.m.r. not to result in base-methylation.[68] When a sample of the S-methyl-O-methyl triester of AMPS and dAMPS obtained by methyl iodide methylation of the corresponding O-methyl diester was examined by ^1H n.m.r. spectroscopy signals characteristic of the S-methyl and O-methyl protons were observed, (δ 3.8, J_{PH} 12.7Hz and δ 2.2 J_{PH} 15.4Hz respectively), but none attributable to a $CH_3N<$ group arising from base-methylation, which would be expected to appear as a singlet ca. δ 4.1, by analogy with similar examples in the literature.[123]

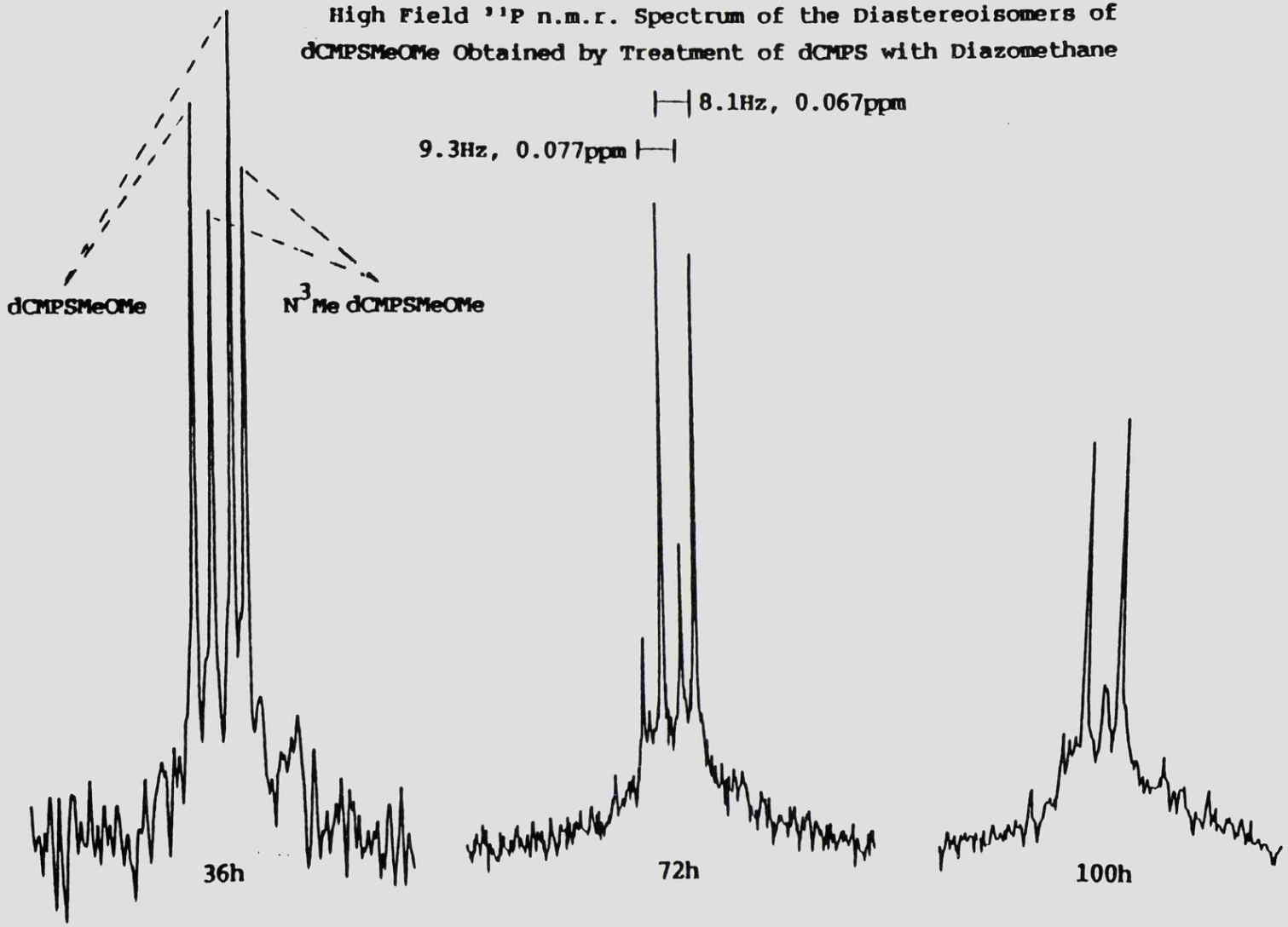
Hence the configurational assignments made using methyl iodide-methylated diester are applicable to material obtained by direct methylation of the phosphorothioate with diazomethane.

2.1.2. 2'-Deoxy cytidine 5'-O-phosphorothioate:

Cytidine is reported as being more susceptible to base-methylation by diazomethane than adenosine, and 2'-deoxy adenosine[122] and hence was expected to show some base-methylation with diazomethane. Treatment of dCMPS with excess ethereal diazomethane for ca. 24h at room temperature led to an apparently quantitative yield of the S-methyl-O-methyl triester, as judged by low-field ^{31}P n.m.r. spectroscopy. However, examination of the triester signal by high-field ^{31}P n.m.r. spectroscopy showed it to comprise of two pairs of overlapping resonances in an intensity ratio of ca 1.2:1, suggesting that partial base-methylation had occurred under these reaction conditions, accompanied by a small shift in the ^{31}P n.m.r. resonances of the diastereoisomers of 0.029 ppm.. For the pyrimidines N^3 is the only methylation site of importance;[122] measurement of the UV

Figure 1.14

High Field ^{31}P n.m.r. Spectrum of the Diastereoisomers of dCMPSMeOMe Obtained by Treatment of dCMPS with Diazomethane



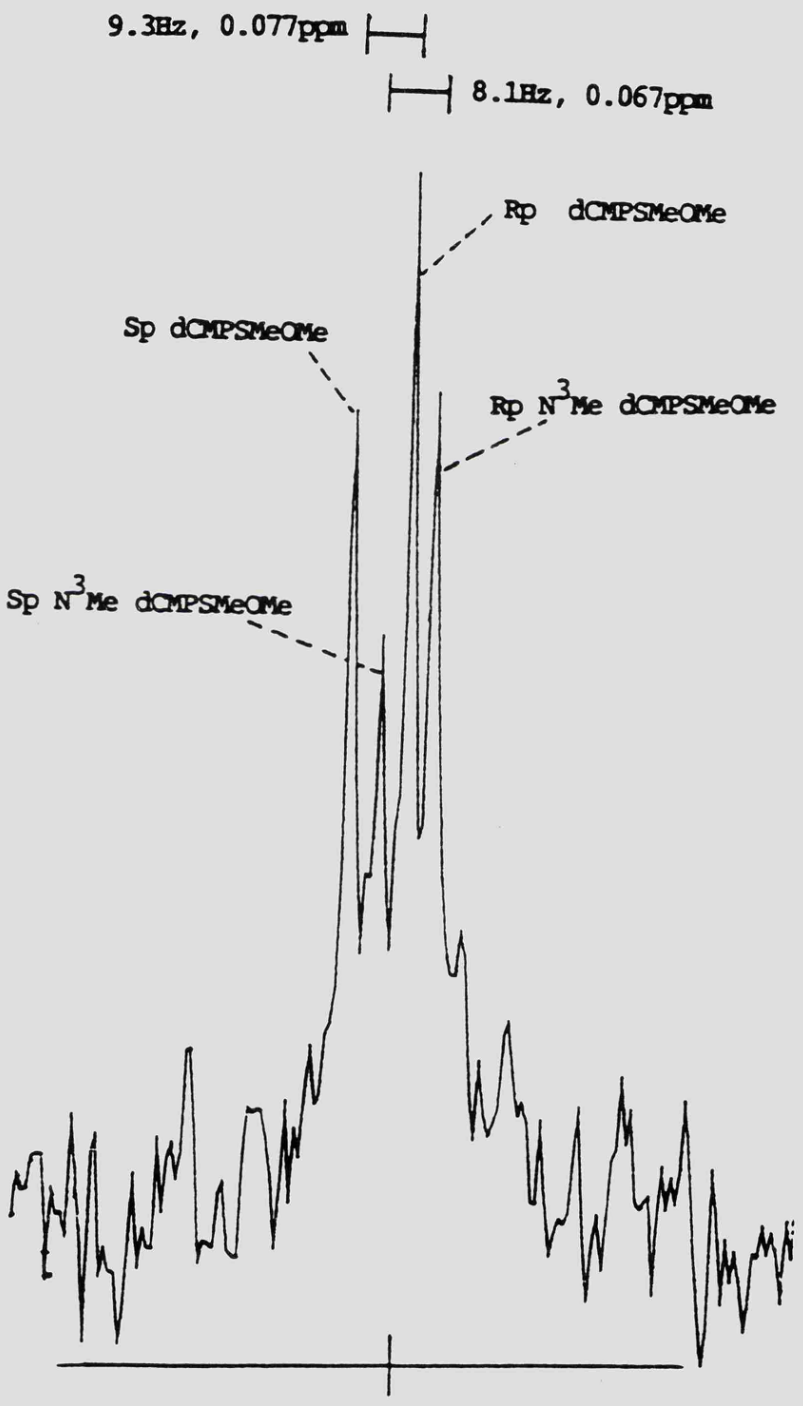
Conc. ca. 50mM dCMPSMeOMe in 3:1 v/v DMF:D_4-MeOH
^{31}P n.m.r. parameters were: field strength 121.5MHz, sweep width 4854Hz, pulse width 5.0μs, aquisition time 1.7s, block size 16K, no. transients 1601.

spectum of the sample showed a λmax of 274nm, which is intermediate between that characteristic of unmodified material, (271nm), and $\underline{N}^3$-methylated material, (278nm).[122] That one of the pairs of resonances was due to the unmodified base was shown by mixing a sample obtained in this way with a sample of methyl iodide-methylated material, which showed a λmax of 271nm, and was not expected to have undergone base-methylation. The ^{31}P n.m.r. spectrum of the resulting mixture showed that one of the pairs of diastereoisomeric resonances had increased in intensity relative to the other, no new peaks appearing. In order to obtain a clean pair of resonances it was necessary to increase the reaction time to ca. 100h, to force more base-methylation, resulting in ca. 80% yield of the $\underline{N}^3$-methylated triester, with a corresponding λmax of ca. 277nm in the UV spectrum. Figure 1.14 shows the ^{31}P n.m.r. signals of the S-methyl-O-methyl triester obtained by various exposures to excess diazomethane in DMF. Methylation of dCMPS with diazomethane using methanol as a solvent gave a clean pair of ^{31}P n.m.r resonances due to the diastereoisomers of the triester, but the overall yield was reduced relative to the reaction in DMF as a result of incomplete O-methylation and desulphurisation.

In order to show that base-methylation does not affect the direction of the ^{31}P n.m.r. resonances of the diastereoisomers of the triester a sample of Sp-deficient triester, obtained as previously described, was subjected to excess diazomethane in DMF, so as to achieve partial base-methylation. The resulting ^{31}P n.m.r. spectrum, shown in figure 1.15, indicates that the direction of resonance is unaffected by base-methylation, the overlapping pairs of resonances showing the same relative intensities.

Figure 1.15

High-field ^{31}P n.m.r. Resonances of a Sample of Sp-deficient S-methyl-O-methyl Triesters of dCMPS Showing Partial Base-methylation by Diazomethane



Conc. ca. 5mM dCMPS-OMe/N^3Me dCMPSMeOMe in 3:1 v/v DMF:D_4-MeOH

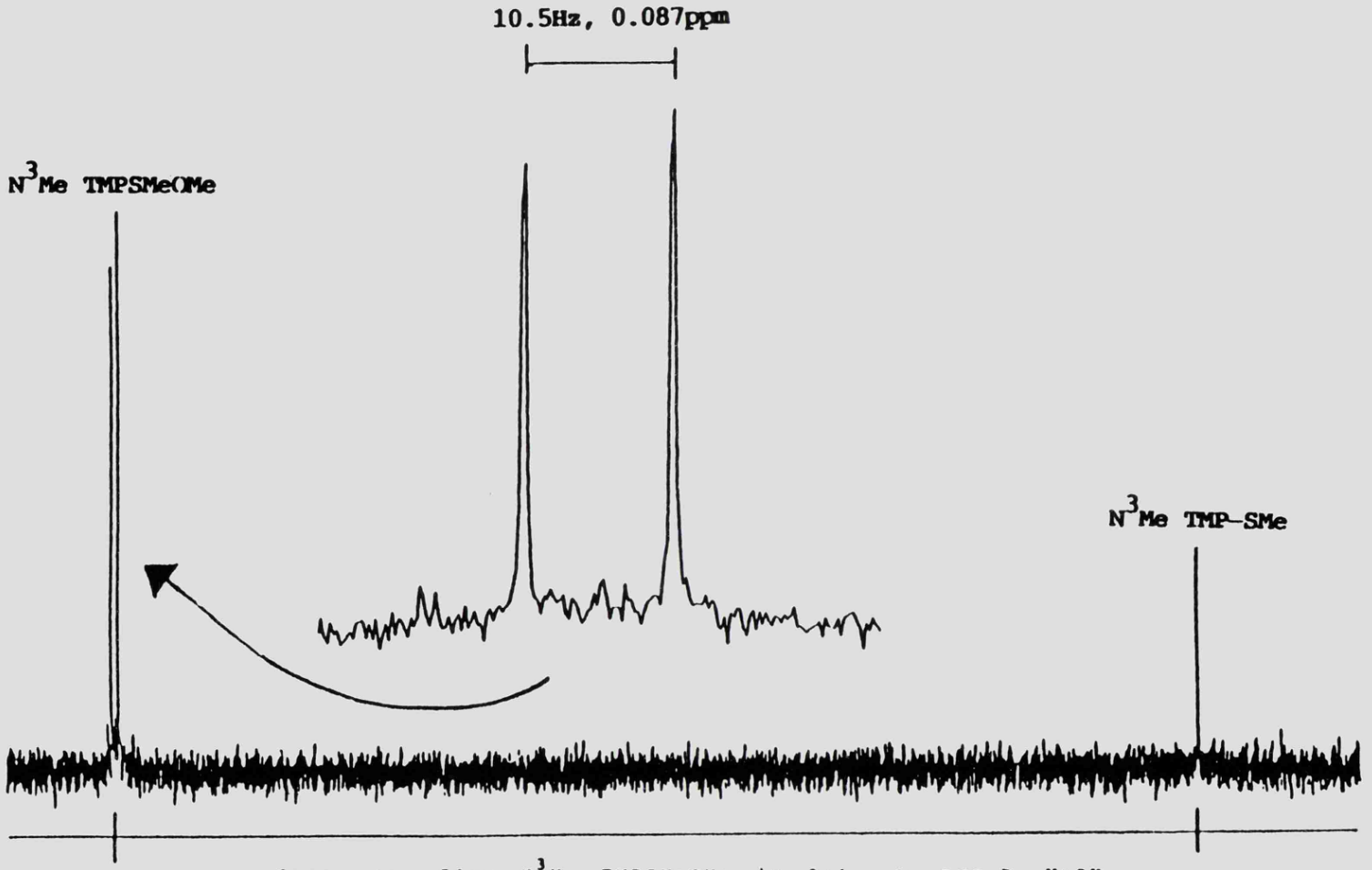
^{31}P n.m.r. parameters were: field strength 121.5MHz, sweep width 6097Hz, pulse width 5.0μs, aquisition time 1.3s, block size 16K, no. transients 6000, line broadening 1.0Hz, Gaussian broadening 0.25Hz.

2.1.3. Thymidine 5'-O-Phosphorothioate:

TMPS was expected to be more susceptible to base-methylation by diazomethane than dAMPS or dCMPS, as judged by the reported relative susceptibilities of the corresponding nucleosides.[122] Treatment of TMPS for 24h with excess diazomethane led to ca. 85% yield of the triester in the presence of the corresponding S-methyl diester, whilst a 48h exposure led to a quantitative yield of the triester, which was expected to be extensively methylated at N^3 of the pyrimidine base. The high-field ^{31}P n.m.r. spectrum showed a clean pair of resonances attributed to the diastereoisomers of the N^3-methylated S-methyl-O-methyl triester, indicating that base-methylation was complete, Figure 1.16. The corresponding UV spectrum showed a λmax of 265nm, characteristic of N^3-methylated thymidine systems, compared with a value of 267nm characteristic of unmodified thymidine systems.[122] When material obtained in this way was combined with a sample of the triester generated by methyl iodide-methylation of the O-methyl diester, the high-field ^{31}P n.m.r. spectrum of the mixture showed two overlapping pairs of resonances. Treatment with methyl iodide was not expected to result in base-methylation,[122] and the UV spectrum of the methyl iodide-methylated material showed a λmax of 267nm, characteristic of un-modified base. In order to show that the direction of resonance of the diastereoisomeric triesters is not altered by base-methylation, the Sp-deficient sample of methyl iodide-methylated material was subjected to excess diazomethane so as to effect N^3-methylation. Re-examination of the high-field ^{31}P n.m.r. spectrum showed that the direction of resonance of the diastereoisomers was unaffected by base-methylation, Figure

Figure 1.16

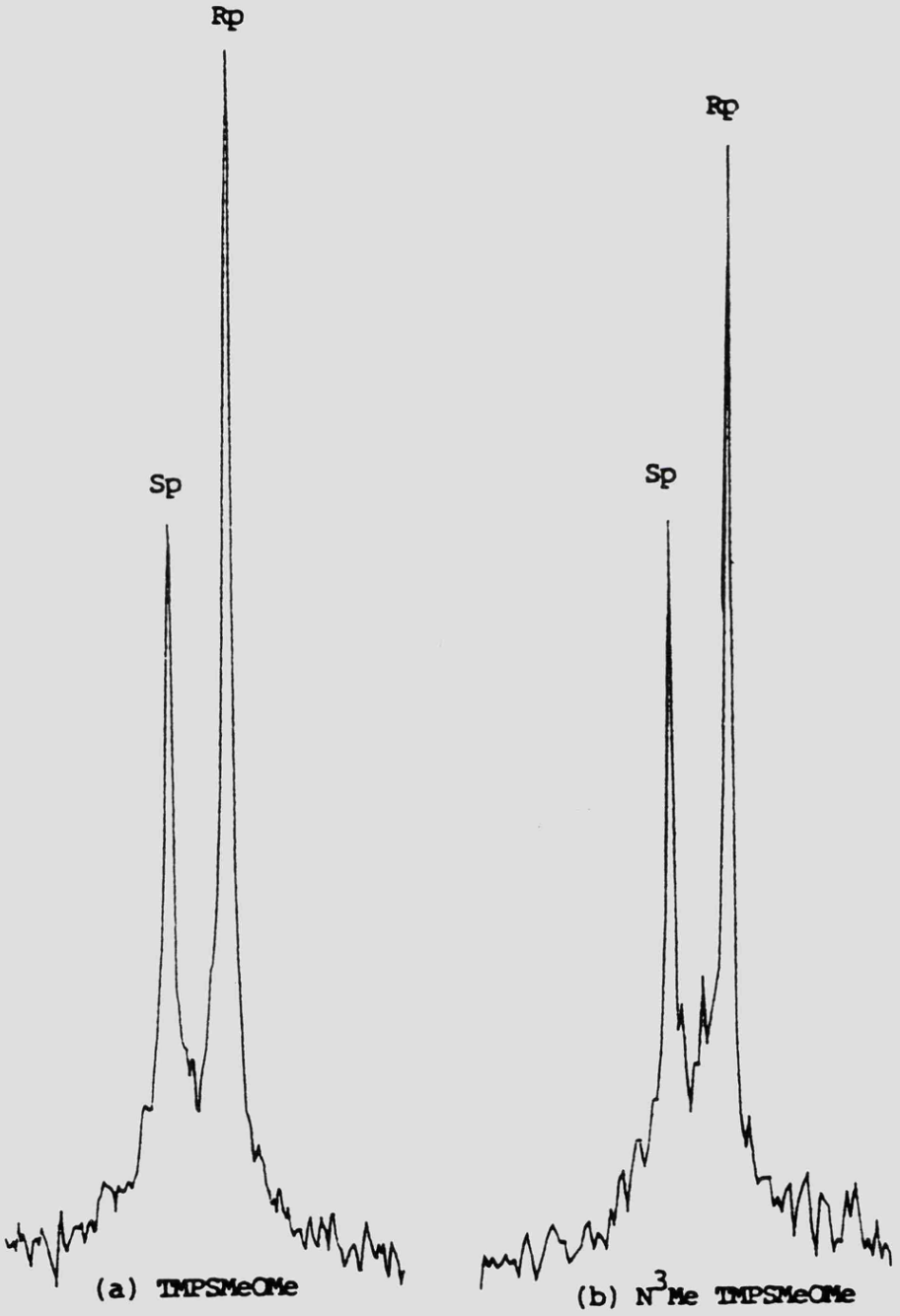
High Field ^{31}P n.m.r. Spectrum of the Diastereoisomers of N^3Me TMPSMeOMe Obtained by Treatment of TMPS with Diazomethane



Conc. ca. 50mM N^3Me TMPSMeOMe in 3:1 v/v DMF:D_4-MeOH ^{31}P n.m.r. parameters were: field strength 121.5MHz, sweep width 3649Hz, pulse width 5.0μs, aquisition time 2.2s, block size 16K, no. transients 200.

Figure 1.17

High Field ^{31}P n.m.r. Spectra of Sp-deficient Samples of N^3Me TMPSMeOMe and TMPSMeOMe

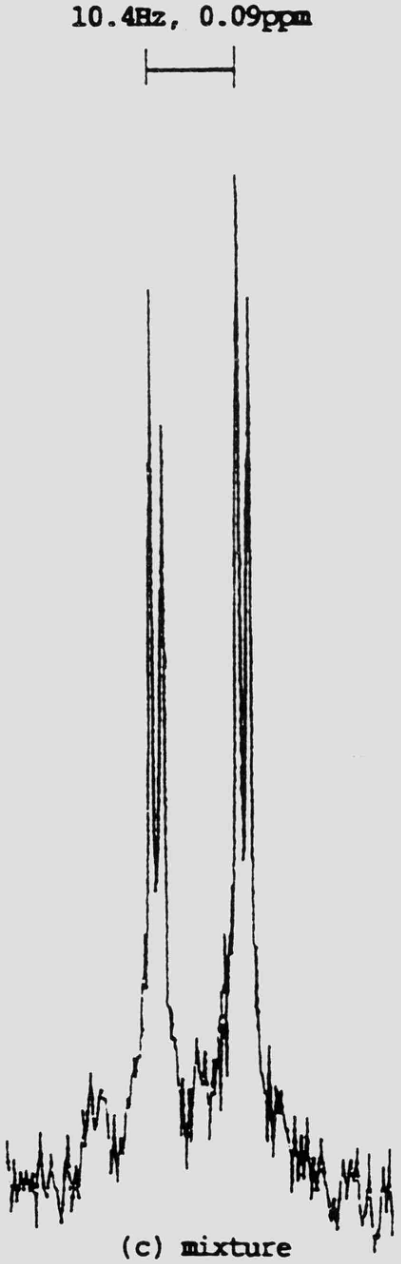


(a). Conc. ca. 8mM TMPSMeOMe in 3:1 v/v DMF: D_4-MeOH ^{31}P n.m.r. parameters were: field strength 121.5MHz, sweep width 7246Hz, pulse width 5.0μs, aquisition time 1.1s, block size 16K, no. transients 49960, line broadening 0.5Hz, Gaussian broadening 0.08Hz.

(b). Conc. ca. 8mM N^3Me TMPSMeOMe in 3:1 v/v DMF: D_4-MeOH ^{31}P n.m.r. parameters were: field strength 121.5MHz, sweep width 6092Hz, pulse width 6.0μs, aquisition time 1.3s, block size 16K, no. transients 42017, line broadening 1.0Hz.

Figure 1.17 cont.

High Field ^{31}P n.m.r. Spectra of Sp-deficient Samples of N^3Me TMPSMeOMe and TMPSMeOMe



(c) mixture

(c). Conc. ca. 4mM N^3Me TMPSMeOMe/TMPSMeOMe in 3:1 v/v DMF:D_4-MeOH
^{31}P n.m.r. parameters were: field strength 121.5MHz, sweep width 6097Hz, pulse width 8.0μs, aquisition time 1.3s, block size 16K zero-filled to 32k, no. transients 28174.

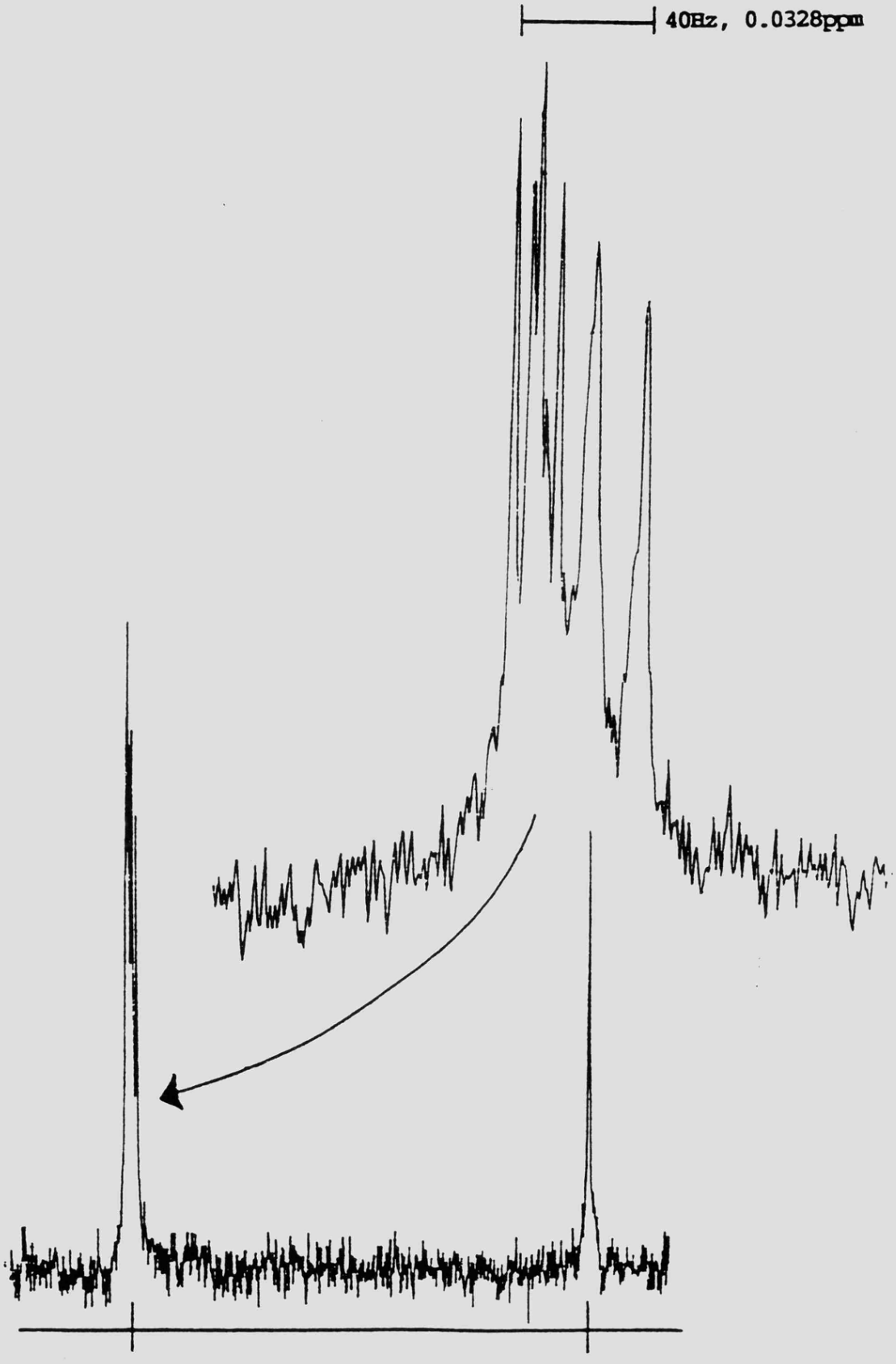
1.17a,b. Hence the configurational assignments made previously for unmodified triester are applicable to $\underline{N}^3$-methylated triester. As a further check the remaining half of the sample of Sp-deficient methyl iodide-methylated triester was combined with an approximately equivalent amount of a 1:1 mixture of diastereoisomers, then this sample divided in half. One half was treated with diazomethane, then the two parts recombined. The high-field ^{31}P n.m.r. spectrum shown in figure 1.17c was obtained, showing that the two pairs of diastereoisomers resonate in the same direction, thus reaffirming that methylation of the pyrimidine base does not affect the configurational assignment of these triesters.

2.1.4. 2'-Deoxyguanosine 5'-O-phosphorothioate:

dGMPS was expected to be the most susceptible of the systems under investigation to base-methylation with diazomethane.[122] A 24h exposure resulted in an apparent yield of ca. 68% if the S-methyl-O-methyl triester in the presence of the corresponding S-methyl diester, as judged by low-field ^{31}P n.m.r. spectroscopy. However, examination of the triester signal at high-field showed it to consist of a complex pattern of overlapping resonances, attributed to various base-modified species, unsuitable for the observation of isotope shifts, Figure 1.18. This is in agreement with the observation by Farmer et. al.[124] of a mixture of products on methylation of dGMP with excess ethereal diazomethane. The corresponding UV spectrum showed a shift in λmax from 259nm, characteristic of unmodified material, to 271nm, consistent with base-methylation.[122] In the case of dGMPS, therefore, diazomethane is not a suitable reagent and stereochemical analysis must be performed using the alternative methylating agent, dimethyl sulphate.

Figure 1.18

High Field ^{31}P n.m.r. Spectrum of a Sample of Diazomethane-methylated 2'-Deoxy Guanosine 5'-O-Phosphorothioate



Conc. ca.40mM methylated dGMPS in 3:1 v/v DMF:D_4-MeOH ^{31}P n.m.r. parameters were: field strength 121.5MHz, sweep width 11627Hz, pulse width 5.0μs, aquisition time 0.7s, block size 16K, no. transients 5205, line broadening 0.5Hz, Gaussian broadening 0.2Hz.

2.2 Dimethyl Sulphate as a Methylating Agent:

The relative susceptibilities of the nucleosides to base methylation with dimethyl sulphate are reported as being;[122]

guanosine > adenosine > cytidine >> thymidine

and the corresponding nucleoside phosphorothioates are expected to show similar relative susceptibilities.

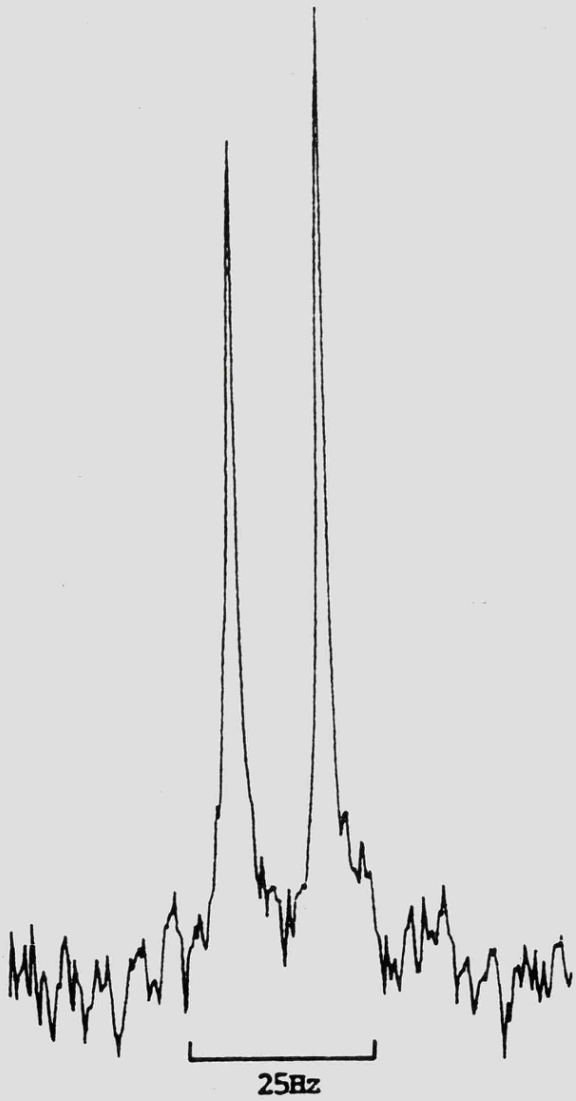
Methylation of the nucleoside 5'-phosphorothioates using dimethyl sulphate was found to give quantitative yields of the desired triester in ca. 2-3h at room temperature. Since dimethyl sulphate is non-volatile, and so could not easily be removed by evaporation, ^{31}P n.m.r. spectra were recorded in its presence. The presence of dimethyl sulphate in the ^{31}P n.m.r. samples did not have a significant effect on the observed shifts of the S-methyl-O-methyl phosphorothioate triesters. Thus samples containing ca. 10-40% dimethyl sulphate in DMF:D_4MeOH showed shifts in the range δ_P +30ppm to δ_P +31ppm, compared with values of ca. δ_P +29.5ppm to δ_P +30.5ppm in the absence of dimethyl sulphate, and ca. δ_P +32ppm in MeOH. Separations of diastereoisomers tended to be slightly increased, by the order of ca. 2 Hz, 0.016ppm, and the direction of resonance of the diastereoisomers of the triesters was unaffected.

2.2.1. Thymidine 5'-O-phosphorothioate:

On the basis of literature reports the thymidine base was expected to be resistant to alkylation with dimethyl sulphate.[122] When a sample of TMPS was treated with an approximately 100-fold

Figure 1.19

High Field ^{31}P n.m.r. Spectrum of the Diastereoisomers of TMPSMeOMe Obtained by Treatment of TMPS with Dimethyl Sulphate



Conc. ca. 15mM TMPSMeOMe in 3:1:1 v/v DMF:D_4MeOH:Me_2SO_4 ^{31}P-n.m.r. parameters were: field strength 121.5MHz, sweep width 4854Hz, pulse width 5.0μs, aquisition time 1.7s, block size 16K, no. transients 680, line broadening 0.5Hz, Gaussian broadening 0.2Hz.

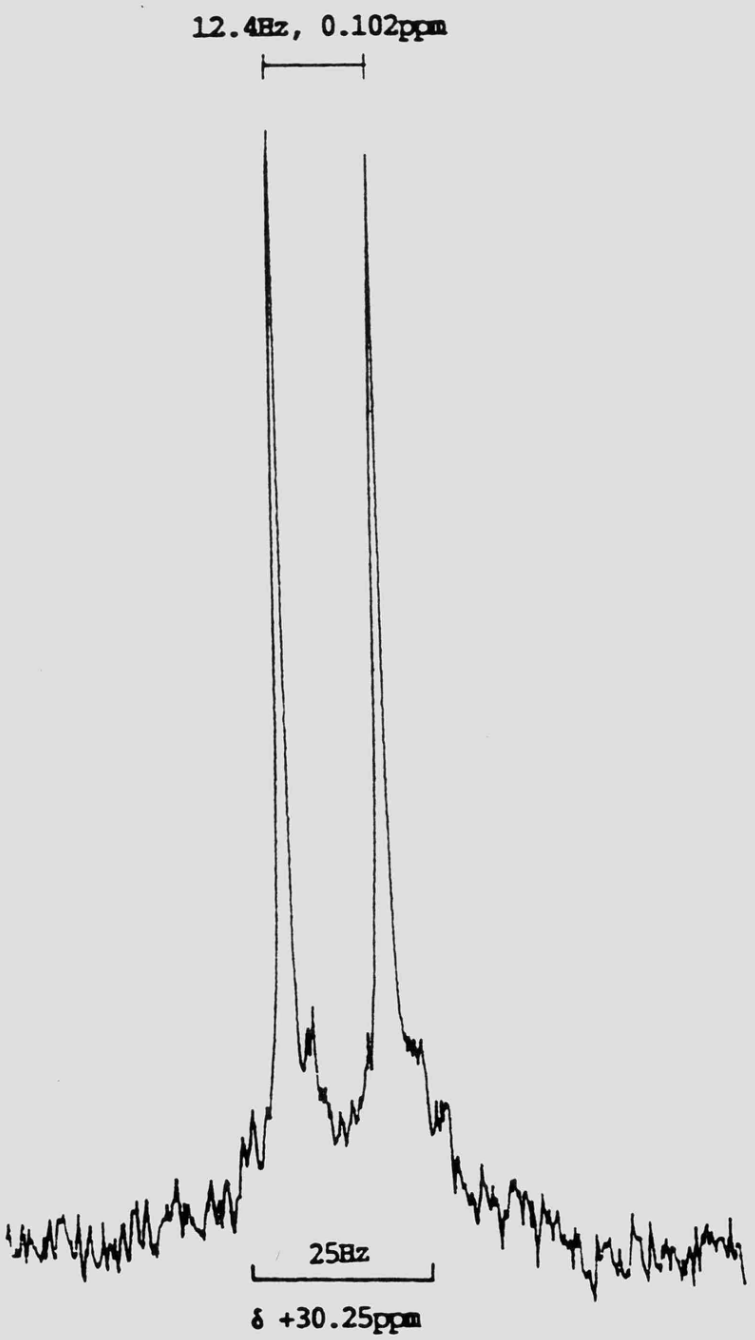
excess of dimethyl sulphate in DMF in the presence of triethylamine, the high-field ^{31}P n.m.r. spectrum of the reaction mixture indicated a quantitative yield of diastereoisomers of the S-methyl-O-methyl triester, appearing as a clean pair of resonances, (Figure 1.19). The literature based assumption that base-methylation had not occurred under these conditions[122] was supported by the observation of a λmax of 267nm in the UV spectrum, characteristic of un-modified material. Hence for the stereochemical analysis of [^{16}O,^{18}O]TMPS either diazomethane or dimethyl sulphate can successfully be employed as a methylating agent to give a quantitative yield of triester, and a clean pair of resonances suitable for the observation of ^{18}O-isotope shifts.

2.2.2. 2'-Deoxycytidine 5'-O-phosphorothioate:

In the case of dCMPS, which was expected to be more susceptible to base-methylation with dimethyl sulphate than TMPS,[122] a 2h exposure to excess dimethyl sulphate in DMF in the presence of triethylamine gave an apparantly quantitative yield of the triester, as judged by low-field ^{31}P n.m.r. spectroscopy. However, examination of the triester resonances at high-field showed that, as was the case when diazomethane was employed as the methylating agent, partial methylation on the pyrimidine base had occurred giving rise to two pairs of diastereoisomers, so that the ^{31}P n.m.r. showed corresponding overlapping pairs of resonances. The UV spectrum of the reaction mixture showed a value for λmax intermediate between that of unmethylated material, (271nm), and N^3-methylated material, (278nm),[122] consistent with partial base-methylation. In order to obtain complete N^3-methylation, and hence a clean pair of

Figure 1.20

High Field ^{31}P n.m.r. Spectrum of the Diastereoisomers of N^3Me dCMPSMeOMe Obtained by Treatment of dCMPS with Dimethyl Sulphate



Conc. ca. 35mM N^3Me dCMPSMeOMe in 3:1:1 v/v DMF:D_4MeOH:Me_2SO_4
^{31}P n.m.r. parameters were: field strength 121.5MHz, sweep width 7246Hz, pulse width 5.0μs, aquisition time 2.3s, block size 16K zero-filled to 32K, no. transients 1233, line broadening 0.3Hz, Gaussian broadening 0.1Hz.

diastereoisomeric resonances suitable for the measurement of ^{18}O-isotope shifts it was necessary to methylate first in the absence of triethylamine. Thus after 30min exposure in the absence of triethylamine methylation on sulphur was complete, as indicated by the low-field ^{31}P n.m.r. spectrum of the reaction mixture which appeared as a single resonance at δ +21ppm and the corresponding UV spectrum showed a characteristic λmax of 278nm, indicating that complete $\underline{N}^3$-methylation had occurred on the pyrimidine base. Subsequent addition of triethylamine to the reaction mixture resulted in rapid methylation on oxygen to give the desired triester quantitatively, which, by high-field ^{31}P n.m.r. spectroscopy, appeared as a clean pair of diastereoisomeric resonances, Figure 1.20. Thus, for the analysis of a sample of [^{16}O,^{18}O]dCMPS, a higher yield of the desired triester is obtainable more rapidly using dimethyl sulphate than with diazomethane, so that dimethyl sulphate is is the favoured reagent.

2.2.3. Adenosine and 2′-deoxyadenosine 5′-O-phosphorothioate:

The principal sites of base-methylation for adenosine and 2′-deoxyadenosine derivatives by dimethyl sulphate are reported as being $\underline{N}^1$- with some alkylation at $\underline{N}^3$- and $\underline{N}^7$-,[122] the relative susceptibilities of these sites being reported as;[125]

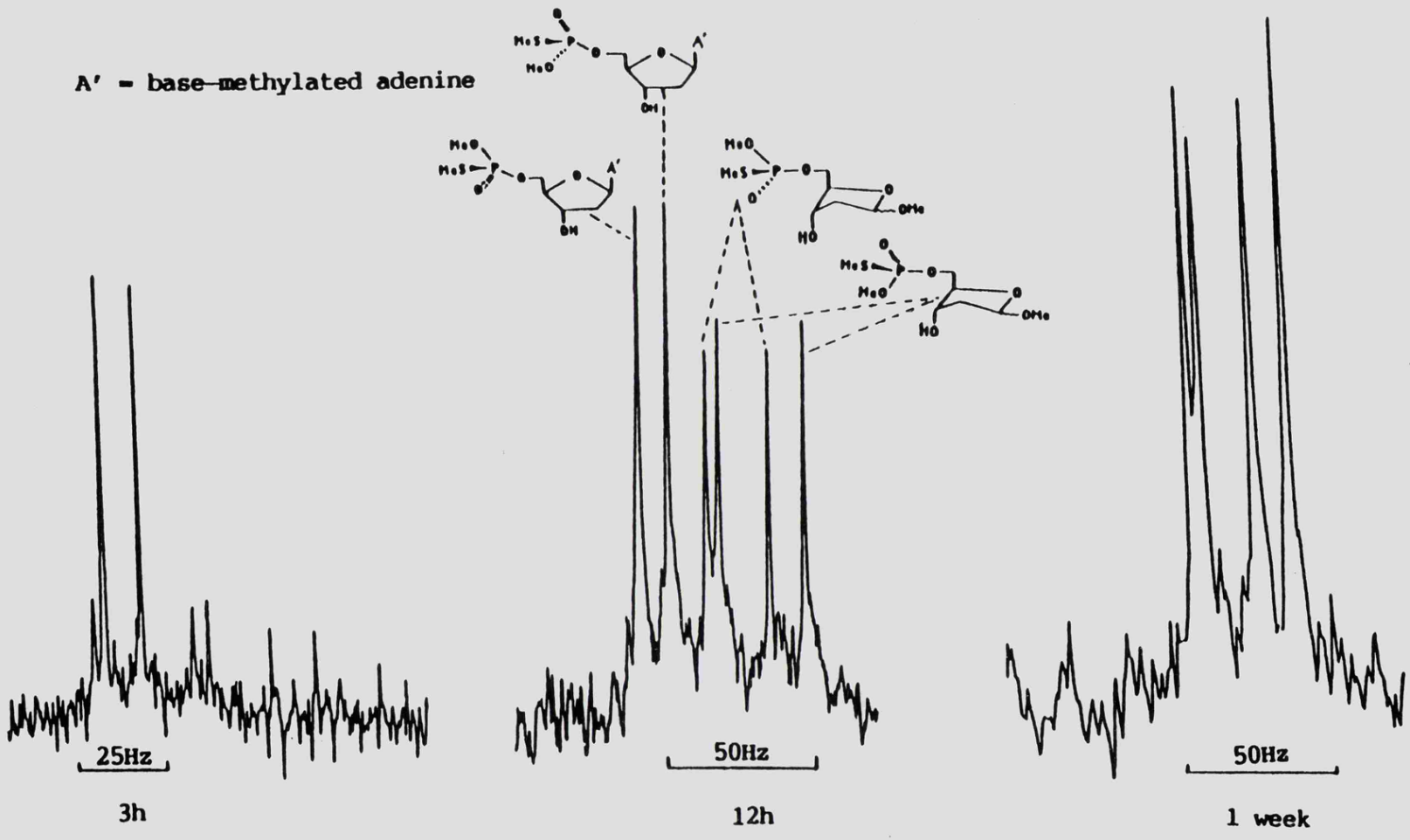
$$\underline{N}^1- > \underline{N}^3- > \underline{N}^7-$$

Treatment of AMPS with 100 equiv. of dimethyl sulphate leads to a quantitative yield of the S-methyl-O-methyl triester, the high-field ^{31}P n.m.r. spectrum of which showed a clean pair of diastereoisomers, separation 14.1Hz,0.12ppm, which were stable over a period of several weeks at room temperature.

Similarly, treatment of dAMPS with excess dimethyl sulphate

Figure 1.21

High Field ^{31}P n.m.r. Spectrum of the Diastereoisomers of the S-methyl-O-methyl Phosphorothioate Triester Obtained by Methylation of dAMPS with Dimethyl Sulphate



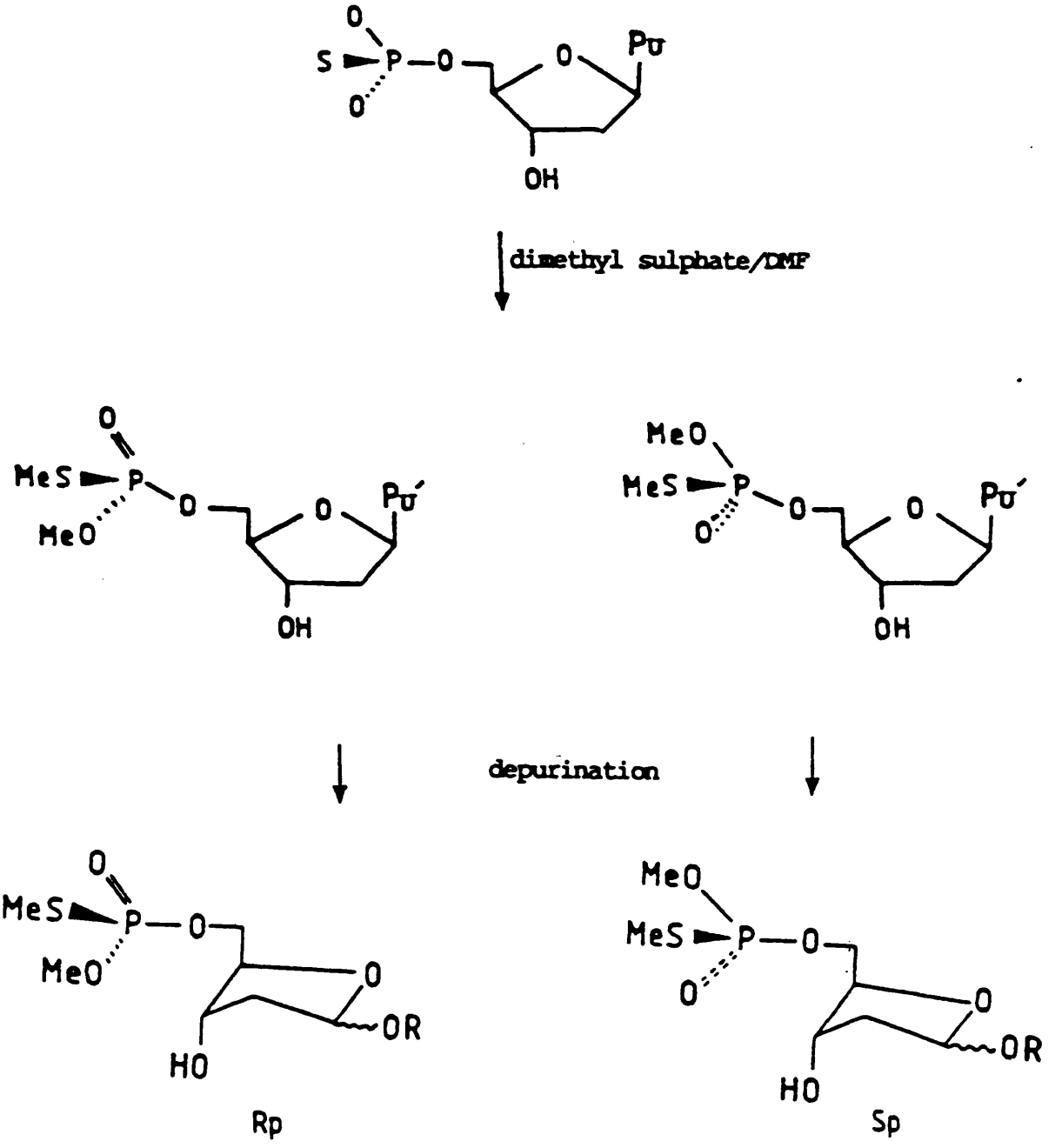
Conc. ca. 50mM in 2:1:2 v/v DMF:D$_2$O:Me$_2$SO$_4$. ^{31}P n.m.r. parameters were: field strength 121.5MHz, sweep width 6067Hz, pulse width 5.0μs, aquisition time 1.7s, block size 16K, no. transients 1276, line broadening 1.0Hz, Gaussian broadening 0.2Hz.

yielded the corresponding S-methyl-O-methyl triester, the diastereoisomers of which appeared as a pair of resonances in the high field ^{31}P n.m.r. spectrum. However, on re-recording the high-field ^{31}P n.m.r. after the n.m.r. sample had been standing at room temperature for ca. 12h, a set of four overlapping resonances downfield of the original signals by ca 0.27ppm were observed, (Figure 1.21). When the spectrum was re-recorded 6 days later the original signals due to the diastereoisomers of the S-methyl-O-methyl triester were no longer detectable, the set of four overlapping resonances being the only signals observed.

It is well known that alkylation of the 2'-deoxyadenosine system at N^3- and N^7- has a destabilising effect on the glycosidic bond,[122,125,126] and that the deoxy-analogue undergoes a more rapid depurination than the ribo- analogue.[127] This destabilisation of the glycosidic bond of adenine methylated at N^3- by dimethyl sulphate is exploited by Maxam and Gilbert in their method of DNA sequencing in order to achieve chain cleavage at adenine residues.[126] In double stranded DNA the N^1- site is not exposed to methylation, but in a single stranded DNA it is, and methylation occurs without glycosidic destabilisation, and without preventing further N^3 methylation. It seems likely, therefore, that methylation of AMPS and dAMPS with dimethyl sulphate is accompanied by methylation at N^1 and N^3 of the adenine base, resulting in formation of the $N^{1,3}$-dimethyl 2'-deoxyadenosine 5'-S-methyl-O-methyl phosphorothioate triester, and the corresponding adenosyl triester. The less stable 2'-deoxyribo- system appears to be undergoing slow depurination in the n.m.r. sample as a result of base-methylation, (Scheme 1.8). That the original pair of diastereoisomers decay to give a

Scheme 1.8

Depurination of Base-methylated 2'-deoxyribo Purine Nucleoside -S-methyl-O-methyl Phosphorothioates



Pu = guanine, adenine

Pu' = N^7-methyl guanine, $N^{1,3}$-dimethyl adenine

R = H or Me

set of four overlapping resonances is attributable to the formation of α and β epimers at C1′ in approximately equal amounts. Although the structure of the depurinated material was not further investigated it seems likely that the epimeric substituent at C1′ is methoxy, in view of the observation that depurination appears to commence from the time at which the n.m.r. sample is prepared by introduction of D_4-MeOH. Alternatively the C1′ substituent may be hydroxyl, from water present in the n.m.r. solvents. The amount of dimethyl sulphate employed in the methylation reaction was reduced to a 10-fold excess in order to induce more selective base-methylation. The high-field ^{31}P n.m.r. spectrum of the resulting S-methyl-O-methyl triester showed a stable pair of diastereoisomeric N^1-methylated triesters, (ca. 65%), which were stable over a week, in the presence of depurinated material presumed to arise from some additional N^3-methylation, (ca. 35%).

In order to check the effect of base-methylation on the configurational assignments for the S-methyl-O-methyl phosphorothioate triesters of AMPS and dAMPS, samples of Sp-deficient triester, obtained from the methyl-iodide methylation of the corresponding Sp-deficient O-methyl diester, were exposed to dimethyl sulphate, and the ^{31}P n.m.r. spectra examined. As expected, the direction of resonance of the triesters were unaffected by base-methylation, the lower intensity Sp diastereoisomer resonating downfield of the Rp diastereoisomer. In view of the sensitivity of the 2′-deoxyadenosine system to depurination when dimethyl sulphate is used as the methylating agent, diazomethane is the strongly favoured reagent for configurational analysis of [^{16}O,^{18}O]dAMPS, whilst for the ribo equivalent either reagent is acceptable.

Dimethyl sulphate has been successfully employed in the configurational analysis of a sample of [^{16}O,^{18}O]AMPS obtained in the stereochemical investigation of bovine intestinal mucosa 5'-nucleotide phosphodiesterase, (Chapter Two).

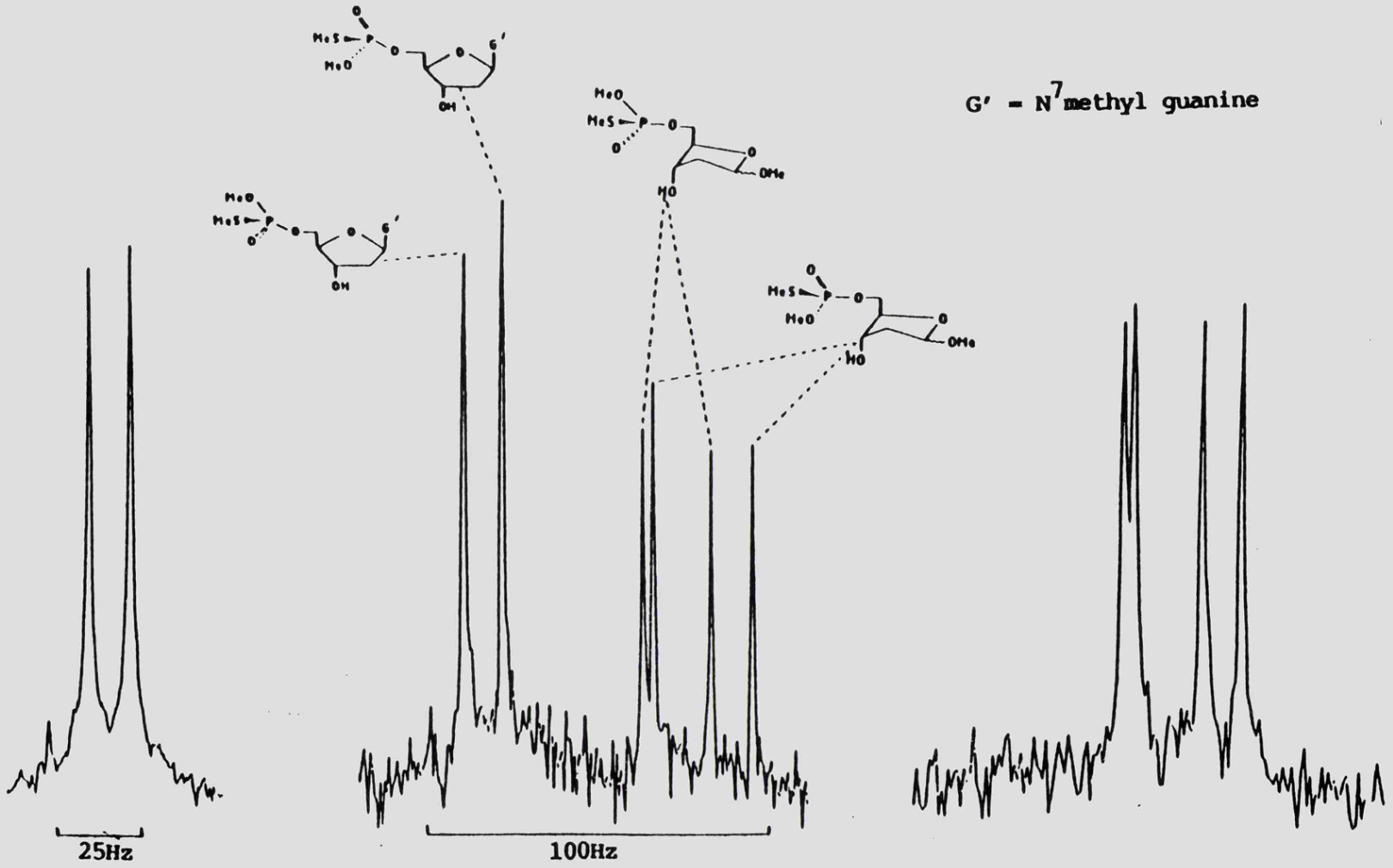
2.2.4. 2'-Deoxyguanosine 5'-O-phosphorothioate:

The major site of alkylation of the guanine base with dimethyl sulphate is reported as being $\underline{N}^7$-,[122] again this is reported to result in destabilisation of the glycosidic bond,[122,126] as exploited in the Maxam-Gilbert method of DNA sequencing for obtaining cleavage at guanine positions. Again the effect is more pronounced in the deoxyribo- than the ribo-series.[126] Treatment of dGMPS with excess dimethyl sulphate in DMF gives a quantitative yield of $\underline{N}^7$-methylated triester as a clean pair of resonances, the corresponding UV spectrum being in agreement with that reported for $\underline{N}^7$-methylated derivatives of guanosine and 2'-deoxyguanosine. As observed for dAMPS, slow depurination of the sample occurs, the diastereoisomeric triester signals decaying with the concommitant increase in the four overlapping upfield resonances due to α and β epimers of the depurinated triesters, Figure 1.22.

Configurational assignments for the material arising from depurination of $\underline{N}^7$-methylated $\underline{S}$-methyl-$\underline{O}$-methyl triesters of dGMPS and $\underline{N}^{1,3}$-dimethylated $\underline{S}$-methyl-$\underline{O}$-methyl triesters of dAMPS could be made by examination of the relative peak intensities in the ^{31}P n.m.r. spectrum of samples of Sp-deficient triester after depurination, (figure 1.23). Although the α and β epimers were not assignable to their corresponding ^{31}P n.m.r. resonances, examination of the relative peak intensities in the spectrum allowed the resonances due to the epimers with the Sp configuration

Figure 1.22

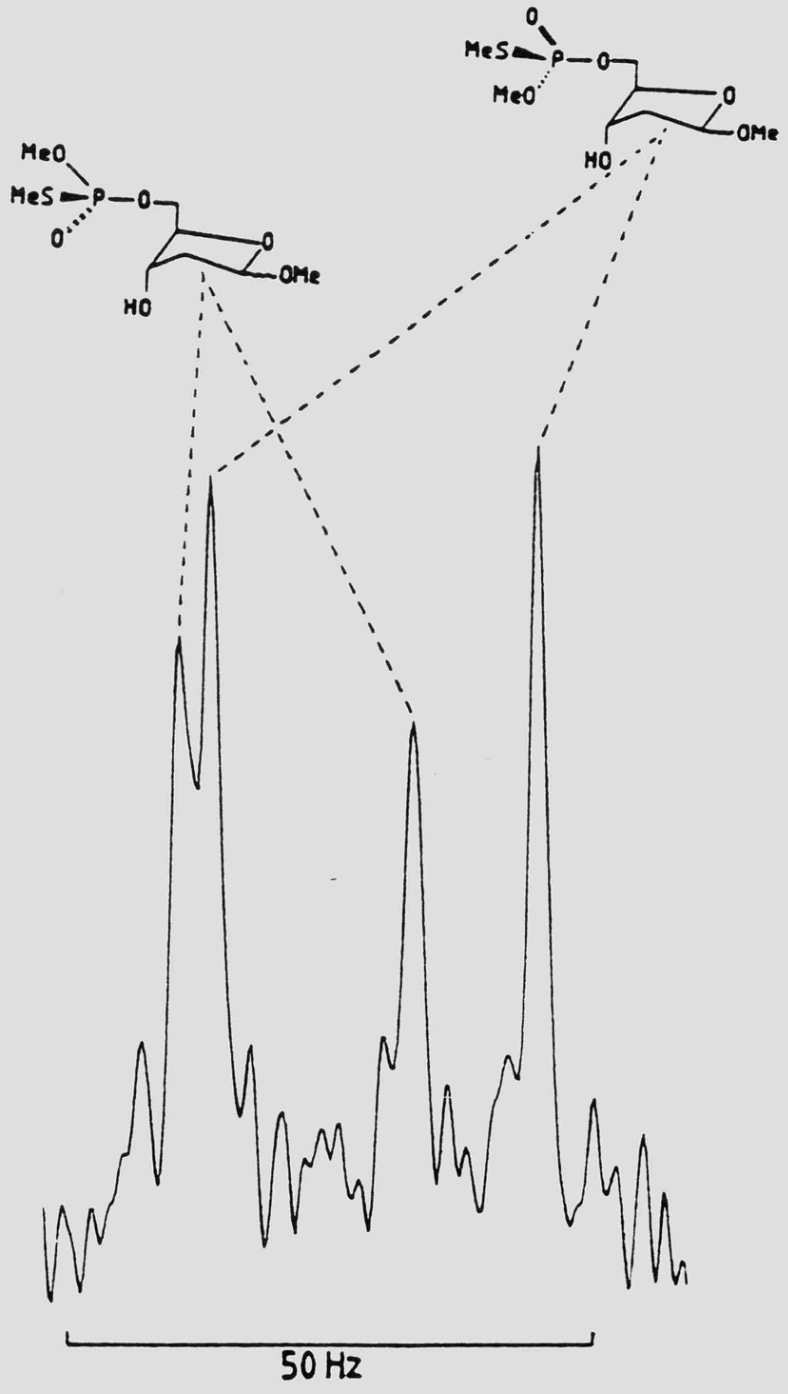
High Field ^{31}P n.m.r. Spectrum of the Diastereoisomers of the S-methyl-O-methyl Phosphorothioate Triester Obtained by Methylation of dGMPS with Dimethyl Sulphate



Conc. ca. 15mM in 2:1:2 v/v DMF:D2O:MeOH:Me2SO4
31P n.m.r. parameters were: field strength 121.5MHz, sweep width 7246Hz, pulse width 5.0μs, aquisition time 1.3s, block size 16K, no. transients 2002, line broadening -0.5Hz, Gaussian broadening 0.2Hz.

Figure 1.23

High Field ^{31}P n.m.r. Spectrum of Depurinated S-methyl-O-methyl Phosphorothioate Triesters



^{31}P n.m.r. parameters were: field strength 121.5MHz, sweep width 4854Hz, pulse width 5.0μs, aquisition time 1.7s, block size 16K, no. transients 8000, line broadening -0.5Hz, Gaussian broadening 0.08Hz.

at the phosphorothioate centre to be identified. It can be seen that, of the two well-separated upfield resonances, the Sp diastereoisomer resonates downfield of the Rp as for all other systems investigated here. Since these resonances are well separated isotope shifts are expected to be clearly detectable on these resonances, so that configurational analysis can still be performed in the event of depurination.

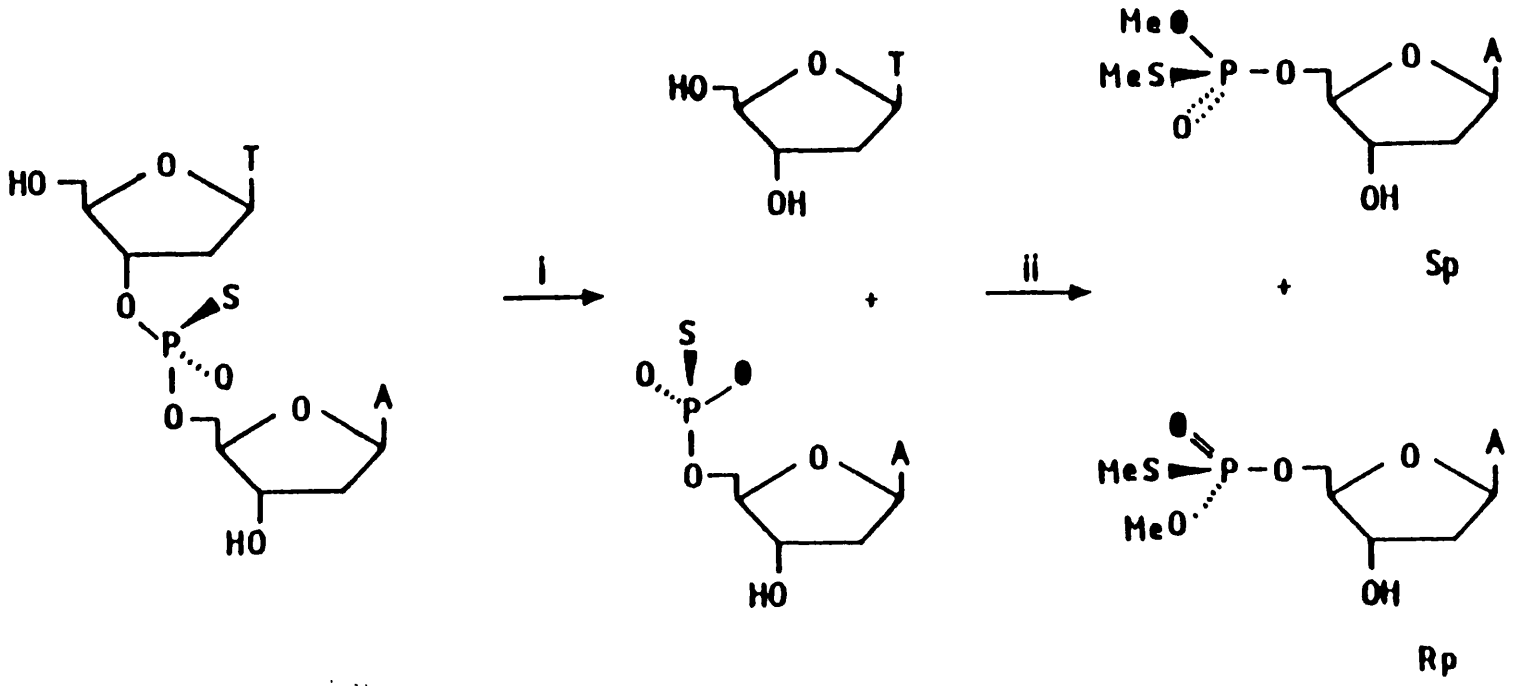
3. VALIDATION OF THE ANALYSIS METHOD:

In order to demonstrate the validity of the analysis method a sample of 2'-deoxyadenosine 5'-[^{16}O,^{18}O] phosphorothioate of known absolute configuration at phosphorus, generated by the stereospecific cleavage of a sample of (Sp)-5'-O-(2'-deoxyadenosyl)-3'-O-thymidyl phosphorothioate in ^{18}O-labelled water using Nuclease P1, (scheme 1.9), was provided by Dr. B.V.L. Potter. This cleavage has already been shown to proceed with inversion of configuration at phosphorus,[68] so that the nucleoside 5'-[^{16}O,^{18}O] phosphorothioate isolated from the digestion mixture was known to have the Sp configuration.

Methylation of the sample of ^{18}O-labelled material with diazomethane in the presence of unlabelled material led to the formation of the corresponding phosphorothioate S-methyl-O-methyl triester, resonating at δ_p(DMF) +29.9ppm,s. Examination of the high-field ^{31}P n.m.r. spectrum in a solvent system of 3:1 v/v DMF:D_4MeOH gave the spectrum shown in figure 1.24. The higher intensity resonances are due to the diastereoisomers of the unlabelled triester, each showing a lower intensity isotope-shifted resonance. The isotope shift on the

Scheme 1.9

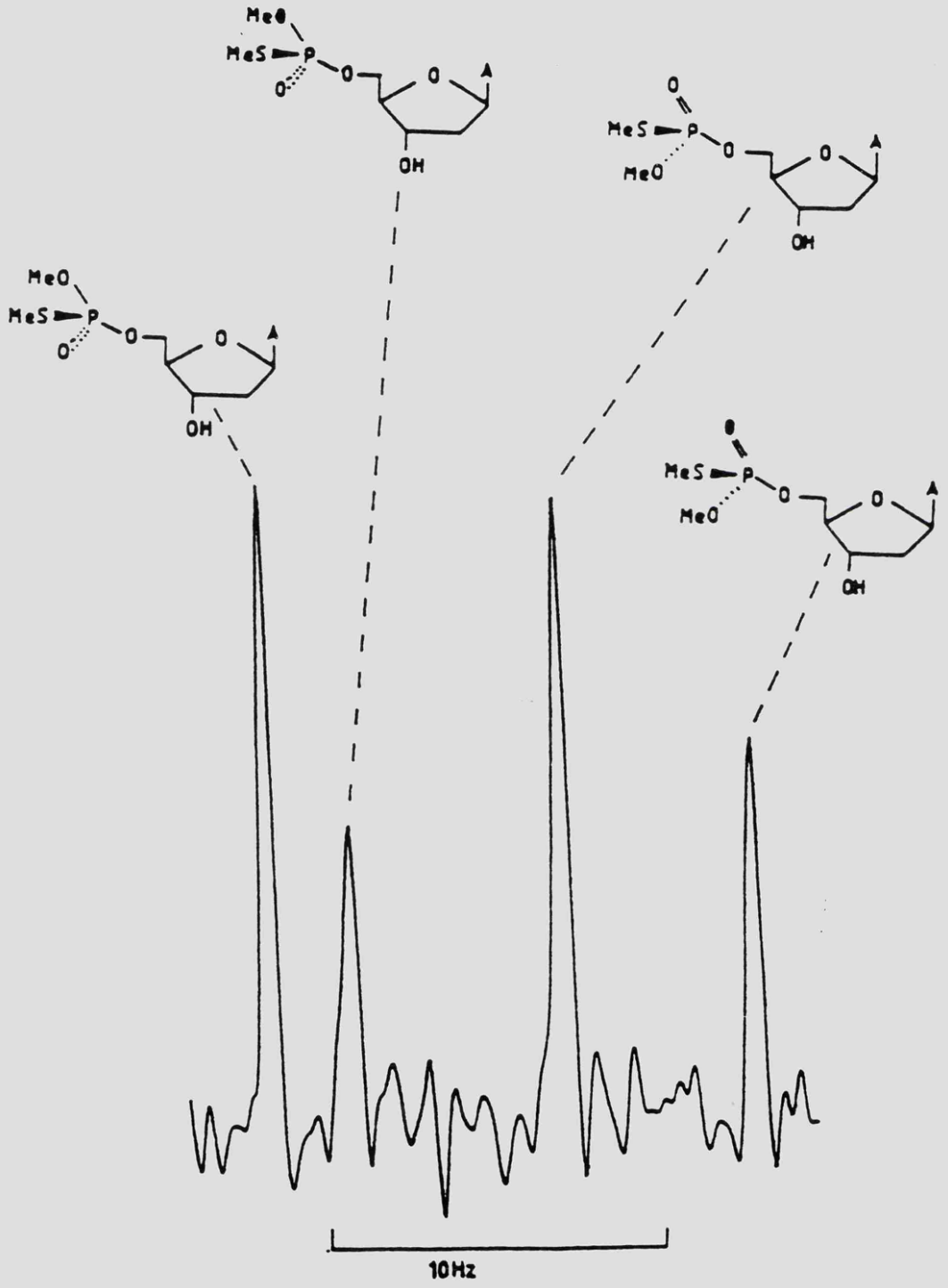
Summary of the Validation of the Analysis Method



REAGENTS: I) Nuclease P1-H_2O; II) CH_2N_2-MeOH

Figure 1.24

High Field ^{31}P n.m.r. Spectrum of the Diastereoisomers of $[^{16}O_2]$ dAMPSMeOMe and $[^{16}O,^{18}O]$ dAMPSMeOMe



Conc. ca. 10mM in 3:1 v/v DMF:D_4-MeOH
^{31}P n.m.r. parameters were: field strength 121.5MHz, sweep width 2000Hz, pulse width 8.0μs, aquisition time 8.4s, block size 16K, no. transients 6600, line broadening -0.5Hz, Gaussian broadening 0.1Hz.

downfield resonance is small, (0.019ppm, 2.3 Hz), relative to that on the upfield resonance, (0.047ppm, 5.7 Hz), indicating that the ^{18}O-label must be singly-bonded to phosphorus in the P-OMe position in the former case, and doubly-bonded in the P=O position in the latter, since the magnitude of the isotope shift is a function of bond order.[111,112] Since the configuration of the 2'-deoxyadenosine 5'-[^{16}O,^{18}O] phosphorothioate starting material is known, and hence the position of the ^{18}O-isotope in the product, the diastereoisomers of the S-methyl-O-methyl phosphorothioate triesters can be independently assigned to their appropriate ^{31}P n.m.r. resonances, as shown on the spectrum. These assignments made by examination of $^{31}P(^{18}O)$ isotope shifts in a sample of known configuration at phosphorus are in agreement with those made indirectly using the stereoselectivity of snake venom phosphodiesterase.

Hence the analysis method is validated, and becomes applicable to the determination of configuration of samples of unknown configuration at phosphorus.

4. SUMMARY

For the stereochemical analysis of ^{18}O-labelled AMPS and TMPS either diazomethane or dimethyl sulphate may be successfully employed to generate the corresponding S-methyl-O-methyl triesters cleanly, and in high yield, (80-100%). The advantages of dimethyl sulphate over diazomethane are that it is much easier to handle in that it can be used straight from the bottle, whilst diazomethane must be prepared prior to the methylation, and cannot be stored; reaction times are much shorter with dimethyl

sulphate, of the order of 2-3h compared with 24-48h; and yields are quantitative. On the other hand dimethyl sulphate cannot easily be removed from the triester sample should further analysis be required, (e.g. by h.p.l.c. or F.A.B. mass spectrometry), whilst diazomethane is a very 'clean' reagent and any excess can quickly and easily be removed by evaporation. Methylation with diazomethane has been successfully applied to the configurational analysis of a sample of [^{16}O,^{18}O]TMPS of unknown configuration at phosphorus isolated from the hydrolysis of d[Ap(S)T] by mung bean nuclease in ^{18}O-labelled water, (Chapter Three). Methylation with dimethyl sulphate has been successfully applied to the configurational analysis of a sample of [^{16}O,^{18}O]AMPS of unknown configuration at phosphorus, isolated from the hydrolysis of AMPS-OMe by bovine intestinal mucosa 5'-nucleotide phosphodiesterase in ^{18}O-labelled water, (Chapter Two).

Diazomethane is the favoured reagent for the stereochemical analysis of [^{16}O,^{18}O]dAMPS, the S-methyl-O-methyl triesters being obtained cleanly in high yields, (ca. 80%), and has been used in the validation of the analysis method to methylate sample of [^{16}O,^{18}O]dAMPS of known configuration at phosphorus, (see below). Dimethyl sulphate may also be used as a quick and easy alternative, but it is recommended that a large excess be used and that the isotope shifts are examined on depurinated material; although this means a loss of signal intensity due to the splitting of the signal, the resulting spectrum is likely to give clearer results, the resonances due to the depurinated material being characteristically sharp.

For the stereochemical analysis of dCMPS both reagents may be used, but dimethyl sulphate has been found to give a clearer

triester signal in higher yield more rapidly, (ca.3h cf. ca. 100h), and hence is strongly recommended where a simple n.m.r. analysis is all that is required of the sample.

For the configurational analysis of dGMPS dimethyl sulphate is the only reagent that can be used, diazomethane resulting in multiple methylations which complicate the ^{31}P n.m.r spectrum to the extent that ^{18}O-isotope shifted resonances would be unidentifiable. Again it is recommended that depurination be allowed to go to completion before examination of the ^{18}O-isotope shifts, unless a relatively large sample is available for which a reasonable spectrum can be obtained without a long period of accumulation.

CHAPTER TWO

STEREOCHEMICAL ANALYSIS OF THE HYDROLYSIS REACTION CATALYSED BY BOVINE INTESTINAL MUCOSA 5′-NUCLEOTIDE PHOSPHODIESTERASE

Bovine Intestinal Mucosa 5'-Nucleotide Phosphodiesterase

Mammalian small intestine[129] is a good source of 5'-nucleotide phosphodiesterase, (EC 3.1.4.1.), and preparations of bovine intestinal mucosa 5'-nucleotide phosphodiesterase are available commercially. The enzyme was first isolated from bovine intestine, and from commercial preparations of alkaline phosphatase, with which it shares several properties, by Butler *et. al.* in 1975,[130] and has been shown to catalyse the hydrolysis of a range of phosphodiesters and phosphonate monoesters in addition to the naturally occurring nucleotide substrates.[130] The enzyme demonstrates exonuclease activity, catalysing the hydrolysis of single-stranded nucleic acids to 5'-nucleotides by degradation from the 3'- end, and the hydrolysis of NAD, ADP, ATP, and cyclic 3',5'-AMP to 5'-AMP.[130] Phosphate diesters, such as bis(4-nitrophenyl)phosphate, are hydrolysed to phosphate monoesters, and phosphonate monoesters, such as 4-nitrophenyl phenyl-phosphonate,[129,130] are hydrolysed to the corresponding acids. Also substrates are phosphorothioate and phosphonoamidate compounds, these classes of substrate being hydrolysed at significantly lower rates than comparable oxycompounds.[131]

The enzyme has a molecular weight of 108,000 daltons as determined by sedimentation equilibrium,[130] (107,000 by gel filtration[132]), contains 21% carbohydrate, and consists of identical dimer subunits.[130]

Kinetic studies of the bovine intestinal mucosa 5'-nucleotide phosphodiesterase using pre-steady state kinetics and by determining the steady state kinetic constants of systematically varied substrates indicate the probable involvement of a nucleotidyl-enzyme intermediate in the reaction pathway.[133] Thus

when bis(4-nitrophenyl)phosphate was used as a substrate a transient pre-steady state "burst" of 4-nitrophenol was observed on initial mixing, using a stopped flow spectrometer. This is characteristic of mechanisms in which the rate-limiting step occurs after release of the first product, in this case 4-nitrophenol.[133]

When a group of 4-nitrophenyl and 2-naphthyl esters of the same phosphonic acid were investigated as substrates using steady state kinetics Vmax was found to be independent of the nature of the ester group, indicating that the rate-limiting step occurs after cleavage of the ester linkage. Vmax was found to be strongly dependent on the nature of the phosphonic acid, consistent with the occurrence of a covalent intermediate containing the phosphoryl group whose hydrolysis is rate-limiting.[133]

Stoichiometric amounts of the enzyme were incubated with cyclic 3',5'-[^{3}H]AMP, then quenched with liquified phenol to extract the protein into the phenol phase. After multiple aqueous washes to remove unbound substrate and product 8% of the enzyme was recovered as a nucleotidyl enzyme, its radioactivity migrating with the protein on gel filtration under denaturing conditions.[134] The amount of radioactivity incorporated was found to be proportional to the specific activity of the enzyme, and was increased when the reaction was inhibited by lowering pH and on inclusion of an uncompetitive inhibitor, imidazole, in the reaction mixture. Increasing the incubation period diminished the extent of labelling, as did inclusion of competitive inhibitiors when complete hydrolysis of the substrate was permitted labelling was abolished.[134]

Similarily, studies using a more recently developed and sensitive assay method showed that when [^{3}H]dTTP was used as a

substrate, and the reaction was quenched with acid, more than 50% of the protein was isolated as a thymidylyl-enzyme,[135] demonstrating a stable covalent linkage between denatured enzyme and a phosphoryl group of the substrate. Again incorporation of radioactivity from substrate into the enzyme was found to be reduced by competitive inhibitors, and enhanced by the uncompetitive inhibitor, imidazole.[135]

Evidence therefore suggests that the observed enhancement of nucleotidyl-enzyme formation on lowering of pH and inclusion of imidazole is a result of inhibition of the hydrolysis of the nucleotidyl-enzyme intermediate, i.e. the transfer of the covalent nucleotidyl group to water with the formation of a non-covalent complex between the product nucleoside 5'-monophosphate, and the enzyme. Yields of the thymidylyl-enzyme intermediate were optimal at pH 4, whereas optimum catalytic activity was observed[135] at pH > 9. Investigation of the radioisotope-labelled enzyme-bound intermediate showed it to be stable in mild acid, and at neutral pH, but labile in alkali. Since phosphoramidates, (e.g. phosphohistidine), and acyl phosphates are unstable in acid, whilst O-alkyl phosphoesters, (e.g. phosphoserine), are unstable at basic pH, the pH stability of the intermediate suggests a phosphodiester linkage. When active enzyme was added to the denatured labelled enzyme the label was lost, indicating that the covalent linkage between enzyme and nucleotidyl group was hydrolysed by the phosphodiesterase.

These properties suggest that the intermediate is a hydroxylic amino acid, such as serine or threonine, in a phosphodiester linkage with the substituted phosphoryl group of the substrate.[132] The recent isolation of a phosphoryated

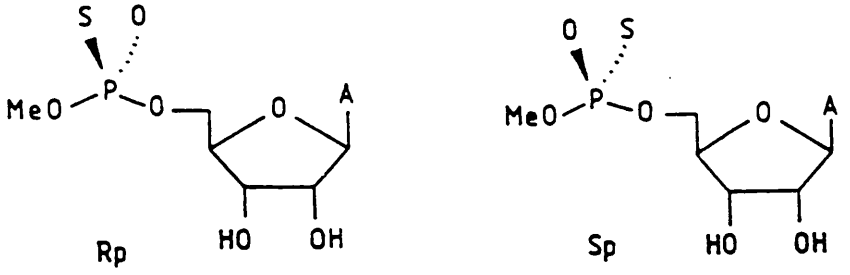
active-site peptide by cyanogen bromide cleavage has suggested that a THREONINE residue becomes attached to the nucleotide during the catalytic cycle.[136] Thus, the active site of the bovine intestinal mucosa 5'-nucleotide phosphodiesterase was labelled using thymidine 5'-α[^{32}P] triphosphate as a substrate and the radioactive active site peptide was isolated and sequenced using automated Edman degredation. Phosphoaminoacid analysis indicated that the labelled residue was threonine.
To provide further, more definitive, evidence as to the involvement or not of a nucleotidyl-enzyme intermediate on the reaction pathway a stereochemical study of the cleavage reaction catalysed by the 5'-nucleotide phosphodiesterase from bovine intestinal mucosa was undertaken using a phosphorothioate substrate analogue, adenosine 5'-O-methyl phosphorothioate and the analysis method described in Chapter One.[137,138]

Adenosine 5'-O-methyl Phosphorothioate, AMPS-OMe, as a Substrate.

The diastereoisomers of AMPS-OMe have been synthesised, and distinguished by high-field ^{31}P n.m.r. spectroscopy, and reverse-phase h.p.l.c., as described in Chapter One. The absolute configurations have been assigned in both cases by examination of mixtures of diastereoisomers after partial selective hydrolysis of the Sp diastereoisomer by snake venom phosphodiesterase, (Chapter One), and it has been shown that the Sp diastereoisomer resonates to higher field than the Rp in the ^{31}P n.m.r. spectrum, and that it has a faster retention time on reverse-phase h.p.l.c., under the solvent conditions used.

Figure 2.1

Diastereoisomers of Adenosine 5′-O-methyl Phosphorothioate

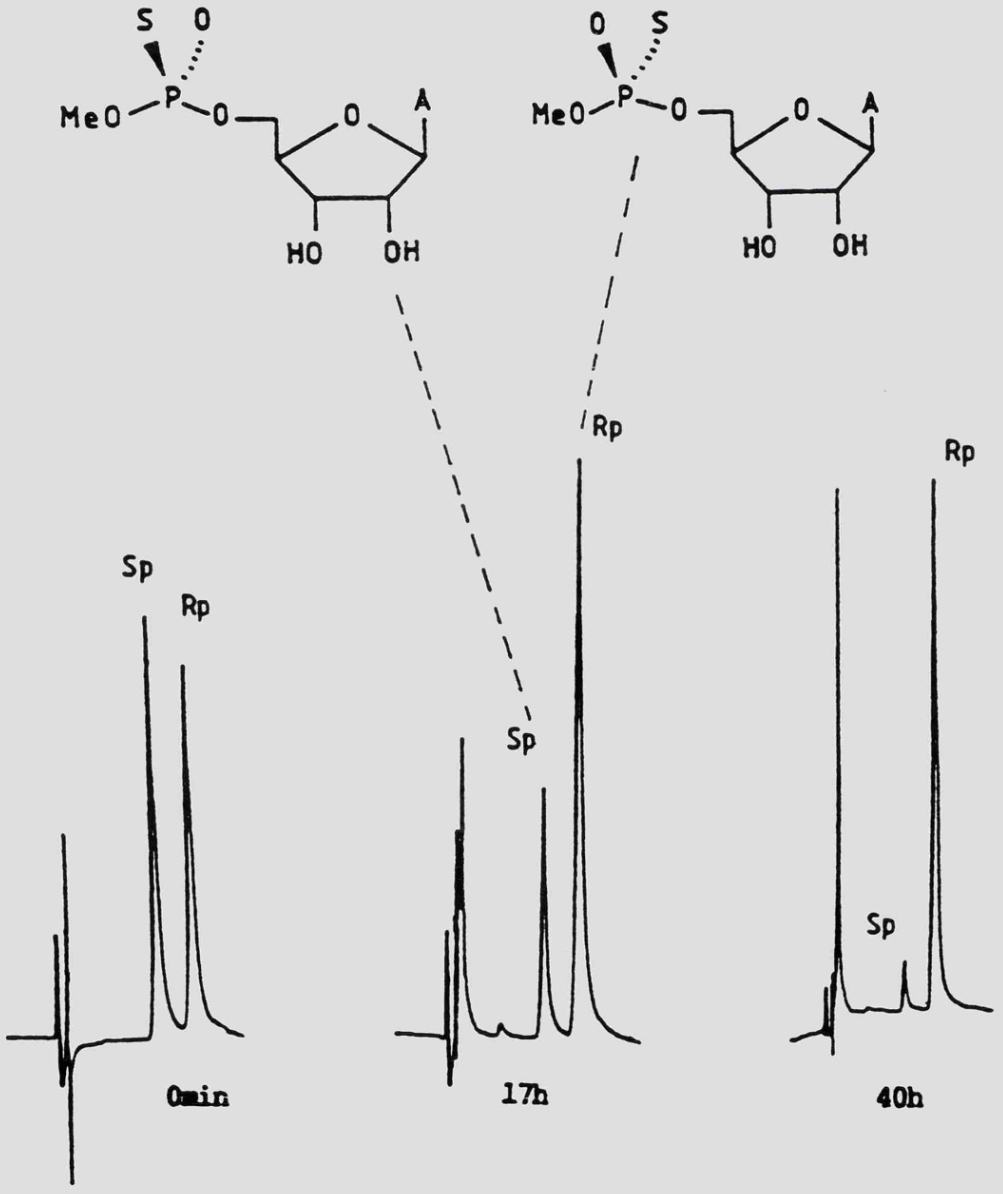


When the diastereoisomers of AMPS-OMe were incubated with bovine intestinal mucosa phosphodiesterase it was found, on monitoring by h.p.l.c., that the Sp diastereoisomer was slowly cleaved by the enzyme. On prolonged incubation the Sp diastereoisomer was completely hydrolysed, leaving the Rp diastereoisomer intact, (Figure 2.2). The initial product of hydrolysis was identified as AMPS by coinjection with a standard sample, but as incubation proceeded desulphurisation was observed to occur with the formation of AMP, which was subsequently slowly dephosphorylated, presumably due to phosphatase activity in the enzyme preparation, with release of free adenosine. Such behaviour has been frequently observed during studies of the interactions of phosphorothioates with snake venom phosphodiesterase.[6,7,139] By varying incubation conditions with respect to temperature, ionic strength of the reaction medium, and concentration of substrate and enzyme, conditions were established for which an adequate yield of AMPS for stereochemical analysis was obtainable. It was found that the rate of desulphurisation was reduced relative to the rate of digestion at lower temperatures, in the presence of large amounts of enzyme.

Bovine intestinal phosphodiesterase has already been shown to

Figure 2.2

H.p.l.c. Analysis of the Hydrolysis of Adenosine 5'-O-methyl Phosphorothioate Catalysed by Bovine Intestinal Mucosa Phosphodiesterase



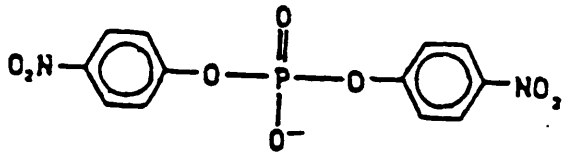
Isocratic elution with 6% MeCN in 50mM TEAB pH 6.8 using an APEX 5μ ODS reverse phase column, UV detector at λ=259nm, temp=20°C, flow=1.5ml min^{-1}

accept phosphorothioates as substrates, and cleaves bis(4-nitrophenyl)phosphorothioate, although at a slower rate than the corresponding phosphate diester.[131] It can also discriminate between the enantiomers of 4-nitrophenyl phenyl-phosphonothioate, only one enantiomer being cleaved, although the specificity has yet to be established.[131] Snake venom phosphodiesterase has also been shown to cleave selectively one enantiomer of racemic 4-nitrophenyl phenyl-phosphonothioate, and this enantiomer has been shown to have the Sp configuration.[61] In view of the observation that snake venom phosphodiesterase and bovine intestinal phosphodiesterase show the same specificity for the Sp diastereoisomer of AMPS-OMe, it seems likely that the enantiomer of 4-nitrophenyl phenyl-phosphonothioate which is cleaved by bovine intestinal phosphodiesterase has the Sp configuration,[131] as has been shown for the snake venom enzyme,[61] since both substrates are expected to be bound similarily by the same enzyme.

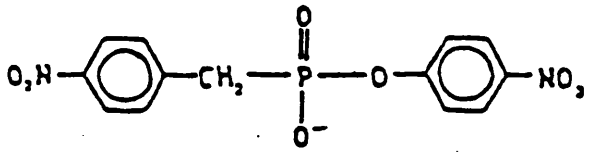
Discrimination between the diastereoisomers of AMPS-OMe, and between the enantiomers of 4-nitrophenyl phenyl phosphorothioate, with preferential cleavage of the Sp isomer, may be due to steric effects arising from the larger size of the sulphur atom than oxygen; unfavourable interactions of active site groups with one isomer may occur as a result. Alternatively, electronic factors may be important; in phosphorothioate diesters charge is thought to be localised mainly upon the sulphur atom,[11a] whilst in phosphate diesters it is equally shared between the two oxygen atoms. This localisation of charge on sulphur might result in unfavourable electronic interactions of the phosphorothioate group of one of the diastereoisomers with the active-site groups that normally stablize the negative charge equally shared between

Figure 2.3

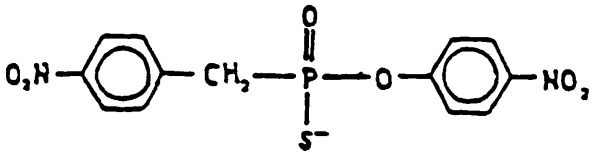
Kinetic Constants For Several Phosphonate Monoesters and Phosphate Diesters

	Km/mM	Vmax/μmol $min^{-1}mg^{-1}$
	1.0	11

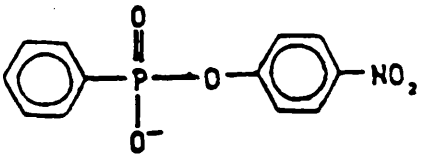
Bis(4-nitrophenyl) phosphate

	6.7	3.4

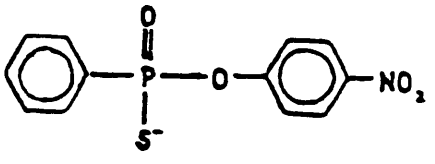
4-nitrophenyl 4-nitrobenzyl-phosphonate

	22	0.08

4-nitrophenyl 4-nitrobenzyl-phosphonothioate

	11	418

4-nitrophenyl phenyl-phosphonate

	21	180

4-nitrophenyl phenyl-phosphonothioate

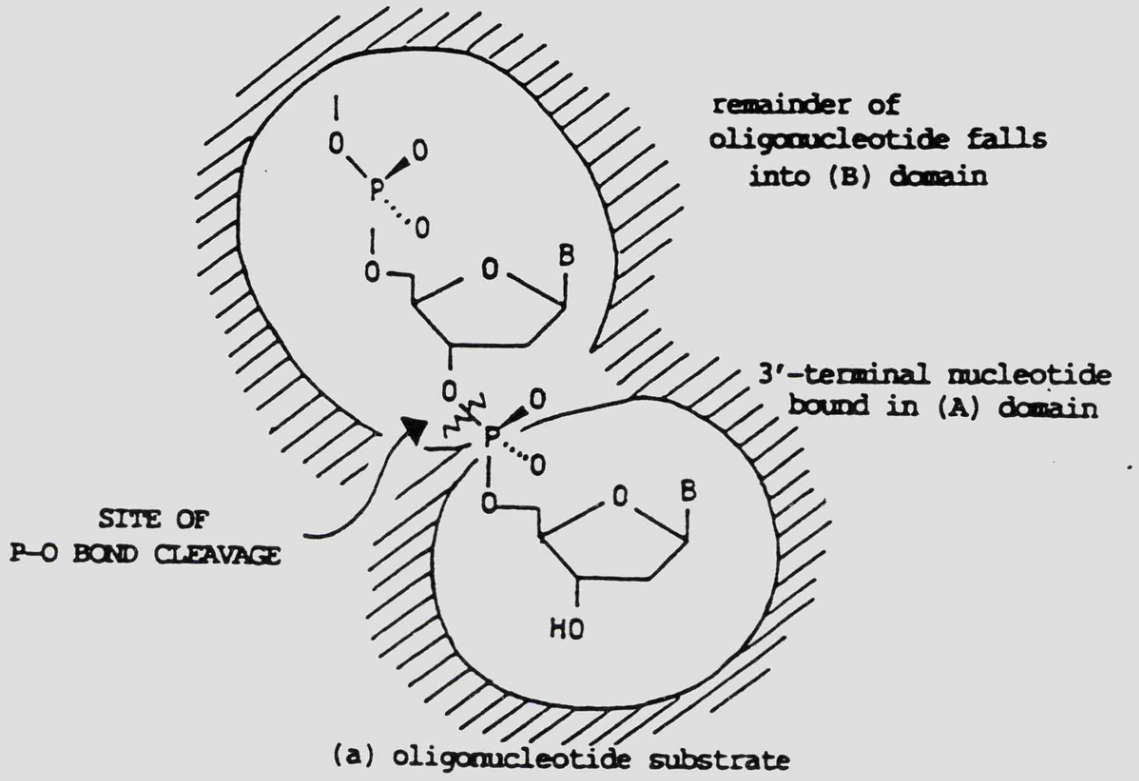
two phosphate oxygens, so that an enzyme-catalysed protonation of a phosphate oxygen necessary for catalysis cannot occur for one diastereoisomer. Alternatively steric and electrostatic effects may prevent the isomer that is not a substrate from being bound at all to the active site. If steric or electronic factors in the active site are responsible for the selection of a specific phosphorothioate or phosphonothioate, then the similar stereochemical specificity of the snake venom enzyme and intestinal enzyme may reflect certain active site analogies.

Studies with non-nucleotidic phosphonate substrates show a consistent decrease in Vmax as the internal molecular symmetry increases.[131] It is expected that these compounds can bind in two different modes, only one of which is productive; incidence of non-productive binding is increased by increasing the molecular symmetry of the substrates. For example, a comparison of the symmetrical bis(4-nitrophenyl) phosphate with the unsymmetrical but isosteric 4-nitrophenyl 4-nitrobenzyl-phosphonate, Figure 2.3; for the phosphonate one mode of binding places the $PhCH_2$-P bond in the active site whilst the other places the PhO-P bond in the active site. Only the latter mode is productive, so that the other must be inhibitory. We see a 3-fold slower rate and a 6.7 fold greater Km for the phosphonate than for the symmetrical phosphate, which can be hydrolysed equally well regardless of binding orientation, and these rate and affinity decreases are attributable to non-productive wrong-way binding.[131]

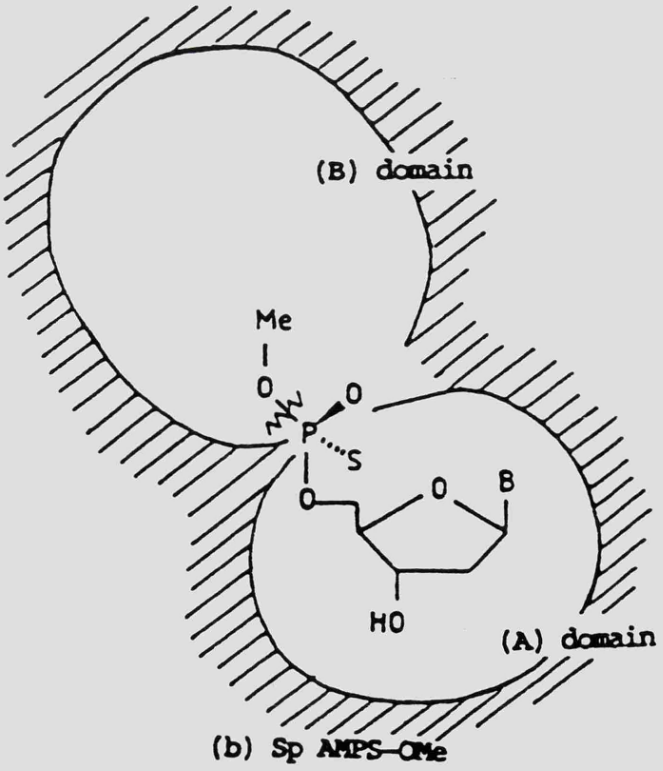
The active site of bovine intestinal mucosa phosphodiesterase has been postulated to include two separate binding domains, one of which contains a binding site (A) for the leaving nucleotide (that at the 3'- terminus of an oligonucleotide substrate), the

Figure 2.4

Representation of the Role of Binding Domains at the Active Site of Bovine Intestinal Mucosa 5'-nucleotide Phosphodiesterase



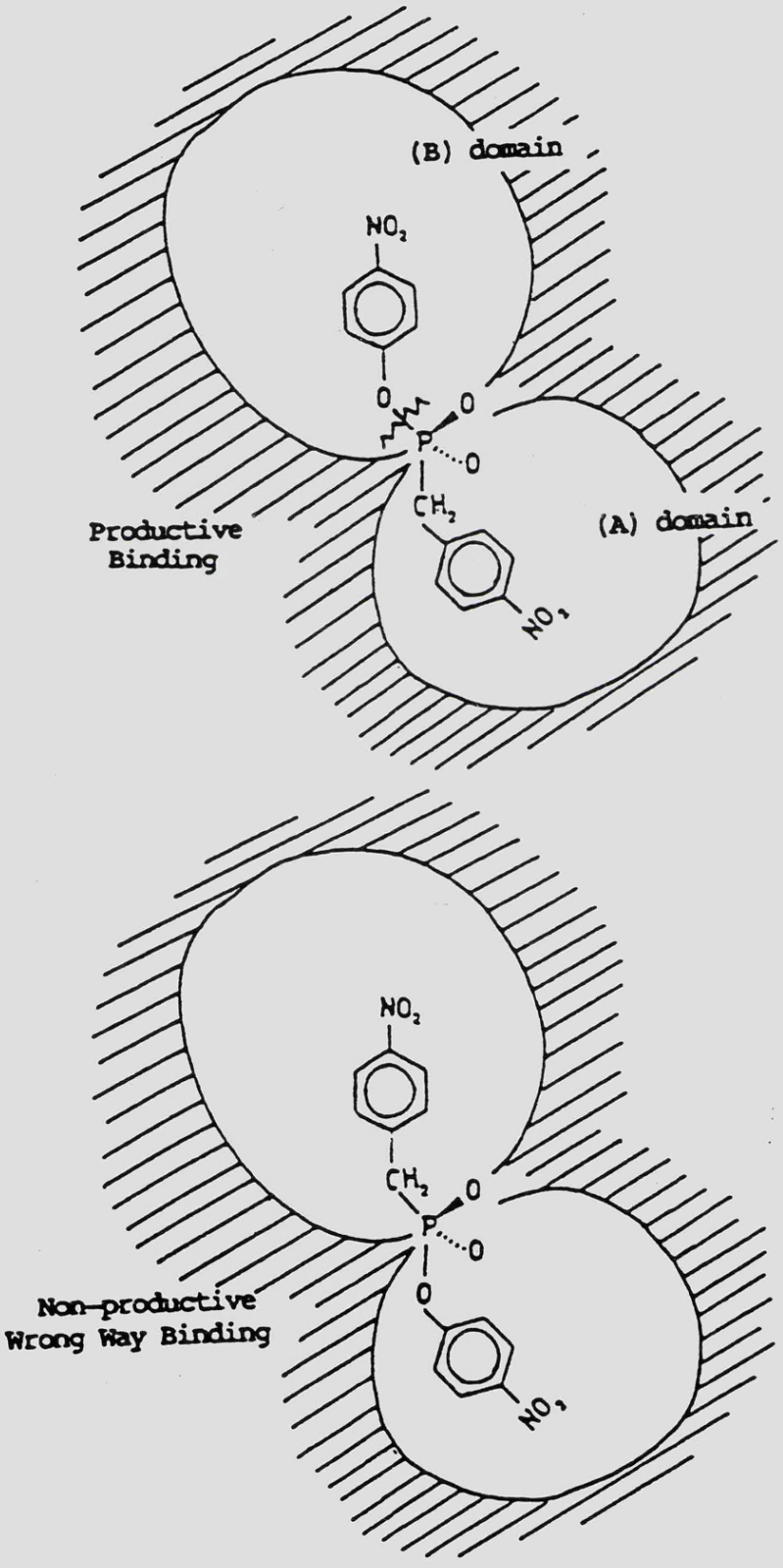
(a) oligonucleotide substrate



(b) Sp AMPS–OMe

Figure 2.5

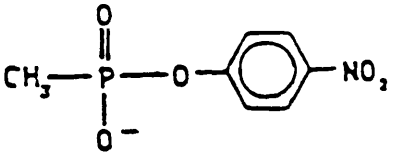
Wrong Way Binding of Symmetrical Phosphonates



other containing a binding site (B) for the penultimate nucleotide, and possibly others along the oligomer.[131] The amino acid residue to which the terminal nucleotide becomes attached in the course of the hydrolysis reaction is interposed between these two domains, with P-O bond cleavage occuring on the (B) side. Figure 2.4 shows a representation of the positioning of a substrate oligonucleotide with respect to the active site of the bovine intestinal mucosa 5'-nucleotide phosphodiesterase. In order to explain the observed symmetry effects for phosphonate substrates it has been postulated that the (B) site has a much greater affinity for aromatic groups than does the (A) site.[131] Wrong way binding which puts the phosphonate moiety into the (B) site, Figure 2.5, would be expected to be minimal for phosphonate esters when the phosphonate substituent, which falls into the A site in productive binding mode, is least aromatic, and the ester group most aromatic, as, for example, in nitrophenyl methyl-phosphonate. This explains why aliphatic esters of phosphonates are not hydrolysed[131], as the aliphatic ester's substituent would have little affinity for the (B) domain of the active site.

Figure 2.6

Nitrophenyl Methyl-phosphonate



Further evidence for the existence of the hydrophobic binding domain A is provided by the observation of competitive inhibition by product analogues. Thus Ki values for 5'-AMP and 5'-CMP (8.0μM and 13.3μM respectively[143]), are ca. 4,000-fold lower than that for ribose 5'-phosphate, (32,000μM), and ca. 100-fold lower

than the values obtained for the non-natural product analogues, such as 4-nitrophenyl phosphate and 4-nitrobenzyl-phosphonate, (0.69mM and 0.68mM respectively[143]). Also significant is the observation of a Ki for 3'-AMP (736μM), which is ca. 100-fold higher than that of 5'-AMP[143], indicating the existence of spatial requirements between the phosphate and the hydrophobic domain. This suggests that wrong-way binding, if it occurs at all for nucleotides, is much less significant than it is for the more symmetrical and poorer fitting phosphonate monoesters. Thus, wrong way non-productive binding of AMPS-OMe is considered to be unimportant in view of the affinity of the nucleoside portion of the molecule for site (A), even though the methoxy-substitutent is likely to have little affinity for the (B) site into which it should fall for productive binding.

The strong binding of the products of hydrolysis of nucleotides by the enzyme[143] explains the observation of higher values of Vmax for hydrolysis of non-nucleotide phosphonate esters and phosphate diesters than for hydrolysis of nucleotides. In the hydrolysis of phosphonate esters the rate-limiting step appears to be the breakdown of the covalent enzyme intermediate, whilst for nucleotide hydrolysis it is the dissociation of the nucleotide product from the enzyme that is rate-limiting[131].

Comparison of the kinetic parameters for the hydrolysis of bis-(4-nitrophenyl)phosphate, (Km 1.0mM, V_{max}=11μmol min^{-1}(mg protein)$^{-1}$)[131], with those for the corresponding phosphorothioate, Figure 2.3, shows an increase in Km of only a factor of about 2, (2.3mM), but a decrease in Vmax of more than 100-fold, (0.08μmol min^{-1} (mg protein)$^{-1}$[131]. This is consistent with the frequently made observation that a phosphorothioate, although bound well, is usually hydrolysed more slowly than the

corresponding phosphate[5,6,7]. A similar observation of a 2-fold increase in Km on substitution of a non-bridge oxygen for a sulphur has been made in comparing the cleavage of one enantiomer of 4-nitrophenyl phenyl-phosphonate, presumed to be the Sp enantiomer by analogy with the specificity of snake venom phosphodiesterase, (Km=11mM, Vmax=418μmol min^{-1} (mg protein)$^{-1}$ with the corresponding phosphonothioate diester, 4-nitrophenyl phenyl-phosphonothioate, (Km=21mM, Vmax=180μmol min^{-1} (mg protein)$^{-1}$[131], Figure 2.3. The relatively small increase in Km on substitution of a non-bridging oxygen for sulphur suggests that the enantiomer which is not a substrate, or only a very poor one, has little affinity for the active site, and that non-productive binding of this enantiomer has little effect on the kinetics of the reaction[131]. Hence the kinetic parameters obtained for the Sp isomer in the presence of the Rp isomer are expected to be reasonable estimates of the values which would be obtained with pure Sp isomer.

Kinetic constants for the hydrolysis of AMPS-OMe were determined using a 1:1 mixture of the diastereoisomers at 23°C, and monitoring the rate of hydrolysis of the Sp diastereoisomer relative to the Rp by h.p.l.c., using h.p.l.c. integration. The reaction rate at each substrate concentration was determined by a plot of the amount of Sp diastereoisomer hydrolysed against time for the first 0-10% of the reaction. During preliminary studies of hydrolysis rates it was found that TEAB associated with the stock solutions of substrate was inhibitory, (this may be a simple case of inhibition by high ionic strength of the reaction medium), so that it was necessary to remove any TEAB associated with substrate prior to commencing the incubation by repeated co-evaporations with methanol using a speed vac concentrator,

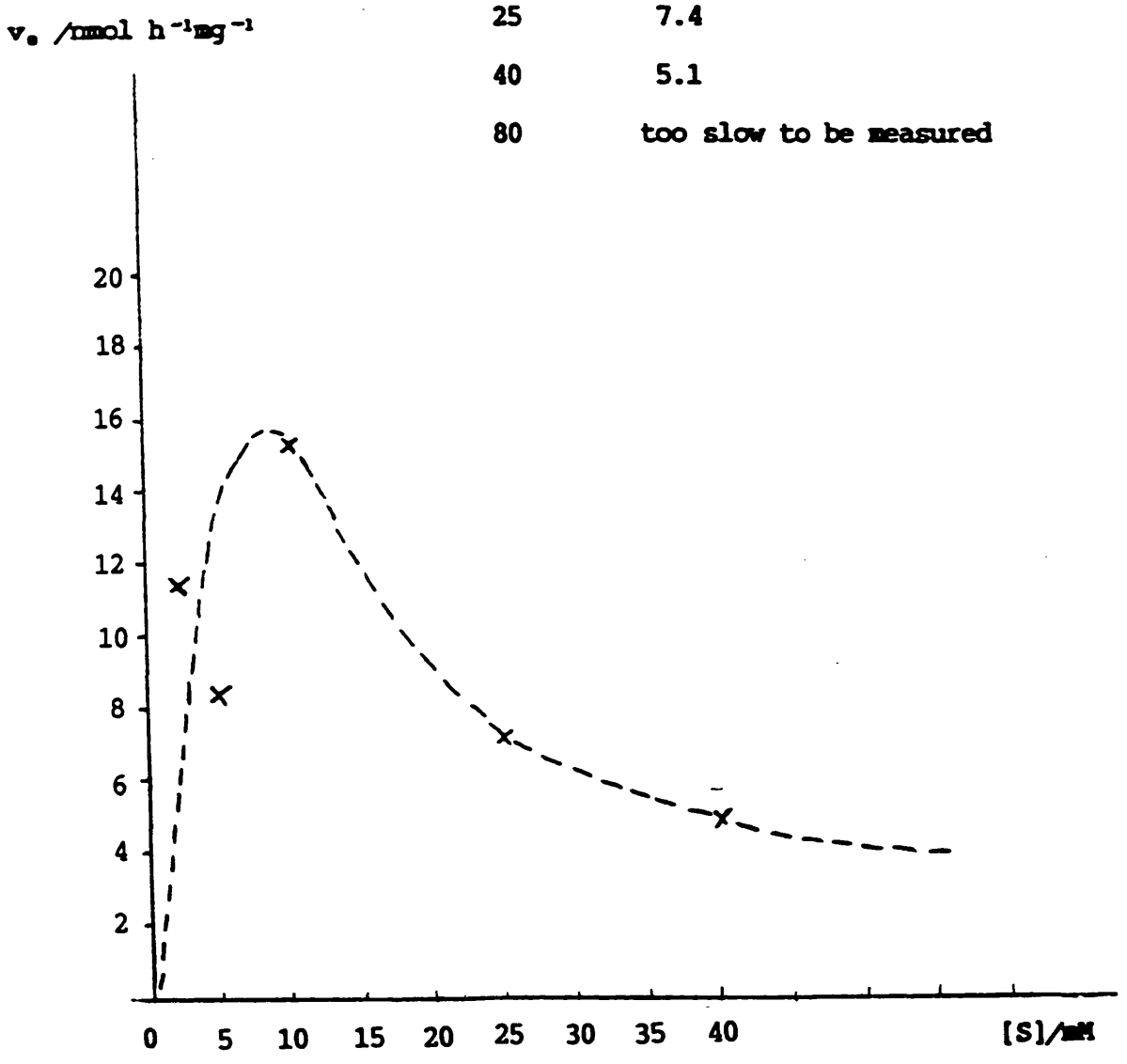
leaving the substrate as a gum in the Eppendorf tube. As a starting point hydrolysis rates were determined at substrate concentrations of 5mM and 10mM and approximate values of Km and Vmax were calculated so as to enable selection of appropriate substrate concentrations for further studies. The preliminary value of Km was determined as ca. 40mM, and the preliminary Vmax as 1.3nmol min^{-1} mg^{-1}. However further experiments conducted in the range of 2.5mM to 80mM substrate showed decreasing rates of hydrolysis with increasing substrate concentration, as illustrated in Figure 2.7. The phenomenon of decreasing reaction rates with increasing substrate concentration is characteristic of substrate inhibition[148], and is likely to have a considerable effect on the determination of Km at high substrate concentrations. Further kinetic studies were carried out using much lower substrate concentrations, in the range of 0.05mM to 0.5mM. Values obtained from a least squares analysis treatment of the data shown in Figure 2.8 were Km=67μM, and Vmax=1.3nmol min^{-1} (mg protein)$^{-1}$. The rather large scatter in the data points obtained from these kinetic studies is attributable to difficulties in measurement of the initial rates of the reaction. Apart from the fact that the hydrolysis reaction is very slow, so that denaturation of the enzyme may occur over the time scale of the reaction, the initial reaction rates were measured in terms of the reduction of the amount of Sp diastereoisomer relative to the Rp diastereoisomer, and, as a small change in a large amount, this was difficult to determine accurately. The Km value of 67μM obtained for Sp AMPS-OMe is comparable to that of thymidine 5'-*O*-(4-nitrophenyl)phosphate, (43μM)[130], and about 30-fold lower than the best simple phosphorothioate investigated to date, bis(4-nitrophenyl)phosphorothioate, reflecting the extra

Figure 2.7

Hydrolysis Rates At High Substrate Concentrations

AMPS-OMe, (a 1:1 mixture of Sp:Rp isomers), incubated with Bovine intestinal mucosa 5′-nucleotide phosphodiesterase (20mg/ml) at 23 °C in 100mM in Tris-HCl, pH 8.

[S]/mM	v_o /nmol $h^{-1}mg^{-1}$
2.5	11.6
5	8.6
10	15.5
25	7.4
40	5.1
80	too slow to be measured



binding of the nucleoside moiety in the former substrates.

A possible explanation for the observation of apparent substrate inhibition at substrate concentrations greater than 10mM is the binding of substrate into the (B) domain of the active site. This may have the effect of inhibiting the binding of substrate into the (A) domain, perhaps by inducing a conformational change in the binding domains of the active site, or by preventing correct positioning of the P-OMe moiety of a substrate molecule bound in the (A) domain, or a mixture of both effects. By preventing or inhibiting binding of substrate into the (A) domain in the correct way for P-O bond cleavage to occur, the reaction rate is reduced. Substrate inhibition has also been reported when Sp ADPαS is used as a substrate for polynucleotide phosphorylase from *Micrococcus luteus*[38]. Thus, Lineweaver-Burk plots were obtained at low (< 5mM) substrate concentrations, whilst at much higher concentrations substrate inhibition was observed.

Alternatively, in view of the fact that the nucleoside monophosphates are strong competitive inhibitors of this enzyme[143], and it seems likely that AMPS and adenosine may be similarily inhibitory, it may be that at higher substrate concentrations significantly inhibitory concentrations of AMP/AMPS accumulate in the reaction mixture more rapidly, and hence affect the initial rate measured for the reactions. This would account for the observed reduction in initial reaction rate with increasing substrate concentration. The idea of competitive inhibition by product would better explain the observed kinetic parameters obtained for the reaction, Km showing an increase of the order of 10^3 at higher substrate concentrations, whilst Vmax does not appear to affected. If the effects are due to substrate

Figure 2.8a

Kinetic Studies At Low Substrate Concentrations

S/μmol	1/S	v_o /nmol $h^{-1}mg^{-1}$	$1/V \times 10^{3}$
65	0.0154	54.3	18.42
80	0.0125	44.3	22.57
100	0.0100	58.7	17.04
125	0.0080	75.0	13.33
150	0.0066	44.3	22.58
225	0.0044	75.0	13.33
300	0.0033	85.7	11.67
400	0.0025	75.0	13.33
500	0.0020	109.3	9.15

AMPS-OMe, (1:1 mixture of Sp:Rp isomers), incubated with bovine intestinal mucosa 5'-nucleotide phosphodiesterase at 30°C in 100mM Tris-HCl, pH 8

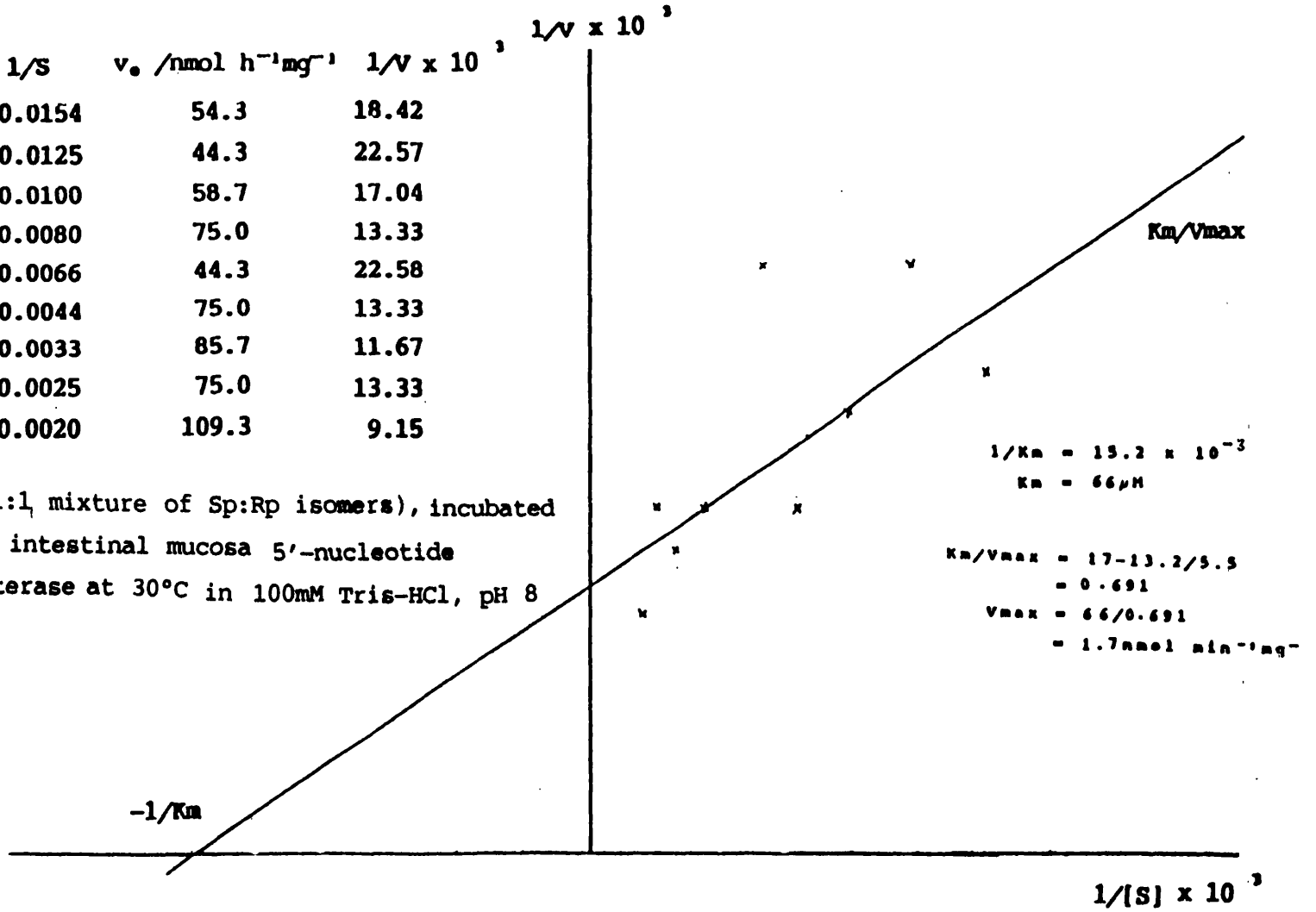


Figure 2.8b

Least Squares Analysis of Kinetic Data

```
LEAST SQUARES FITTING

%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
CALCULATED S
 12.396945075
  8240354O6244
%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
ESTIMATED VARIANCE
 1.4111225684
++++++++++++++++++++++++++++++++
Standard deviation
 1.18790680123
++++++++++++++++++++++++++++++++
VARIANCE/COVARIANCE MATRIX

STANDARD ERROR  1
 .756217644062

STANDARD ERROR  2
 9.94808553284E-2
$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$
OBS-CALC
-3.6170903312
 3.6226123469
-.7472991374
-3.429228325
 8.4020177032
-.4627008625
-1.4962619156
 1.1029664094
-3.3750158875
```

Correlation Coefficient = $\frac{1}{\text{estimated variance}}$

$= \frac{1}{1.411123}$

$= 0.708$

inhibition we would expect to see a reduction in Vmax, with Km unaffected for a non-competitive mechanism, or a change in both Km and Vmax for uncompetitive inhibition.

Comparison of the Vmax value for hydrolysis of AMPS-OMe with that for the hydrolysis of thymidine 5'-*O*-(4-nitrophenyl)phosphate, (270μmol min^{-1} (mg protein)$^{-1}$[130], shows the latter to be some 200,000-fold higher. We have already seen that decreases in Vmax of the order of up to 100-fold are to be expected on replacing non-bridging oxygen for sulphur, although for the snake venom enzyme the Vmax values for thymidine 5'-*O*-(4-nitrophenyl)phosphate and the corresponding phosphorothioate differ only by a factor[106] of 3. This extra large rate difference may be accounted for by the different leaving group properties of methanol and 4-nitrophenol, methanol being a relatively poor leaving group. Alternatively it may be that the rate of displacement of MeO^- in P-O bond cleavage is much slower than that for 4-nitrophenolate due to the absence of stabilisation of negative charge on oxygen in MeO^- relative to 4-nitrophenolate, so that the rate-limiting step in the hydrolysis of AMPS-OMe is hydrolysis of the P-O bond with formation of the covalent enzyme intermediate.

The Rp diastereoisomer of AMPS-OMe is expected to be an extremely poor substrate for bovine intestinal enzyme, in view of the observation that, with snake venom enzyme, thymidine 5'-*O*-(4-nitrophenyl)phosphorothioate shows a rate difference of 1300 between the two isomers, the selected Sp diastereoisomer showing a Vmax of 83μmol min^{-1} (mg protein)$^{-1}$[106]. Assuming a similar rate difference for the intestinal enzyme and the diastereoisomer of AMPS-OMe, and in view of the 64,000-fold lower Vmax for hydrolysis of the favoured diastereoisomer of AMPS-OMe

by the intestinal enzyme, Vmax for the hydrolysis of the Rp diastereoisomer must be exceedingly low, of the order of 10^{-2} pmol min^{-1} $(mg\ protein)^{-1}$.

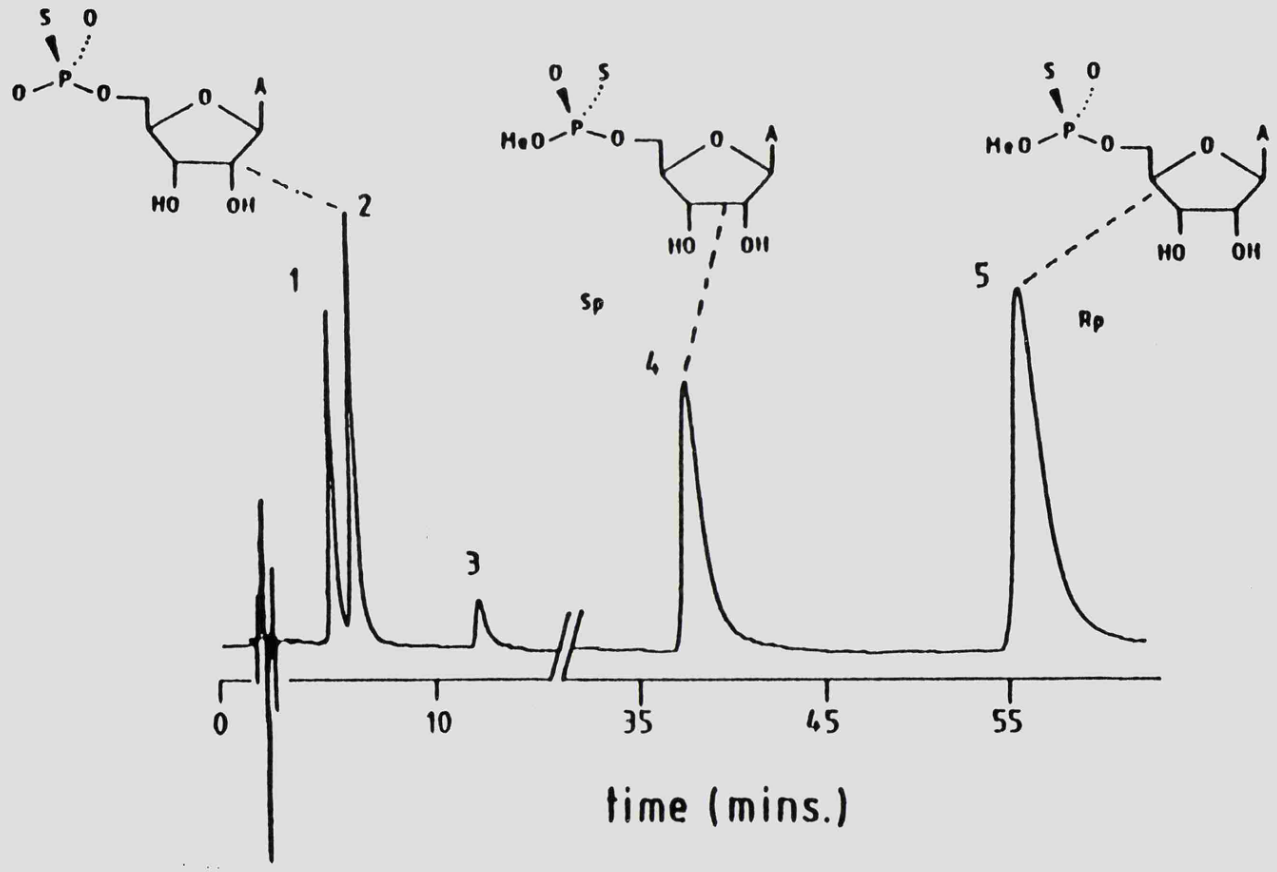
Stereochemical Evidence for a Nucleotidyl-enzyme Intermediate.

When AMPS-OMe was incubated with bovine intestinal mucosa 5'-nucleotide phosphodiesterase in ^{18}O-labelled water, [^{16}O,^{18}O]AMPS of unknown configuration at phosphorus was generated. Figure 2.9 shows an h.p.l.c. analysis of the hydrolysis reaction. This sample was combined with [$^{16}O_2$]AMPS, and the resulting [^{16}O,^{18}O]AMPS isolated from the incubation mixture by ion-exchange chromatography. The UV detector trace obtained is shown in Figure 2.10. Figure 2.11 shows the high-field ^{31}P n.m.r. spectrum of the undigested substrate isolated from the column. Methylation with dimethyl sulphate led to the diastereoisomeric $\underline{N}^{1,3}$- dimethyladenosine 5'-[^{16}O,^{18}O] $\underline{S}$-methyl-$\underline{O}$-methyl phosphorothioate triesters, scheme 2.1, the absolute configurations of which have already been assigned by ^{31}P n.m.r., (Chapter One)[137,138].

The high-field ^{31}P n.m.r. spectrum is shown in figure 2.12. It can be seen that there are two lower-intensity isotope-shifted resonances in the presence of the resonances due to the diastereoisomers of the unlabelled triester. The shift on the downfield resonance, assigned to the Sp diastereoisomer, is smaller, (2.4 Hz), than that on the upfield resonance of the Rp diastereoisomer(5.7 Hz). The relative peak heights indicate an ^{18}O-enrichment of 32%, in good agreement with expectations. Since ^{18}O-isotope shifts are larger in a doubly bonded system than in a singly bonded system[112], the ^{18}O-label is located in the P-$\underline{O}$Me

Figure 2.9

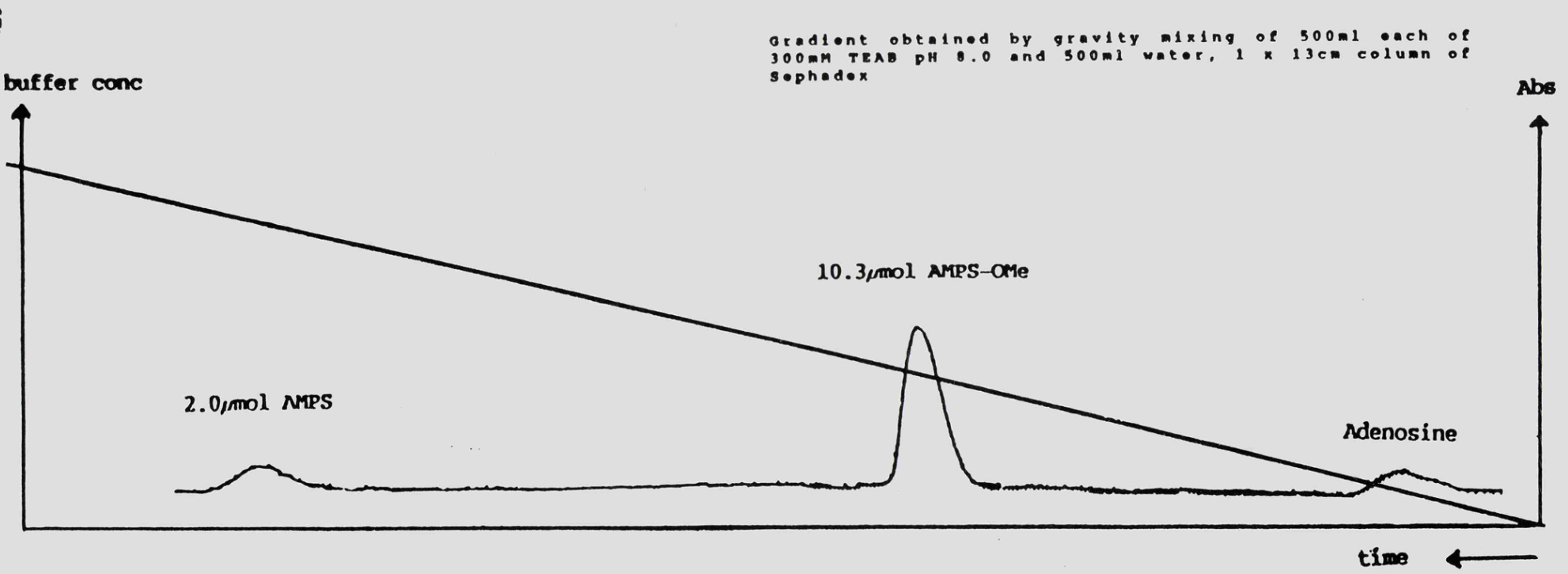
H.p.l.c Analysis of the Hydrolysis of Sp Adenosine 5'-O-Methyl Phosphorothioate in ^{18}O-labelled Water



Isocratic elution with 2.5% MeCN in 50mM TEAB pH 6.8 using an APEX 5μ ODS reverse phase column, UV detector at λ=259nm, temp=20°C, flow=1.7ml min^{-1} increased to 2.3ml min^{-1} after 10min

Figure 2.10

U.V. Detector Trace Obtained From the Ion-Exchange Chromatographic Separation of the Components Of the Hydrolysis of AMPS-OMe By Bovine Intestinal Mucosa 5'-Nucleotide Phosphodiesterase in ^{18}O-labelled Water



position in the Sp diastereoisomer, and in the P=O position in the Rp diastereoisomer. Hence the [^{16}O,^{18}O]AMPS isolated from the enzyme reaction must have had the Sp configuration, and therefore the enzyme reaction has proceeded with RETENTION of configuration at phosphorus.

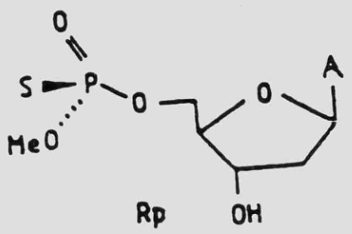
Since it is now generally accepted that single displacements at phosphorus in enzyme-catalysed reactions proceed with inversion of configuration,[5-7,16,26] the observation of overall retention of configuration is strong evidence for a double displacement pathway, involving the formation and breakdown of a covalent enzyme-bound intermediate.

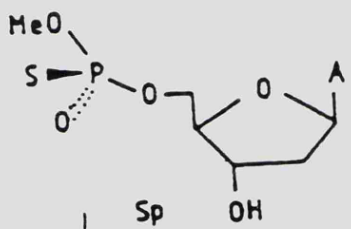
Since there is already strong evidence in favour of a nucleotidyl-enzyme intermediate in the hydrolysis reaction catalysed by this enzyme, this observation of retention of configuration for the reaction may be considered as conclusive evidence for its involvement, stereochemistry currently providing the most definitive proof of the involvment of a phosphoryl-enzyme intermediate, apart from the isolation of the intermediate.

This result can be compared with that obtained from a stereochemical study of snake venom phosphodiesterase, which was also found to catalyse hydrolysis with overall retention of configuration, implicating a covalent enzyme-bound intermediate in the reaction pathway.[61,63,64,106] Active site labelling of the 5'-nucleotide phosphodiesterase from the venom of *Crotalus durissus terrificus* and *Crotalus adamanteus* using ($\alpha^{32}P$)TTP as a substrate showed the phosphorylated active site residue to be THREONINE in both enzymes,[145] as observed for bovine intestinal mucosa 5'-nucleotide phosphodiesterase. The 5'-nucleotide phosphodiesterases represent the first group of enzymes to be

Figure 2.11

High-field ^{31}P n.m.r. Spectrum of Sp-deficient Sample of Adenosine 5'-O-methyl Phosphorothioate Isolated From the Hydrolysis by 5'-nucleotide Phosphodiesterase in ^{18}O-labelled water

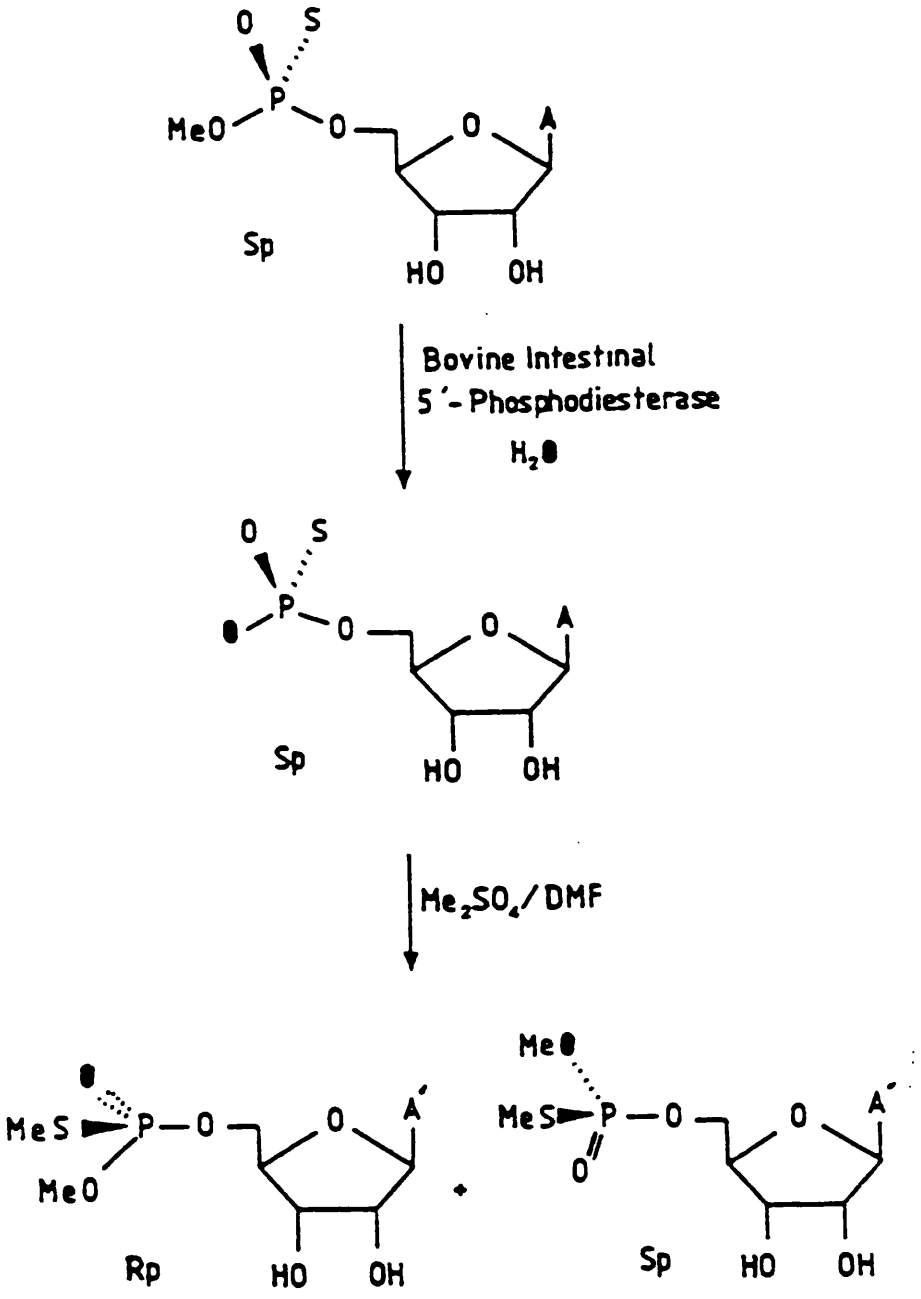




conc. ca. 20mM in 3:1 v/v DMF:d_4-MeOH ^{31}P n.m.r. parameters were:
sweep width 3650Hz, pulse width 5.0μs, aquisition time 2.2s, block size 16K, zero-filled to 32K, no. transients 796.

Scheme 2.1

Summary of the Stereochemical Analysis of the Hydrolysis of Sp Adenosine 5'-O-Methyl Phosphorothioate by Bovine Intestinal Mucosa 5'-Nucleotide Phosphodiesterase

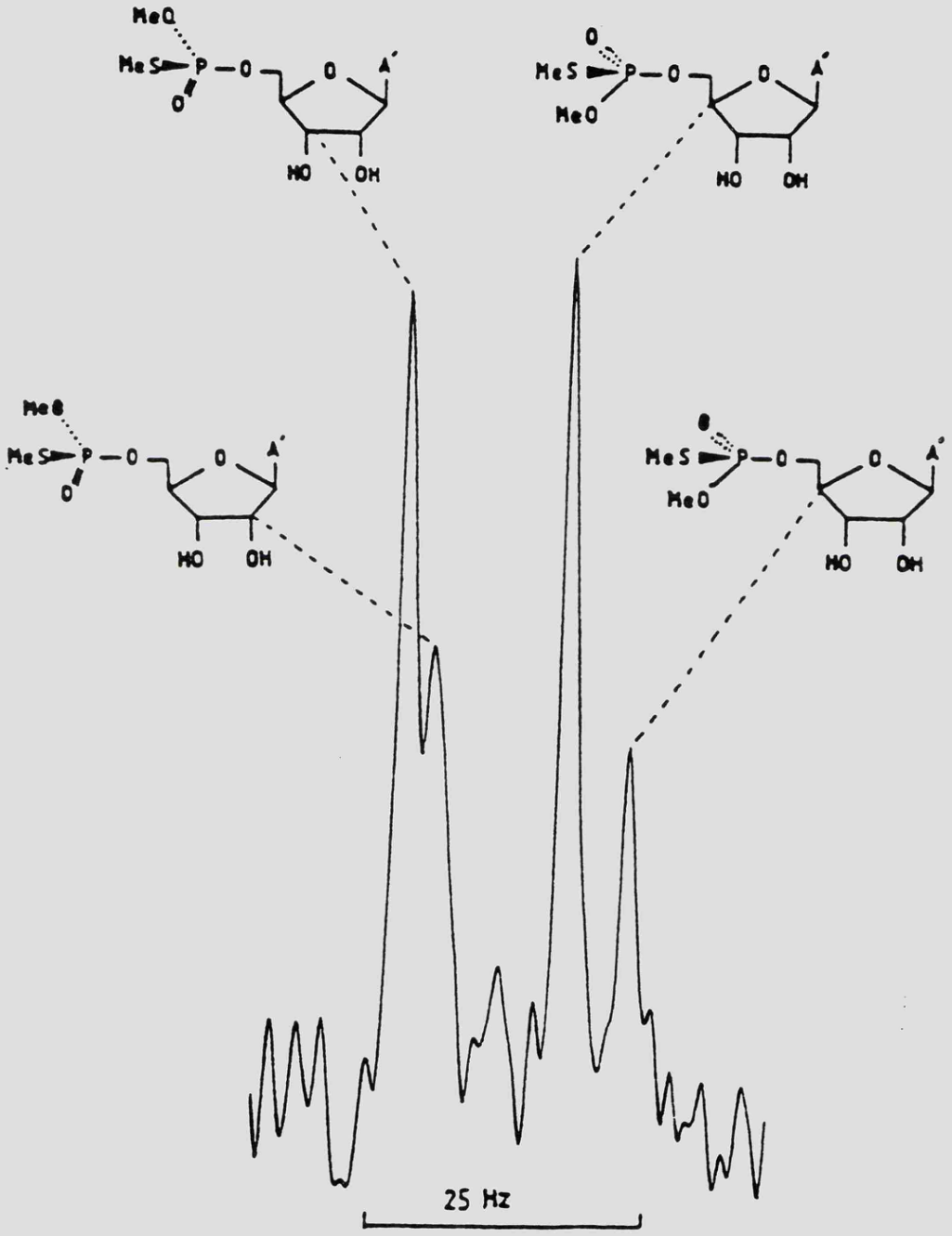


found to have threonine as the active site residue[146]. The possible significance of the occurrence of threonine as the active site residue, rather than serine, may be illustrated by comparison of the catalytic activity of alkaline phosphatase, also shown to involve the formation of a covalent enzyme intermediate, but with SERINE as the phosphorylated active site residue. The reactions catalysed by alkaline phosphatase include the transfer of the phosphoryl group to acceptors other than water, whilst for the 5'-nucleotide phosphodiesterase this has not been observed. The bulkiness of the nucleoside substrate group may have a significant effect in reducing the accessibility of the phosphorus centre in the phosphodiesterase intermediate, no such group being present in the phosphoryl-enzyme intermediate of alkaline phosphatase. This 'congestion' at the active site of the phosphodiesterase intermediate may be further enhanced by the fact that the phosphoryl group is esterified to the secondary hydroxyl of threonine. The phosphoryl group of the alkaline phosphatase intermediate is esterified to the primary hydroxyl of serine and, in that it lacks both the nucleoside substrate group and the extra methyl group of threonine, is expected to be more accessible than that of the phosphodiesterase intermediate, thus permitting access of phosphorus acceptors other than water.[145]

pH studies[143] on the hydrolysis of 4-nitrophenyl phenylphosphonate by bovine intestinal mucosa 5'-nucleotide phosphodiesterase indicated the involvement of a group with pKa = 6.85 in the rate-limiting step of the hydrolysis, the unprotonated form being ca. 14 times more active than the conjugate acid. The value of pKa = 6.85 is consistent with a histidine residue, thus indicated the involvement of the imidazole of an active-site histidine residue in the hydrolysis

Figure 2.12

High-field ^{31}P n.m.r. Spectrum of S-methyl-O-methyl triesters of ^{18}O-labelled and Unlabelled Adenosine 5'-Phosphorothioate isolated From the Hydrolysis of Sp AMPS-OMe Catalysed by 5'-Nucleotide Phosphodiesterase in ^{18}O-labelled Water

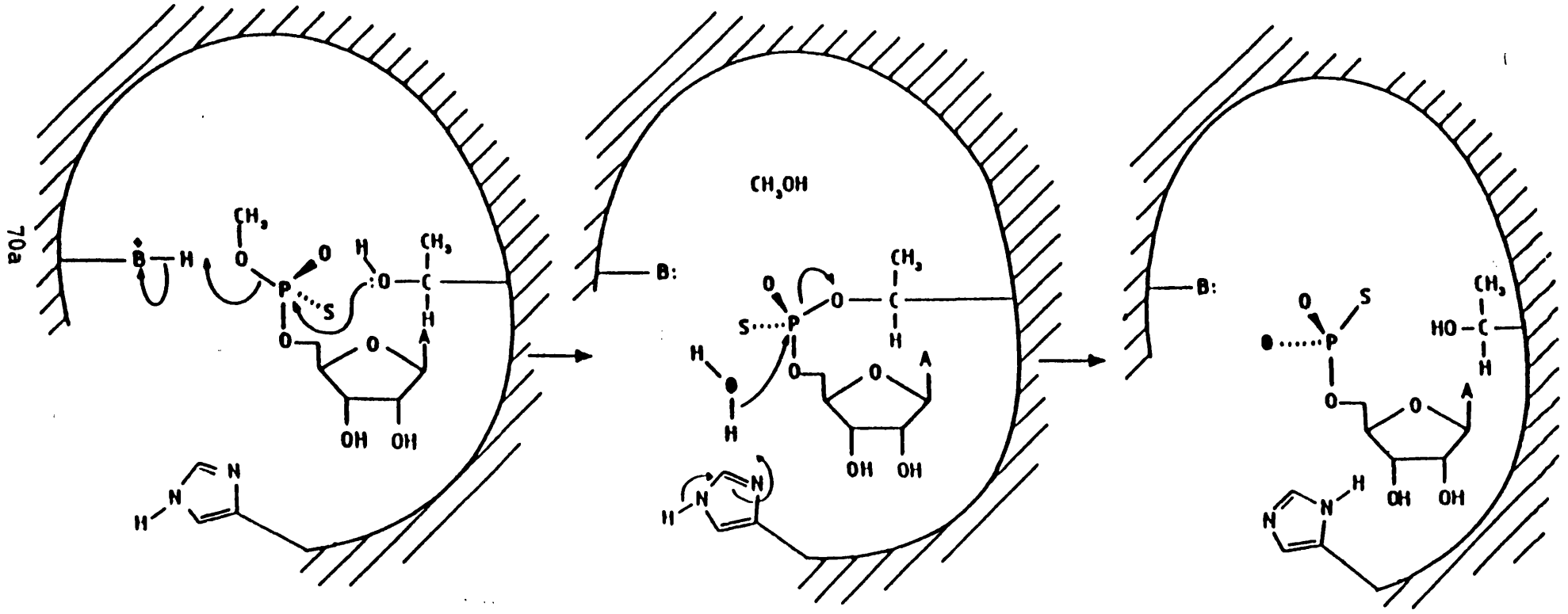


conc. ca. 4mM in 3:1 v/v DMF:D_4MeOH ^{31}P.n.m.r. parameters were:
sweep width 5618Hz, pulse width 5.0μs, aquisition time 1.5s, block size 16K, zero-filled to 32K, no. transients 40,000.

mechanism. Its role is presumed to be that of a general base, enhancing the nucleophilicity of a bound water molecule in its attack on the nucleotidyl-enzyme intermediate formed from the initial attack of the active-site threonine on the substrate in the first step.[136] Scheme 2.2 shows a representation of the proposed mechanism of cleavage at the active site of this enzyme, based on the data currently available.

Scheme 2.2

Proposed Mechanism For the Cleavage of Sp Adenosine 5'-O-Methyl Phosphorothioate in ^{18}O-labelled water by 5'-Nucleotide Phosphodiesterase



CHAPTER THREE

STEREOCHEMICAL ANALYSIS OF THE HYDROLYSIS REACTION CATALYSED BY MUNG BEAN NUCLEASE

Mung Bean Nuclease;

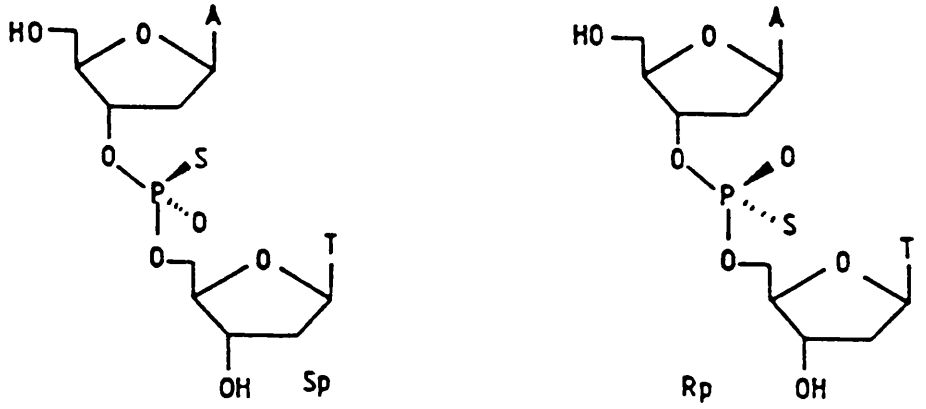
Mung bean nuclease, (EC 3.1.30.1), is the common name for the single-strand specific endonuclease activity isolated from *Phaseolus aureus*, (mung bean sprouts[150]). The enzyme is one of the group of single-strand specific nucleases widely distributed in nature, which show high selectivity for the single-stranded nucleic acids.[149] Mung bean nuclease is a glycoprotein of Mr 39,000 daltons, requiring Zn^{2+} and Mg^{2+} ions for activity, and catalyses the hydrolysis of single-stranded DNA and RNA into mono- and oligo-nucleotides, mononucleotides being the sole product when large amounts of enzyme are used.[150] Due to its specificity for single-stranded DNA, mung bean nuclease is a useful tool in the tailoring of DNA for genetic manipulation. Its properties and methods for its isolation and purification have been reviewed,[151] but little is known of the mechanism by which it interacts selectively with single-stranded nucleic acids, and catalyses the hydrolysis of the phosphodiester linkage.

A useful insight into the mechanism of enzyme action is provided by a stereochemical study of the reaction. Such an investigation allows the distinction to be made between a mechanism involving direct attack of water on the phosphodiester linkage, with the outcome of inversion of configuration at the phosphorus centre, and a mechanism involving a covalent enzyme intermediate, characterised by retention of configuration at phosphorus.[5-7,16,26] The phosphorothioate approach was applied here to the investigation of the stereochemistry of the reaction catalysed by mung bean nuclease, and the substrate chosen was a deoxy- dinucleoside phosphorothioate, 5'-O-thymidyl

3'-O-(2'-deoxyadenosyl) phosphorothioate, d[Ap(S)T]. Dinucleoside phosphorothioates have already been successfully employed in stereochemical studies of nucleases,[65,68] and mung bean nuclease is known to have a preference for AT-rich regions of nucleic acids, being reported to cleave at d[T↓pN] and d[A↓pN] sites.[152] Figure 3.1 shows the diastereoisomers of d[Ap(S)T].

Figure 3.1

Diastereoisomers of d[Ap(S)T]

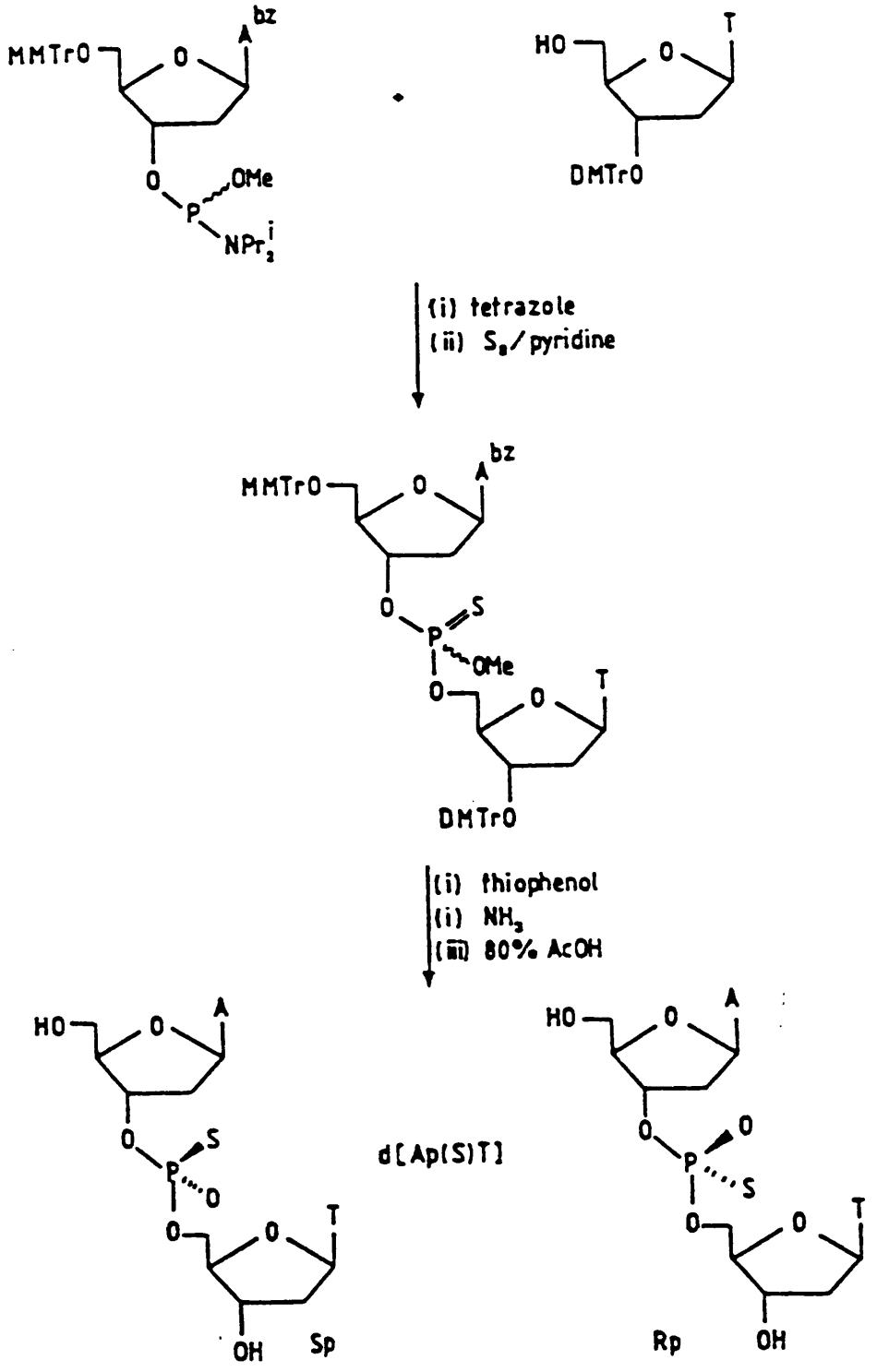


d[Ap(S)T] as Substrate;

The diastereoisomers of d[Ap(S)T] were synthesized in this laboratory by Dr. M.R. Hamblin by the coupling of N6-benzoyl 5'-O-monomethoxytrityl-2' -deoxyadenosine 3'-O-di-isopropyl-aminomethoxyl-phosphoramidite with 3'-O-dimethoxyltrityl-thymidine, and subsequent oxidation to the fully blocked dinucleoside phosphorothioate triester, Scheme 3.1.[101] The diastereoisomers could be partially separated by silica-gel flash chromatography, and subsequently deblocked. Partial selective digestion with snake venom phosphodiesterase, which digests the Rp diastereoisomer,[6,7] and nuclease P1, which selectively cleaves the Sp diastereoisomer,[6,7,68] allowed assignment of absolute configuration by examination of relative peak heights by

Scheme 3.1

Summary of the Synthesis of the Diastereoisomers of d[Ap(S)T]



^{31}P n.m.r. spectroscopy and reverse-phase h.p.l.c. As observed for other dinucleoside phosphorothioates, the Rp diastereoisomer has the 'fast' retention time on reverse-phase h.p.l.c., and resonates at low-field in the ^{31}P n.m.r. spectrum, whilst the Sp diastereoisomer is 'slow' on h.p.l.c., and resonates at high-field.[6,68,153-158]

When a mixture of the diastereoisomers of d[Ap(S)T] was incubated with mung-bean nuclease, h.p.l.c. showed that the Sp diastereoisomer was selectively hydrolysed to give TMPS and 2'-deoxyadenosine as products. Experiments using samples of pure isomers showed that the Rp diastereoisomer is not cleaved by the enzyme.

When this hydrolysis was performed in ^{18}O-labelled water [^{16}O,^{18}O]TMPS of unknown configuration at phosphorus was generated, and was isolated from the incubation mixture by ion-exchange chromatography.

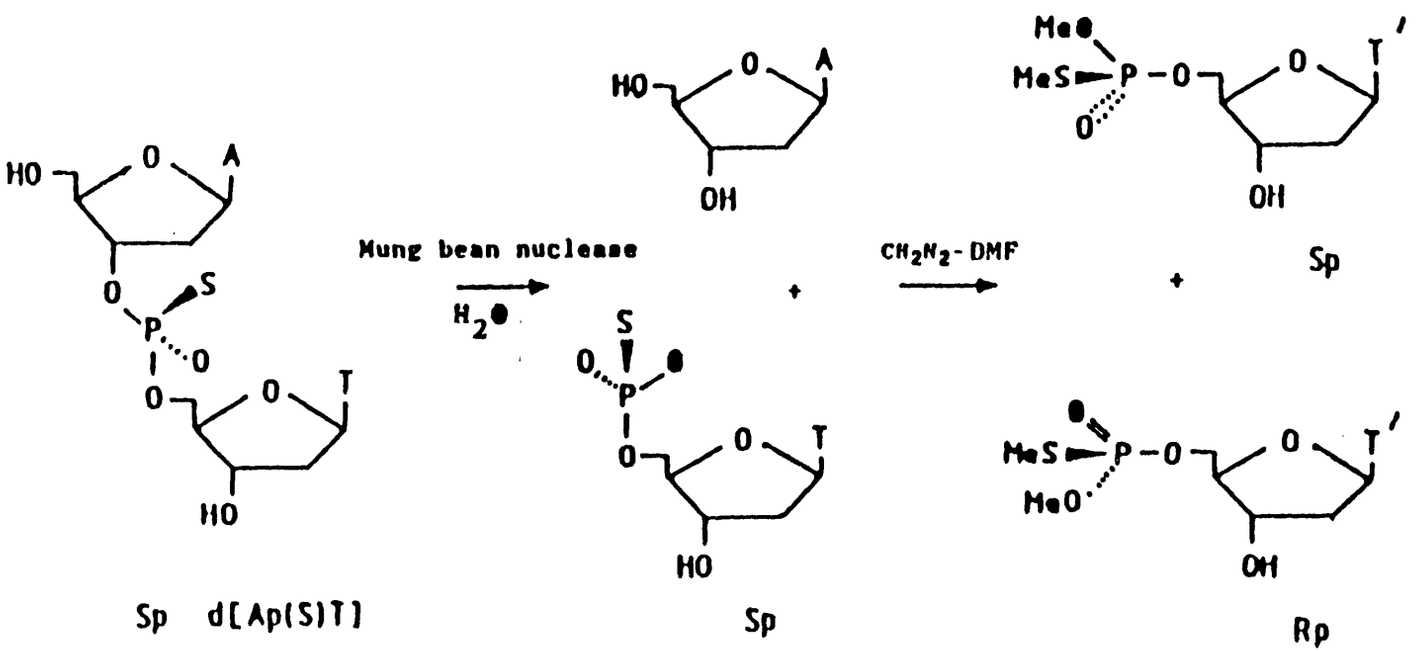
Configurational Analysis of [^{16}O,^{18}O]TMPS;

The sample of [^{16}O,^{18}O]TMPS, isolated by Dr. M.R. Hamblin from the hydrolysis of d[Ap(S)T] using mung bean nuclease in ^{18}O-labelled water, was subjected to configurational analysis using the method described in Chapter One, in order to determine its absolute configuration at phosphorus. A knowledge of the absolute configuration of the substrate d[Ap(S)T] allows the stereochemical course of the hydrolysis reaction to be deduced.

The sample of [^{16}O,^{18}O]TMPS was combined with an approximately equivalent amount of [$^{16}O_2$]TMPS in order to provide a reference for the measurement of ^{31}P(^{18}O) isotope shifts in the ^{31}P n.m.r. spectrum of the corresponding S-methyl-O-methyl triester. The combined sample was then methylated with excess ethereal

Scheme 3.2

Summary of the Configurational Analysis of the Hydrolysis Reaction Catalysed by Mung Bean Nuclease



Sp d[Ap(S)T]

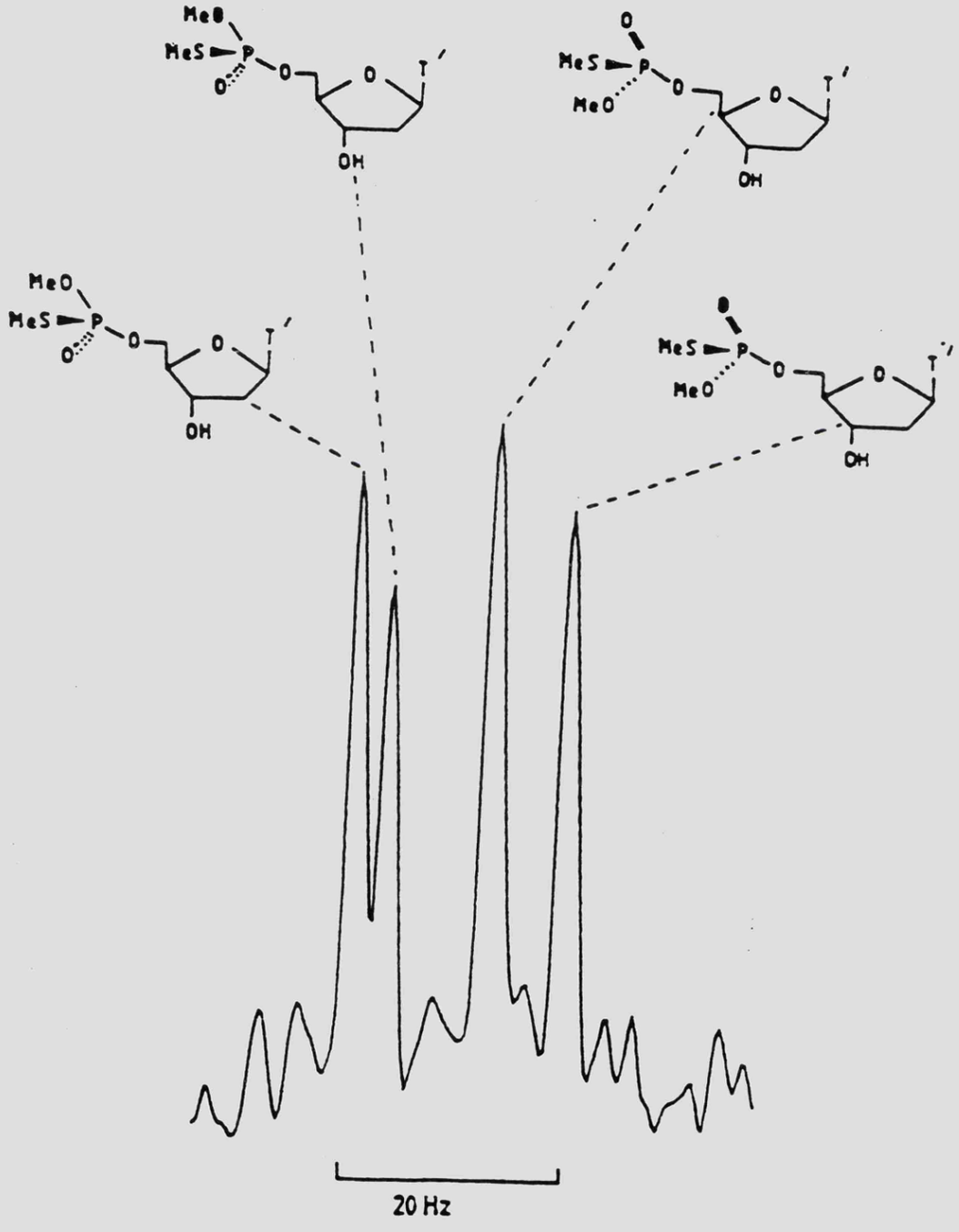
diazomethane for 36h at room temperature in DMF to generate the diastereoisomers of $\underline{N}^3$-methylthymidine 5'-[^{16}O,^{18}O] -$\underline{S}$-methyl-$\underline{O}$-methyl phosphorothioate triester in the presence of the corresponding unlabelled triester. The route to the configurational analysis is summarised in Scheme 3.2. Figure 3.2 shows the high-field ^{31}P n.m.r. spectrum of the resulting methylated material, recorded in the previously described solvent system of 3:1 v/v DMF:D_4MeOH. The higher intensity resonances are due to the diastereoisomers of the unlabelled triester, the configurations of which have already been assigned, (Chapter 2), as shown on the spectrum. Two $^{31}P(^{18}O)$ isotope shifted resonances are observable; a smaller one, (2.5Hz), on the downfield Sp diastereoisomer, and a larger one, (5.7Hz), on the Rp diastereoisomer. Since the magnitude of the $^{31}P(^{18}O)$ isotope shift is a function of bond order,[112] being larger in doubly-bonded systems than singly-bonded systems, the ^{18}O-atom can be located in the P-$\underline{O}$Me position in the Sp diastereoisomer, and in the P=$\underline{O}$ position in the Rp diastereoisomer. It can thus be seen that the [^{16}O,^{18}O]TMPS isolated from the mung bean nuclease incubation must have had the Sp configuration. Comparison of the structure of the Sp diastereoisomer of d[Tp(S)A] which was hydrolysed with that of the Sp diastereoisomer of [^{16}O,^{18}O]TMPS which is the product of hydrolysis it can be seen that the reaction has proceeded with INVERSION of configuration at the phosphorus centre.

Stereochemical Evidence for a Single Step Mechanism;

The simplest interpretation of this stereochemical result is that the hydrolysis catalysed by mung bean nuclease occurs by a direct 'in-line' attack of water opposite the

Figure 3.2

High-field ^{31}P n.m.r. Spectrum of the Diastereoisomers of $\underline{N}^3$-methyl thymidine [^{16}O, ^{18}O] -$\underline{S}$-methyl-$\underline{O}$-methyl Phosphorothioate Triester



Total conc ca. 5 mM in 3:1 v/v DMF: D_4-MeOH
^{31}P n.m.r. parameters were:
field strength 121.5MHz, sweep width 4854Hz, pulse width 5μs, aquisition time 1.7s, data collection in 32K, no. transients 12,000. Isotope shift on δp = 2.5Hz, on Rp = 5.7Hz.

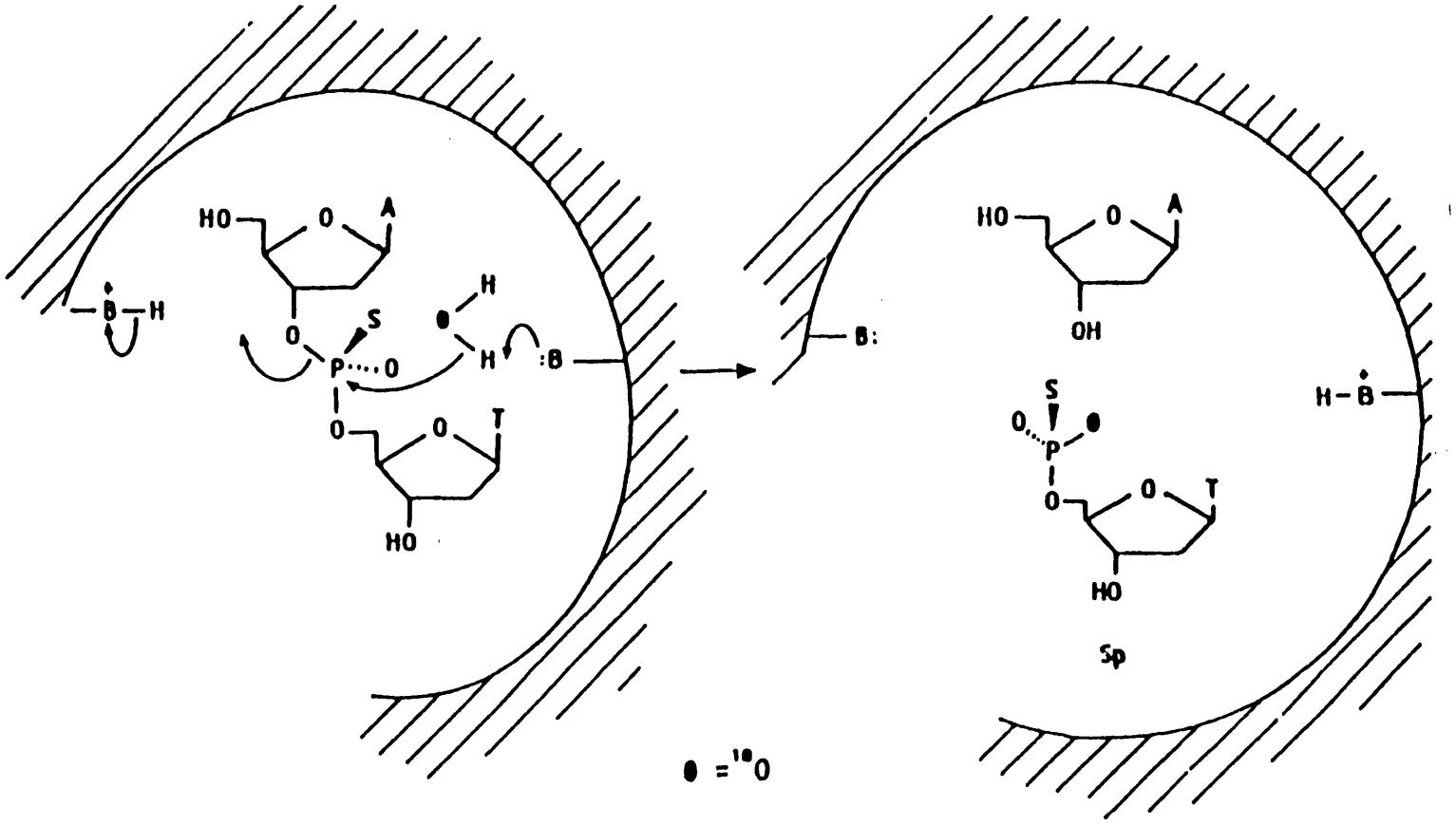
nucleotide-3'-O-linkage, as shown in Scheme 3.3, rather than by the formation of a covalent nucleotidyl-enzyme intermediate, (involving a double displacement at phosphorus, and hence overall retention of configuration).[5-7,16,26] Scheme 3.4 represents the proposed mechanism for hydrolysis of an oligomeric nucleotide substrate.

The result for this enzyme can be compared with stereochemical results for other members of the single-strand specific nuclease family; nuclease S1 from *Aspergillus oryzae*,[65] and nuclease P1 from *Penicillium citrinum*,[68] both recognise the Sp diastereoisomer of a dinucleoside phosphorothioate, and catalyse its cleavage with INVERSION of configuration at phosphorus. In addition to the common stereochemistry of their catalytic mechanism these nucleases exhibit other similarities, all three being glycoproteins, requiring zinc ions for activity, and having multiple metal ion-binding sites. In contrast, snake venom phosphodiesterase,[61,62,64] and spleen phosphodiesterase,[178] recognise the Rp diastereoisomer, and, like bovine intestinal phosphodiesterase, (Chapter 3), which would also be expected to recognise the Rp diastereoisomer of a dinucleoside phosphorothiote, show RETENTION of configuration, hence implicating nucleotidyl-enzyme intermediates in their catalytic mechanisms.

Although it is not generally understood why both types of mechanism should be used by the phosphodiesterases and nucleases it may be that the degree of selectivity of the enzyme for its substrates is related to the mechanism by which it handles them. High selectivity, as observed in the single-strand specific nucleases, seems to be associated with a single step mechanism whilst the relatively unselective phosphodiesterases appear to

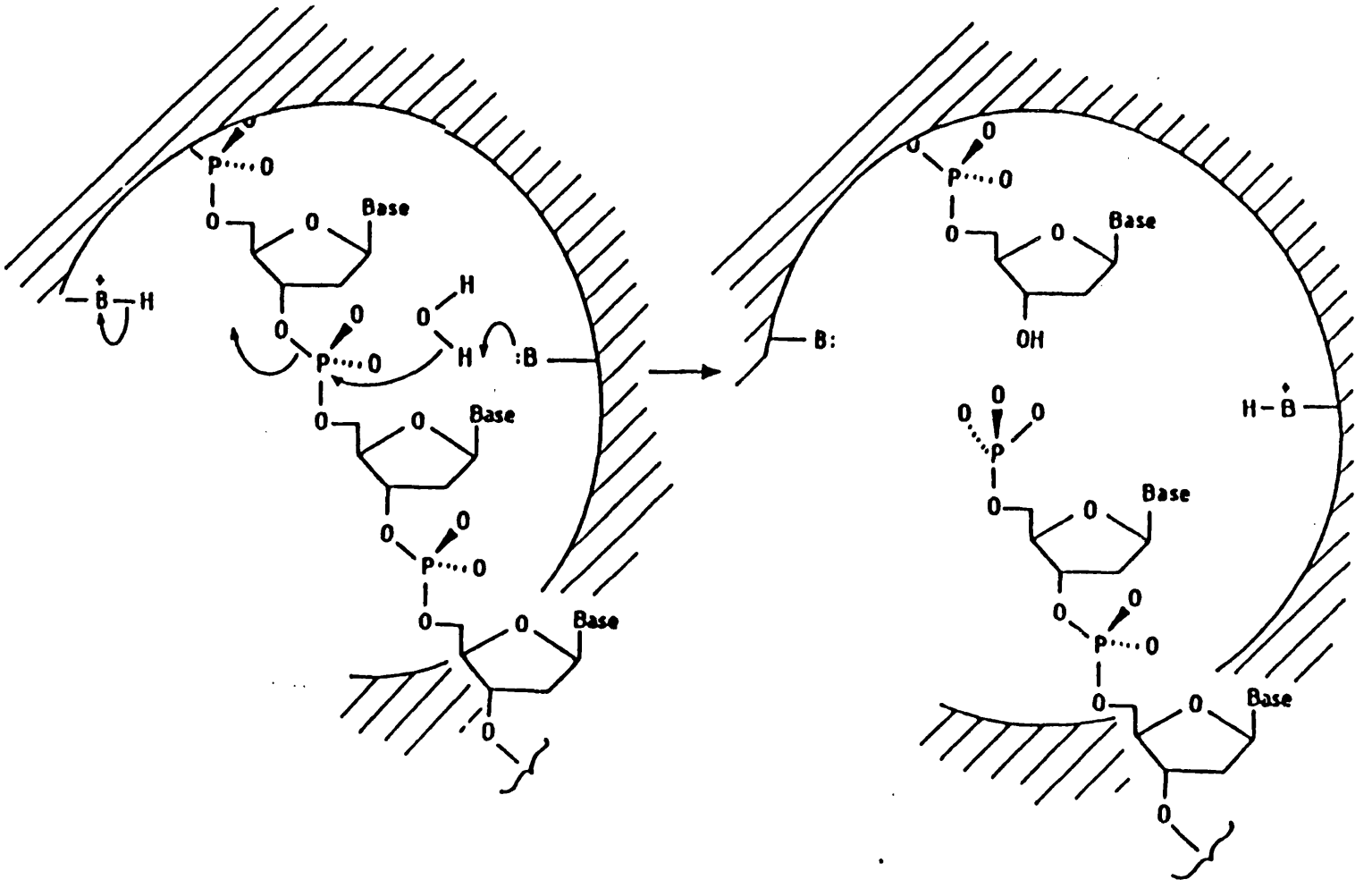
Scheme 3.3

Proposed Mechanism for the Hydrolysis of d[Ap(S)T]
by Mung Bean Nuclease



Scheme 3.4

Proposed mechanism for the Hydrolysis of Single Stranded DNA by Mung Bean Nuclease



use a two step mechanism involving a covalent enzyme-substrate intermediate. This latter is conceivably less sterically demanding than a mechanism in which all participating groups must achieve the correct positioning in a single step.

CHAPTER FOUR

STEREOCHEMICAL ANALYSIS OF THE IODINE-MEDIATED DESULPHURISATION OF A DINUCLEOSIDE PHOSPHOROTHIOATE.

Background

The development and syntheses of P-chiral isotopically labelled phosphate[13,16] and thiophosphate[5-7]esters has made possible the determination of the stereochemical course of enzymatic reactions at phosphorus for such classes of enzymes as phosphokinases, phosphodiesterases, phosphatases, and nucleotidyl transferases. Although the use of chiral phosphorothioate substrate analogues was the first developed and often experimentally the easiest approach, enzyme-catalysed thiophosphoryl transfer almost invariably occurs more slowly than phosphoryl transfer, and in some cases not at all. In this situation the oxygen chiral phosphate approach must be used for stereochemical investigations, where the substrate is made chiral by substitution of the heavy isotopes of oxygen, isotopic substitution having no effect on the enzymology of the substrate analogue.

Initially methods for stereoselective introduction of the stable oxygen isotopes into nucleotidyl phosphates were of limited applicablility; the first, applicable to the synthesis of nucleoside phosphates, involves the use of specifically-labelled 5-membered P-chiral isotopomers of cyclic phosphates,[59,162]or phosphoramidates,[78] and their condensation with the appropriate nucleoside or nucleotide. The second method employs diastereoisomeric nucleoside phosphoroanilidates,[164] and their reaction with isotopically-labelled carbonyl compounds such as benzaldehyde,[165] or carbon dioxide.[166] A more recent, and generally applicable method, for the synthesis of oxygen chiral dinucleoside monophosphates involves oxidation of the

dinucleoside monophosphite triesters with iodine in ^{18}O-labelled water.[167a]

In view of the relative accessibility of *P*-chiral phosphorothioate esters considerable interest has been centred on their conversion into the corresponding [^{17}O] or [^{18}O]-phosphates. Since diastereoisomeric oxygen chiral phosphates cannot be separated by physical methods their synthesis must exhibit high stereoselectivity to be useful. Thus the stereospecific conversion of a single diastereoisomer of a chiral phosphorothioate into the corresponding isotopically chiral phosphate represents a relatively simple route to these compounds. Methods include the activation of sulphur in nucleoside phosphorothioates with bromine,[161] *N*-bromosuccinimide,[160] cyanogen bromide,[168] and the simultaneous replacement of the activated sulphur with ^{17}O or ^{18}O derived from ^{17}O- or ^{18}O-labelled water respectively. ^{18}O-labelled DMSO[169] can also be used, these methods proceeding with inversion of configuration at phosphorus. Other methods include the methylation of sulphur with methyl iodide, followed by displacement with ^{18}O-NaOH[170], and the direct reaction of phosphorothioates with [^{18}O]-chloral [171,172] or [^{18}O]-styrene oxide [171,173] these proceeding with retention of configuration at phosphorus. The latter methods have the disadvantage that the ^{18}O-labelled reagents must be prepared from commercially available ^{18}O-labelled H_2O.

In some cases, however, side reactions can occur with nucleotide bases, particularly with guanine, which would have serious consequences if the desulphurisation of an oligonucleotide phosphorothioate was being attempted.

Reports exist of the use of iodine as a mild reagent for the

desulphurisation of oligonucleotides containing phosphorothioate internucleotidic linkages.[38,156] Thus the $[pAp(S)U]_n$ copolymer generated by the co-polymerisation of UDP and Sp ADPαS by polynucleotide phosphorylase was desulphurised using an aqueous ethanolic bicarbonate solution of iodine, as a method of characterisation of the copolymer[38] by comparison of desulphurised material with authentic samples. Similarily the octanucleotide $d(GG_sAATTCC)$ containing the recognition sequence of the *Eco*RI restriction endonuclease with a phosphorothioate internucleotidic linkage at the cleavage site was characterised by desulphurisation to d(GGAATTCC) using iodine in aqueous pyridine for 45min at room temperature.[156] The desulphurisation reaction was found to proceed quantitatively and to be free from side reactions, as demonstrated by the complete hydrolysis of the resulting d(GGAATTCC), by the *Eco*RI restriction endonuclease to the expected products.

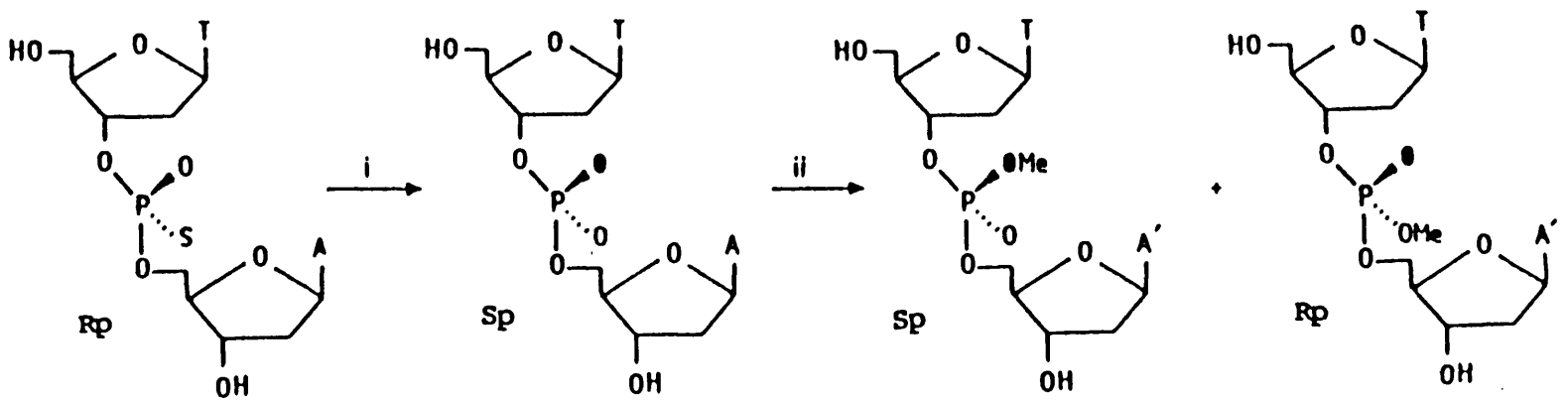
This mild desulphurisation is potentially a useful route to ^{17}O, or ^{18}O-containing oligonucleotides via the corresponding phosphorothioate-containing oligonucleotide, depending upon the stereochemical course of the replacement.

Stereochemical Analysis of the Desulphurisation Reaction.

An investigation of the stereochemical course of the iodine-mediated desulphurisation reaction was undertaken using a dinucleoside phosphorothioate, as the simplest unit containing an internucleotidic phosphorothioate linkage. In order to determine the stereochemistry of the conversion a method for location of the ^{18}O-isotope in the resulting dinucleoside phosphate was required. The method employed here for the configurational

Scheme 4.1

Summary of Route To O-methyl Esters of Sp [^{18}O] d[TpA]



REAGENTS: i) N-bromosuccinimide, $H_2{}^{18}O$-dioxan

ii) (a) K + Dowex (b) 18-crown-6 (c) MeI-DMSO

A'=N'-methyladenine

^{18} = ^{18}O

analysis of the ^{18}O-labelled dinucleoside phosphate is based on that described by Potter et al.,[68] in the determination of the stereochemistry of the desulphurisation of Rp-d[Tp(S)A] with N-bromosuccinimide in dioxane and ^{18}O-labelled water. This involves preparation of the diastereoisomeric methyl esters of the ^{18}O-labelled dinucleoside phosphate by treatment with methyl iodide, followed by examination of the $^{31}P(^{18}O)$-isotope shifts in the high-field ^{31}P n.m.r. spectrum of the resulting triester. Provided that the diastereoisomers of the unlabelled material have been assigned to their corresponding resonances, examination of the relative magnitude of the observed isotope shifts on each resonance permits the ^{18}O-isotope to be located, and hence configuration of the [^{18}O] dinucleoside phosphate.

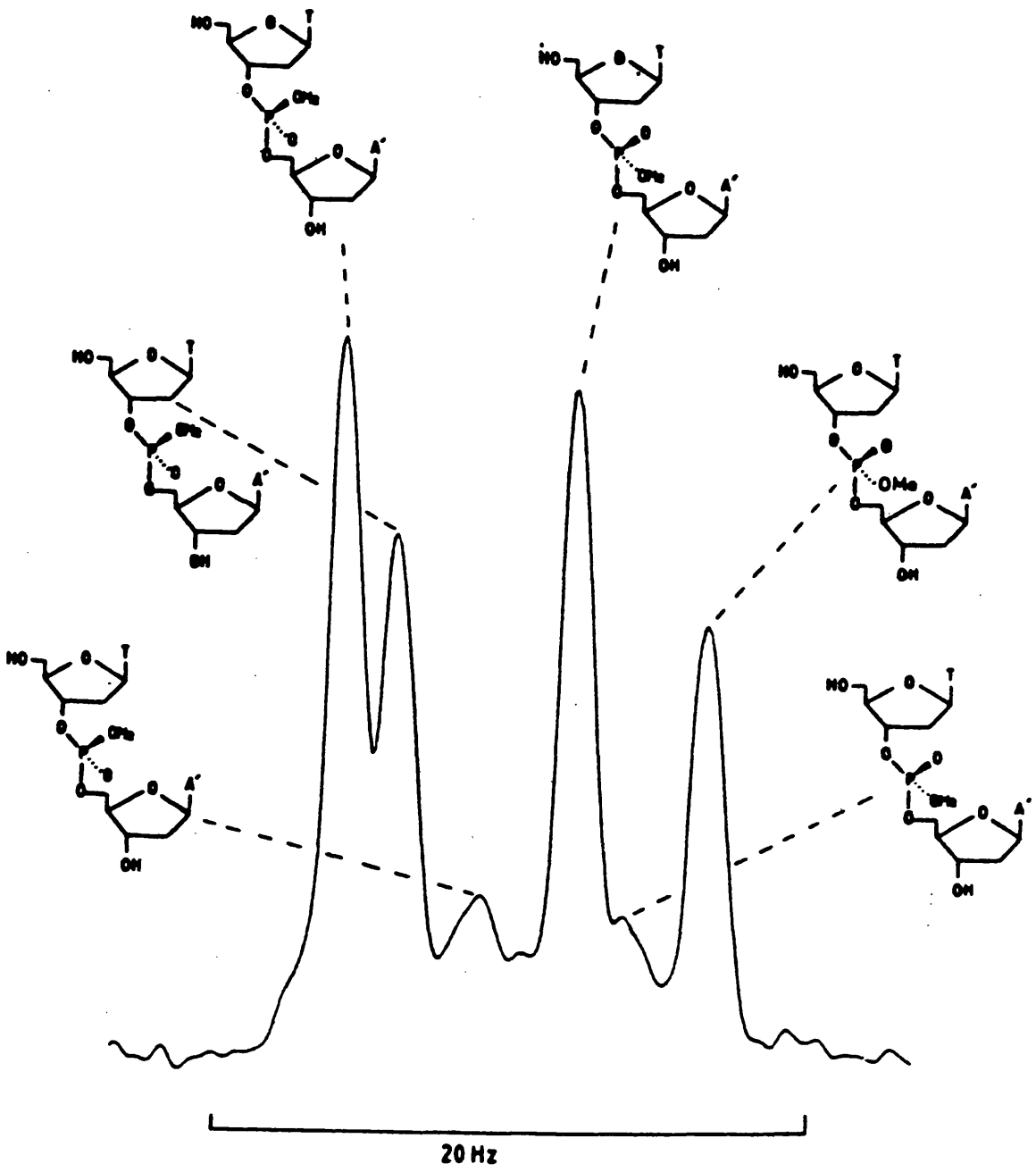
Configurational Assignment of the Methyl Esters of d[TpA];

In order to determine the stereochemistry of the iodine-mediated desulphurisation using this approach it was first necessary to assign the diastereoisomers of the methyl esters of the dinucleotide to their corresponding ^{31}P n.m.r. resonances. This was achieved by methylation of a sample of ^{18}O d[TpA] of known configuration in the presence of unlabelled d[TpA], and examining the resulting high-field ^{31}P n.m.r. spectrum.

A sample of Sp ^{18}O-d[TpA] was provided by Dr. B.V.L. Potter, having been obtained from the desulphurisation of Rp d[Tp(S)A][34] using N-bromosuccinimide in ^{18}O-labelled water[160]. The starting material, Rp-d[Tp(S)A], was prepared by Dr. B.V.L. Potter by the method of Romaniuk and Eckstein,[34] and isolated by reverse-phase h.p.l.c. Sp ^{18}O-d[TpA] was combined with an approximately equivalent amount of ^{16}O-d[TpA] and converted to the potassium-18-crown-6 salt. Overnight treatment of the mixture

Figure 4.1

High-field ^{31}P n.m.r Spectrum of O-methyl Esters of Sp [^{18}O]d[TpA]



conc ca. 20mM in 2:1 v/v DMSO-D_6 DMSO containing 8-hydroxyquinoline.
^{31}P n.m.r. parameters were:
sweep width 1000Hz, pulse width 7μs, aquisition time 4s, data collection in 16K, no. transients 11,837.

with methyl iodide in DMSO yielded a mixture of the diastereoisomeric ^{16}O-and ^{18}O- methyl esters of d[TpA]. (Scheme 4.1, ^{16}O-esters not shown). Under these reaction conditions it was expected that methylation of the adenine base would occur, probably[122,177] at $\underline{N}^1$, whilst the thymine base is expected to be unchanged, being reportedly susceptible to alkylation by diazoalkanes only.[122] Examination of the high-field ^{31}P n.m.r. spectrum of the mixture, (Figure 4.1), showed the resonances due to the diastereoisomeric ^{16}O-d[TpA] methyl esters, each with a $^{31}P(^{18}O)$-isotope shift,[110,111] due to the corresponding ^{18}O-labelled diastereoisomer. Since the magnitude of $^{31}P(^{18}O)$-isotope shifts is dependent on bond order,[112] the small isotope shift, (1.95 Hz), on the downfield resonance was assigned to the ester having the ^{18}O-isotope in the P-$\underline{O}$Me position, whilst the larger isotope shift, (4,97 Hz), on the upfield resonance was assigned to the ester having the ^{18}O-isotope in the P=$\underline{O}$ position. Knowing the configuration of the original ^{18}O-d[TpA], and, from the relative magnitudes of the $^{31}P(^{18}O)$-isotope shifts, the positions of the ^{18}O-label in the methyl esters of ^{18}O-d[TpA] configurational assignments could be made. Thus the downfield resonance could be assigned to the Sp diastereoisomer, and the upfield resonance to the Rp diastereoisomer of the methyl esters of d[TpA], (assignments shown on the spectrum). Other small peaks in the spectrum are a result of an approximately 15% contamination of the Rp d[Tp(S)A] starting material with Sp d[Tp(S)A], as estimated by h.p.l.c., yielding contaminating Rp ^{18}O-d[TpA] on desulphurisation.

Having assigned the absolute configurations of the diastereoisomers of the $\underline{O}$-methyl esters of d[TpA] to the corresponding ^{31}P n.m.r. resonances it is possible to locate

^{18}O-labels in a sample of ^{18}O-d[TpA] of unknown configuration, enabling the stereochemistry of the iodine-mediated desulphurisation reaction to be determined.

Stereochemical Analysis of the Desulphurisation:

i) in aqueous pyridine;

In order to determine the stereochemical course of the iodine-mediated desulphurisation a sample of dinucleoside phosphorothioate of known absolute configuration at phosphorus was desulphurised in ^{18}O-labelled water. Location of the ^{18}O-isotope by ^{31}P n.m.r. spectroscopy allowed the absolute configuration of the product to be related to the known configuration of the starting material, thus indicating the stereochemical course of the replacement.

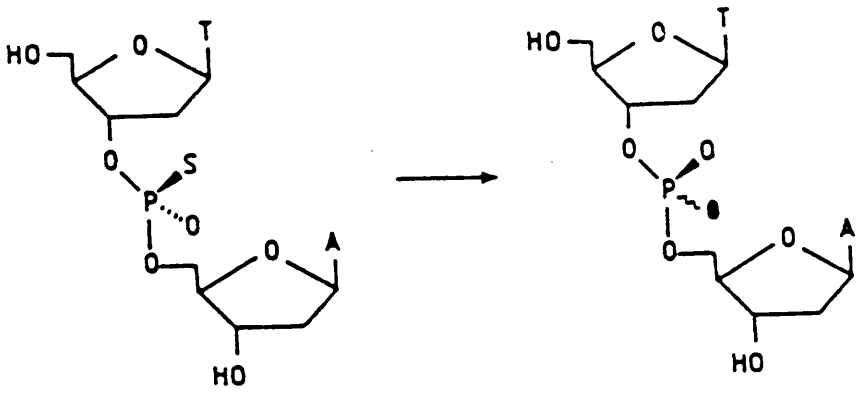
A sample of predominantly Sp d[Tp(S)A] was provided by Dr B.V.L. Potter, having been synthesised[34] by the condensation of monomethoxytritylthymidine with S-2-cyanoethyl phosphorothioate, followed by condensation with N^6,$O^{3'}$-dibenzoyladenosine. After removal of the protecting groups the diastereoisomers of the resulting d[Tp(S)A] were separated by preparative reverse-phase h.p.l.c., and configurations were assigned by hydrolysis of mixtures of diastereoisomers by snake venom phosphodiesterase, known to preferentially hydrolyse the Rp diastereoisomer of dinucleoside phosphorothioates.[139]

The sample was desulphurised by treatment with iodine in pyridine/^{18}O-labelled water for ca. 1.5h at room temperature, Scheme 4.2, after which time iodine was removed by extraction into ether, and pyridine/water was removed by evaporation. In order to provide reference peaks in the ^{31}P n.m.r. spectrum the

^{18}O-labelled dinucleotide was combined with an approximately equivalent amount of unlabelled d[TpA], before conversion to the potassium 18-crown-6 salt, and methylation with methyl iodide in

Scheme 4.2

Iodine-mediated Desulphurisation of Sp d[Tp(S)A] in Pyridine



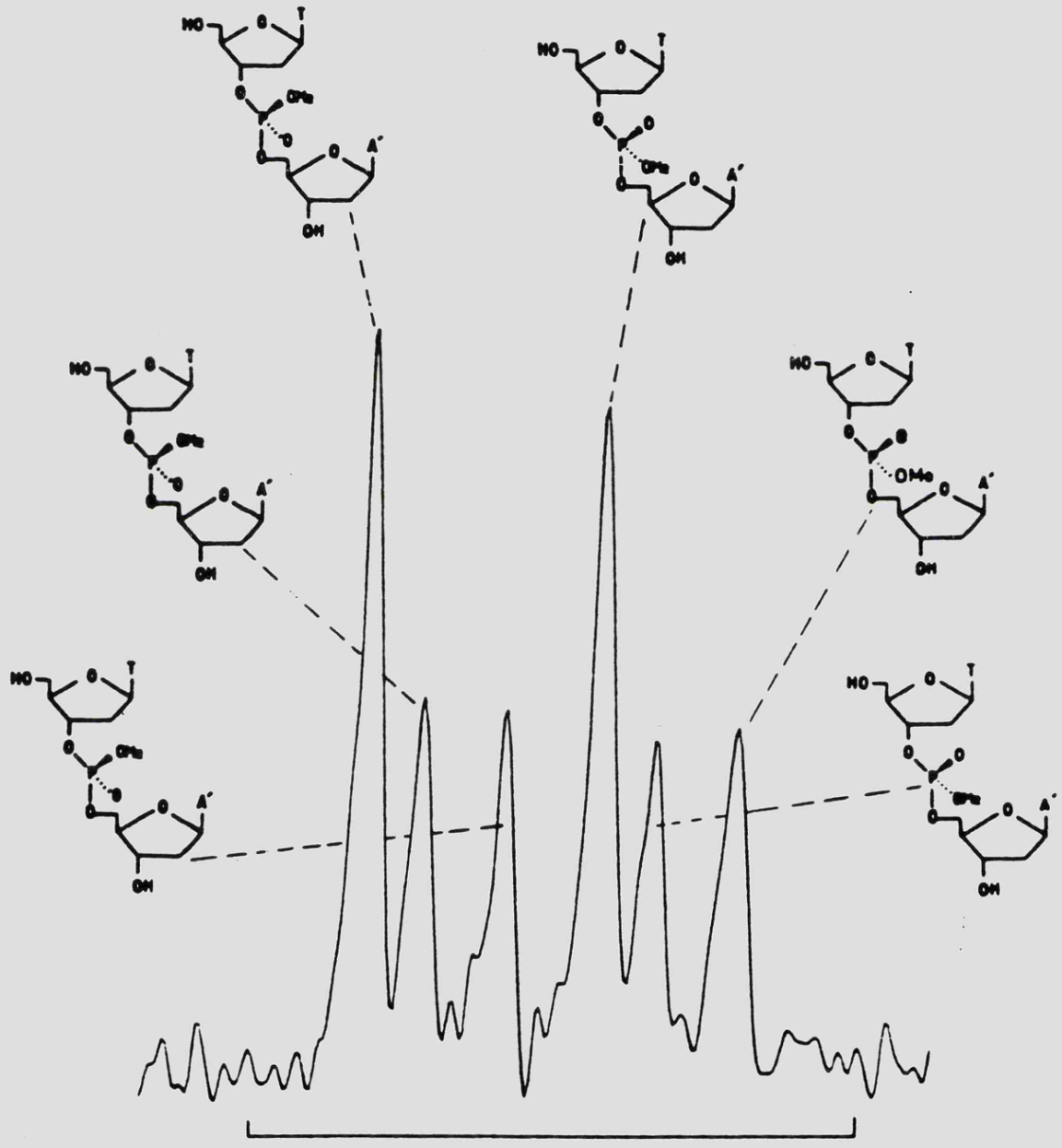
Reagents: Iodine - H_2O/pyridine

DMSO overnight. High-field ^{31}P n.m.r. analysis of the resulting sample of the methyl esters of ^{16}O- and ^{18}O-d[TpA] gave the spectrum shown in Figure 4.2. It can be seen from the spectrum that for each high intensity resonance due to the unlabelled diastereoisomers of the triester there are two equal intensity isotope-shifted resonances. This indicates that the iodine-mediated desulphurisation has proceeded with EPIMERISATION at the phosphorus centre, the ^{18}O-isotope appearing in both the P=O and P-OMe positions in both diastereoisomers in equal amounts. The observation of epimerisation for this method of desulphurisation makes it less useful than if it had proceeded stereoselectively since the diastereoisomers of an isotopically chiral phosphate cannot be separated by physical methods.

This stereochemical result can be compared with that reported by Frey[114] in the stereochemical analysis of phosphoanhydride

Figure 4.2

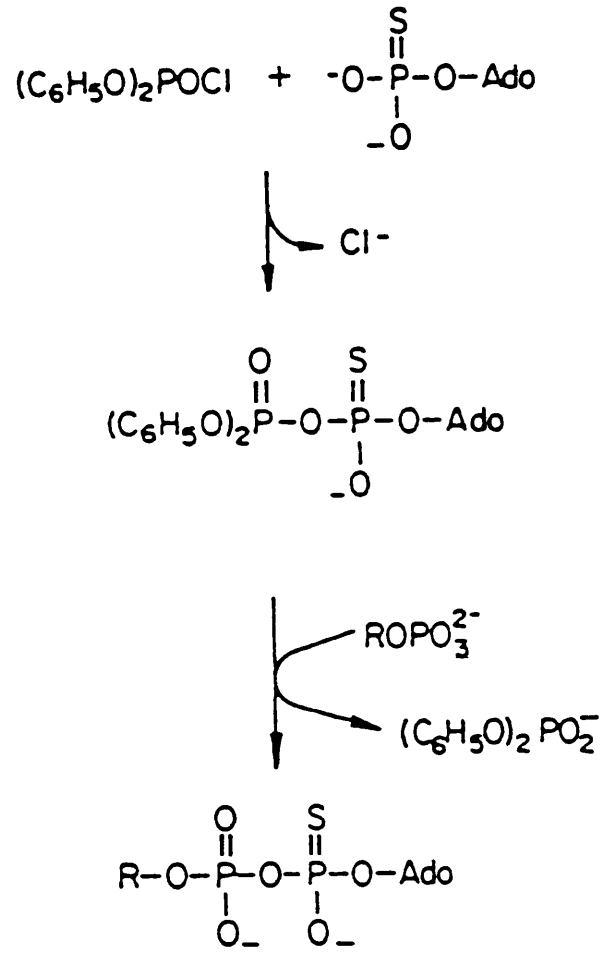
High-field ^{31}P n.m.r. Spectrum of the O-methyl Esters of [^{18}O] d[TpA] Generated by Iodine-mediated Desulphurisation of Sp d[Tp(S)A] in Pyridine.



conc ca. 20mM in 2:1 v/v DMSO-D_6 DMSO containing 8-hydroxyquinoline.
^{31}P n.m.r. parameters were:
sweep width 1000Hz, pulse width 7μs, aquisition time 4s, data collection in 16K, no. transients 11,560.

Scheme 4.3

Summary of General Method of Phosphoanhydride Synthesis

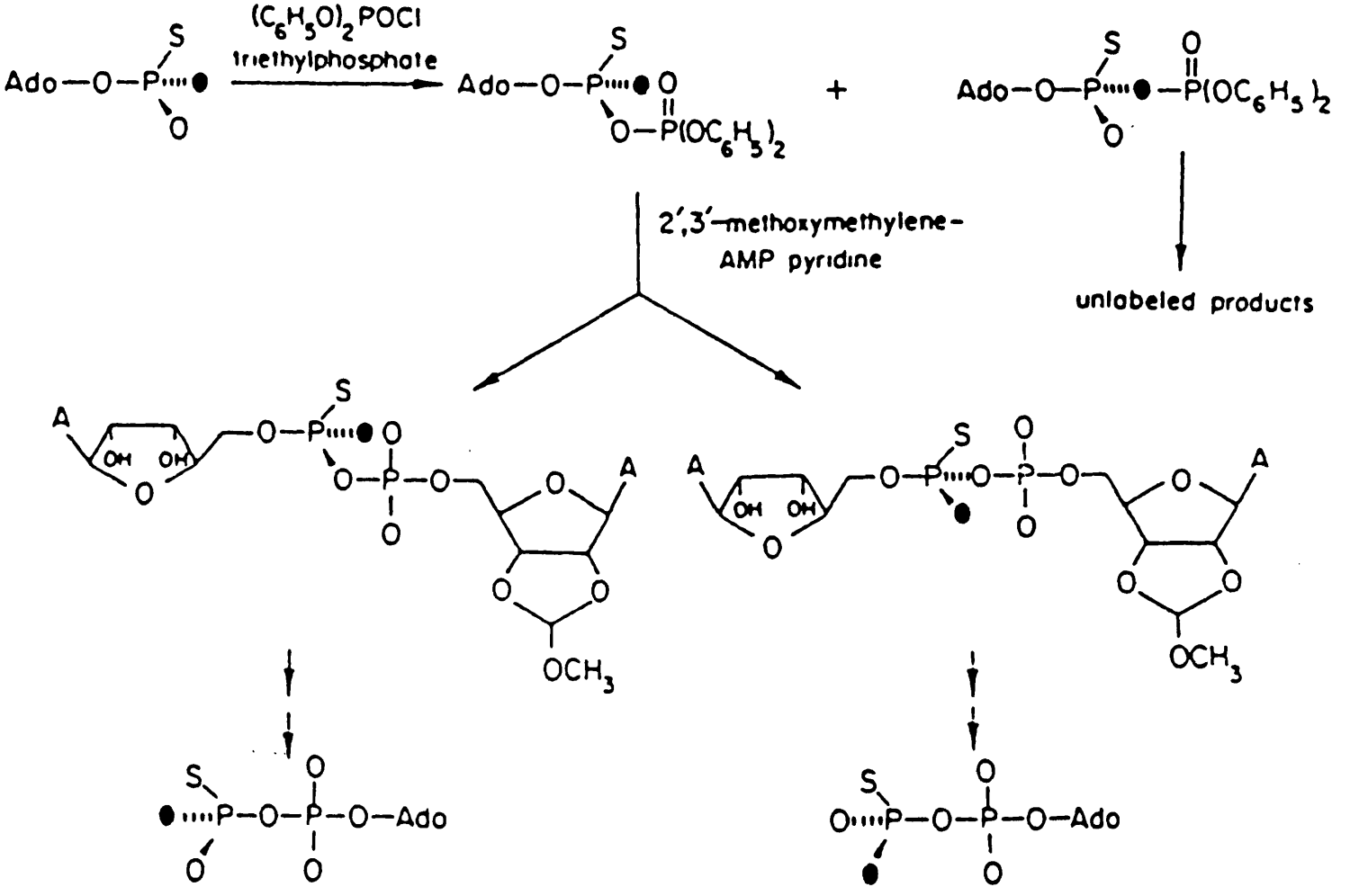


Ado = adenosine

R- = H- , HO-P(=O)-, HO-P(=O)(-O-)-O-P(=O)(-O-)-, alkyl

Scheme 4.4

Summary of Method Employed For Configurational Analysis of Coupling Reaction in Phosphoanhydride Synthesis



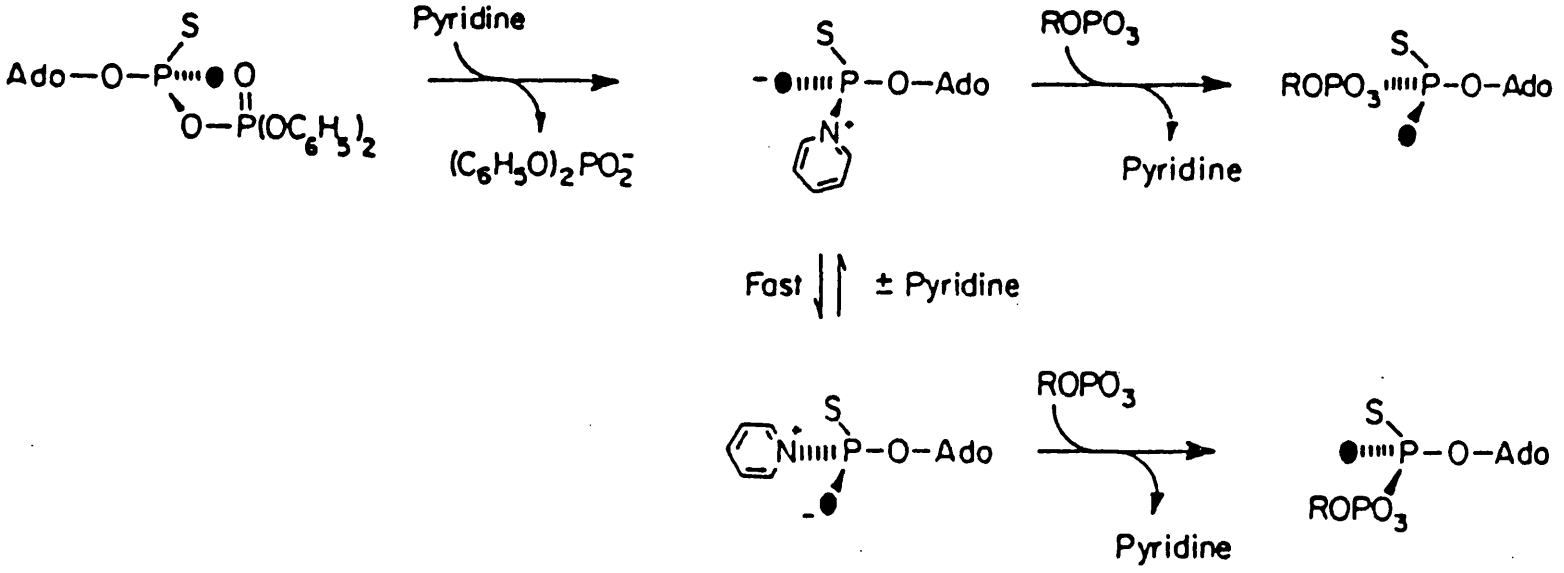
synthesis. Scheme 4.3 shows the general method of phosphoanhydride synthesis first developed by Michelson[120a] which has been widely applied to the synthesis of sulphur-containing analogues of nucleotides. The method involves activation of a nucleoside 5'-phosphorothioate with diphenyl phosphochloridate, followed by reaction in pyridine with the trialkylammonium salts of phosphoric acid, pyrophosphoric acid, or phosphate esters, to produce the desired phosphoanhydride, and diphenyl phosphate.

In order to determine the stereochemistry of the coupling step the reaction was conducted using [^{16}O,^{18}O]AMPS, which was coupled to AMP or 2',3'-methoxymethylidene-AMP. Configurational analysis of the phosphorothioate centre in both products showed the ^{18}O-isotope to be equally distributed between the two non-bridging positions, and thus that the displacement of diphenyl phosphate had occurred with epimerisation at phosphorus, Scheme 4.4. This stereochemical result was accompanied by the observation that pyridine was required as at least a component of the solvent in order for the coupling reaction to occur, and led to the conclusion that pyridine was acting as a nucleophilic catalyst in the reaction, by way of the mechanism shown in Scheme 4.5.[114] Thus initial attack by nucleophilic pyridine at the phosphorus centre displaces diphenyl phosphate, leading to the formation of a reactive pyridinium intermediate, (X). Several further displacements by pyridine may occur prior to final displacement by phosphate, resulting in loss of configuration at the phosphorus centre. By analogy it seems probable that a similar mechanism is involved in the iodine-mediated desulphurisation reaction under investigation.

In order to test this possibility trials with a simple non-nucleoside phosphorothioate, sodium O,O-dimethyl-

Scheme 4.5

Proposed Mechanism of Coupling Reaction in Phosphoanhydride Synthesis



phosphorothioate, $(MeO)_2PSO^-Na^+$, a gift of Dr P.M. Cullis, were conducted using a less nucleophilic analogue of pyridine, and also non-nucleophilic tri-N-butylamine in order to distinguish between the role of pyridine as a nucleophilic catalyst and as a basic catalyst.

When the less nucleophilic 2,6-dimethyl pyridine (lutidine), was employed in place of pyridine for the iodine-mediated desulpurisation of $(MeO)_2PSO^-Na^+$ the substitution proceded rapidly, as indicated by monitoring of the reaction by ^{31}P n.m.r. spectroscopy, desulphurisation being accompanied by a shift in the ^{31}P n.m.r. resonance from δ +52ppm to ca. δ +2ppm, and being almost complete after 20min at room temperature.

When the same reaction was conducted using tri-N-butylamine in place of lutidine, no desulphurisation was observed after 2h. On addition of a few drops of pyridine and rexamination of the spectrum after ca. 10min, extensive desulphurisation was observed, indicating that pyridine was necessary for the desulphurisation reaction.

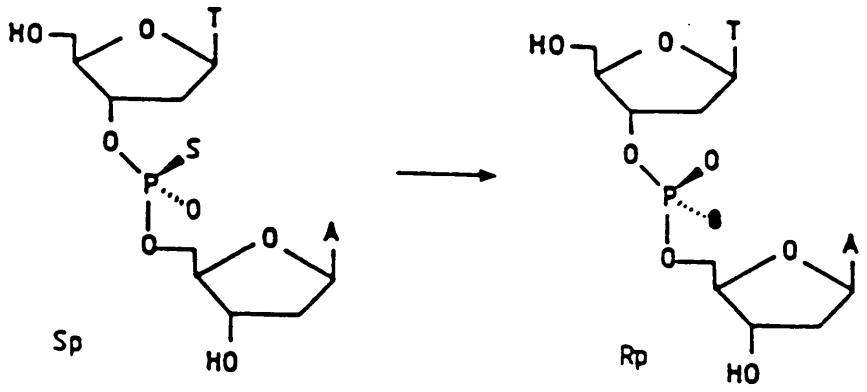
ii) in aqueous lutidine;

When the desulphurisation of Sp d[Tp(S)A] was conducted using the less nucleophilic 2,6-dimethyl pyridine, (lutidine), in place of pyridine, Scheme 4.6, the reaction was found to proceed much more slowly than in pyridine, and was allowed to stand overnight. On methylation of the product, the high-field ^{31}P n.m.r. spectrum, (figure 4.3), showed evidence for stereoselectivity in the desulphurisation.

It can be seen from the relative magnitudes of the isotope-shifts that the patern of resonances is opposite to that observed for the sample of Sp ^{18}O-d[TpA], the ^{18}O-label being

Scheme 4.6

Iodine-mediated Desulphurisation of Sp d[Tp(S)A] in Lutidine



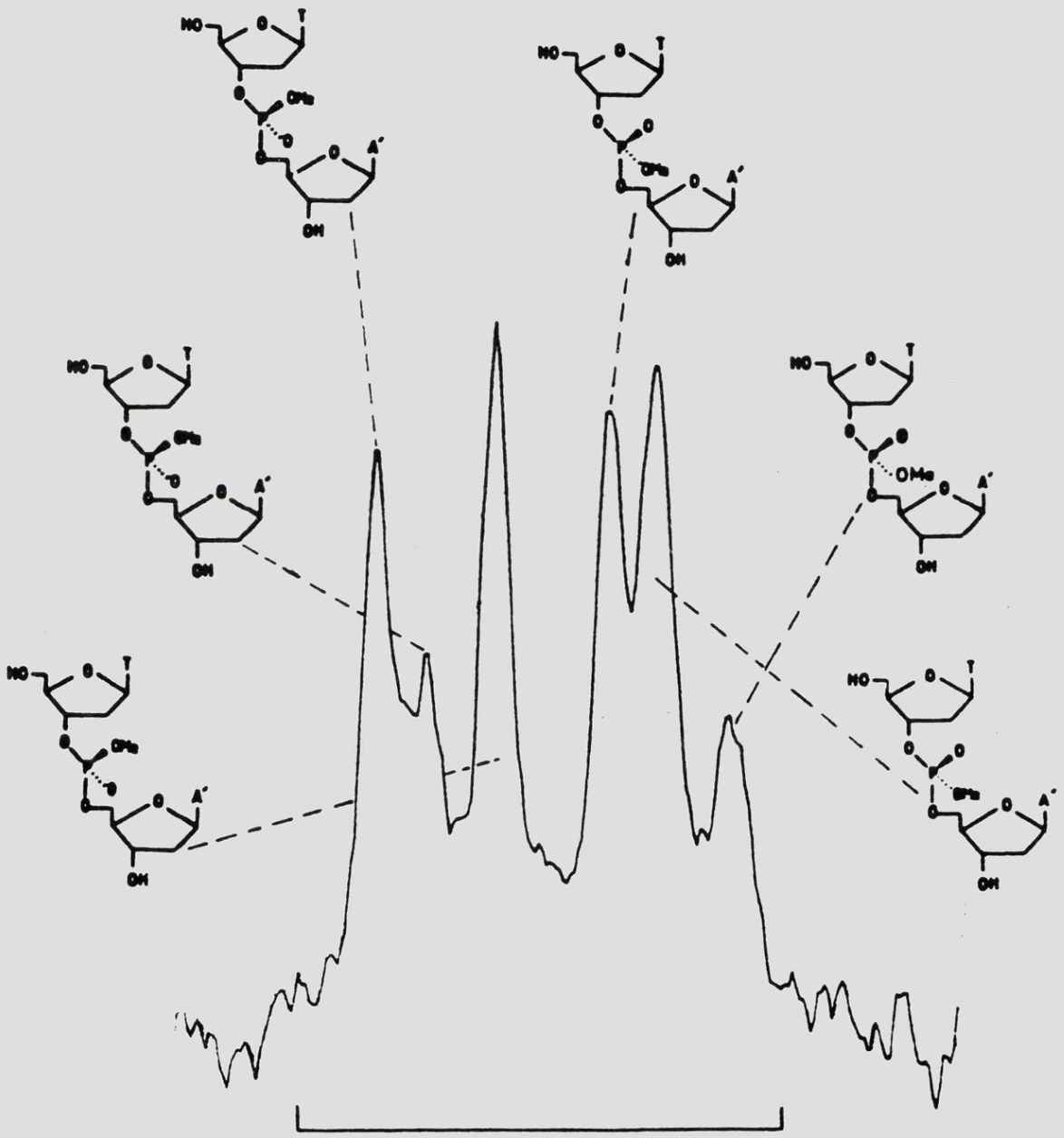
Reagents: Iodine - H_2O/lutidine

located in the P=O position in the Sp diastereoisomer, and in the P-OMe position in the Rp diastereoisomer, implying that the configuration of the ^{18}O-d[TpA] obtained here is predominantly Rp. Allowing for the 15% contamination of the starting material with the opposite diastereoisomer, measurement of relative intensities of the $^{31}P(^{18}O)$-shifted resonances indicates a stereospecificity of ca. 75%. That the iodine-mediated desulphurisation using aqueous lutidine proceeds so much more slowly for d[Tp(S)A] than for $(MeO)_2PSO^-Na^+$ is possibly a result of steric effects; the phosphorus centre of $(MeO)_2PSO^-Na^+$ being much more sterically accessible than that of d[Tp(S)A] so that nucleophilic catalysis by lutidine may still have been the predominant mechanism of desulphurisation in the former case.

The observation of epimerisation at phosphorus in aqeous pyridine, compared to considerable stereospecificity in the presence of the less nucleophilic 2,6-dimethyl pyridine indicates that the pyridine is indeed most likely involved as a

Figure 4.3

High-field ^{31}P n.m.r Spectrum of the O-methyl Esters of [^{18}O] d[TpA] Generated by Iodine-mediated Desulphurisation of Sp d[Tp(S)A] in Lutidine

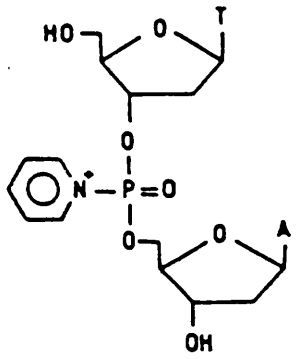


conc ca. 10mM in 2:1 v/v DMSO-D_6 DMSO containing 8-hydroxyquinoline.
^{31}P n.m.r. parameters were:
sweep width 1000Hz, pulse width 7μs, aquisition time 4s, data collection in 16K, no. transients 6,712.

nucleophilic catalyst in the former case, presumably by way of the intermediate species, shown in Figure 4.4.

Figure 4.4

Proposed Intermediate in Iodine-mediated Desulphurisation of d[Tp(s)A] in Aqueous Pyridine.



Similar intermediates have been proposed by both Mikolajczyk,[174] and Frey,[114] and direct observation of a reactive pyridine intermediate consequent on the addition of 4-dimethylamino pyridine to diphenyl phosphochloridate has recently been claimed.[175]

Hence, it seems that initial activation of sulphur occurs by iodine in pyridine, {perhaps by way of the $[(\text{pyridine})_2I^+]\ I_3^-$ complex},[176] the activated sulphur then being displaced by pyridine. Further successive displacements by pyridine occur at the phosphorus centre before the final base-catalysed reaction with water yields the phosphate diester. In less nucleophilic 2,6-dimethyl pyridine the activated intermediate has a much longer lifetime, so that direct base-catalysed displacement of the sulphur by water can occur, without extensive participation of the heterocycle, with a consequence that the displacement reaction proceeds with predominant inversion of configuration at the phosphorus centre, as observed for related reactions with other activating agents.[160,161,168,169]

EXPERIMENTAL DETAILS

MATERIALS AND METHODS

^{18}O-labelled water (98 atom%) was purchased from Amersham. D_4-MeOH and D_6-DMSO were obtained from Aldrich Chemical Co. Ltd., Gillingham, Dorset. DEAE Sephadex A-25 was obtained from Pharmacia Fine Chemicals AB, Uppsala, Sweden. Nucleosides were purchased from Sigma Chemical Co., London, and Pharma Waldhof GmbH, (Dusseldorf, FRG). AMP was purchased from Pharma Waldhof GmbH, (Dusseldorf, FRG).

All other chemicals were Analar grade, or of the highest purity commercially available, and were purchased from Aldrich Chemical Co. Ltd., Gillingham, Dorset; BDH Chemicals Ltd., Poole; Koch Light Laboratories Ltd., Colnbrook, Bucks.; Fisons Scientific Apparatus Ltd., Loughborough; May and Baker Ltd., Dagenham.

Snake Venom Phosphodiesterase, (EC 3.1.4.1], was purchased from Boehringer Mannheim, London, as a suspension in 3.2M ammonium sulphate, (activity of 1.5 units/mg protein). Bovine intestinal mucosa 5'-nucleotide phosphodiesterase, [EC 3.1.4.1.] was purchased from Sigma Chemical Co., London, as a suspension in 3.2M ammonium sulphate, (activity of 0.8 units/mg phosphate per minute at pH 8.8, 37°C). Mung bean nuclease, (EC 3.1.30.1), was obtained from Sigma Chemical Co., London, as a freeze dried powder of specific activity 58000 units/mg, (1 unit solubilises 1μg denatured calf thymus DNA/min at pH 5, 37°C). Nuclease P1 was obtained from Sigma Chemical Co., London, as a freeze-dried powder of specific activity 320 units/mg protein, (1 unit liberates 1μmol acid soluble nucleotides from RNA/min pH 5.3, 37°C).

Diazomethane was prepared from Diazald salt, (*N*-methyl-*N*-nitroso-*p*-toluenesulphonamide), purchased from Aldrich Chemical Co., in a Mini-Diazald apparatus, also from Aldrich, by dripping an ethereal solution of Diazald salt into hot ethanolic potassium hydroxide solution, the diazomethane being collected as an ethereal distillate.

Anhydrous tri-*N*-butylamine was obtained by refluxing with pellets of potassium hydroxide, followed by distillation under reduced pressure, and was stored over pellets of potassium hydroxide. Diphenyl phosphorochloridate was distilled prior to use. Nucleosides were dried by heating to 80°C in a drying pistol under reduced pressure, in the presence of phosphorus pentoxide.

DMF was dried and purified by distillation of benzene-water azeotrope, followed by shaking with activated alumina, stirring overnight with activated magnesium sulphate and distillation under reduced pressure onto Linde-type 4Å molecular sieves. Anhydrous DMSO was obtained by stirring with calcium hydride, followed by distillation under reduced pressure, and was stored over Linde-type 4Å molecular sieves. Dioxan was passed down an alumina column, refluxed with sodium and benzophenone until a permanent deep blue colour was obtained, and then stored over 4Å molecular sieves under nitrogen. Anhydrous methanol was obtained by refluxing with magnesium and iodine, followed by distillation, and was stored over 3Å molecular sieves. Pyridine was dried by refluxing with potassium hydroxide pellets, then distilled on to, and stored over, further potassium hydroxide pellets. Triethyl phosphate was dried by stirring with barium oxide for 2 days, distillation under reduced pressure, and was stored over 4Å molecular sieves. Trimethyl phosphate was dried by standing over

4Å molecular sieves, distillation under reduced pressure, and storage over 4Å molecular sieves.

Dowex-100 cation exchange resin was purchased from Aldrich chemical co., and was converted to the pyridinium form by washing with 1M HCl, rinsing with water until the supernatant was approximately neutral, then stirred for 0.5h at room temperature with a large excess of pyridine, after which it was filtered out of suspension and stored away from light. High-grade de-ionised water, used for preparing all buffers, was obtained from a Fisons Fionex water de-ioniser and a Milli-Q3 water purifying system, supplied by Millipore Corp., Bedford.

Measurements of pH were performed using narrow-range pH papers, obtained from BDH Chemicals Ltd., or a GK2321 C combined glass electrode, supplied by Radiometer, Copenhagen, which was attached to a PHM83 Autocal pH meter.

Ion-exchange chromatography was performed using DEAE Sephadex A-25 resin in the bicarbonate form, using linear concentration gradients of TEAB, pH ca. 8, obtained by gravity mixing. The column effluent was monitored by an LKB Uvichord II continuous flow UV detector at 254 nm, linked to an LKB 2210 Chart Recorder, before passing to an LKB-7000 Ultrorac fraction collector, which was coupled to the chart recorder through an event marker.

UV Spectroscopy was performed on a Shimadzu UV-240 Graphicord UV Visible Spectrometer using quartz cells, (1cm path length).

H.p.l.c. was performed on a Shimadzu LC-4A liquid Chromatograph connected to an SPD-2AS spectrophotometric detector, and a shimadzu C-R2AX Chromatopac, using Apex 5μ ODS, and Hypersil 5μ ODS reverse phase columns, with mobile phases of acetonitrile in 50mM TEAB, pH ca. 6.8, acetonitrile in 25mM TEAA, pH 6.4, and methanol in 50mM potassium phosphate, pH 6.0.

Low-field ^{31}P n.m.r. spectroscopy was performed on a Jeol FX-60 spectrometer, operating at 24.15 MHz, with broad band proton decoupling. Samples were contained within 5mm n.m.r. tubes held within 10mm tubes which contained deuteriated water, (providing a field frequency lock on the deuterium resonance). High-field ^{31}P n.m.r. spectra were recorded on a Bruker AM300 Spectrometer operating at 121.5 MHz, with broad band proton decoupling, with samples contained in 5mm precision n.m.r. tubes, or on a Bruker WH400 spectrometer at Warwick University, operating at 162 MHz. with broad band proton decoupling, (the SERC service, run by Dr O. Howarth). Chemical shifts are quoted relative to external 80% H_3PO_4 and are positive when downfield of this reference.

^{1}H n.m.r. spectroscopy was performed on an EM390 n.m.r. spectrometer, operating at 90MHz, with samples contained in 5mm n.m.r. tubes using a standard of TMS.

Enzyme incubations were thermostated using a Pierce Reacta-Therm Stirring/Heating Module.

Volumes in the range 0.001 ml to 1.000 ml were measured using Gilson micropipettes with disposable plastic tips.

PREPARATION AND PURIFICATION OF NUCLEOSIDE 5'-O-METHYL PHOSPHOROTHIOATE ESTERS.

2'-Deoxyadenosine 5'-O-methyl Phosphorothioate Ester, dAMPS-OMe.

The mono-triethylammonium salt of 2'-deoxyadenosine 5'-phosphorothioate, dAMPS, (200μmol), was converted to the pyridinium form by stirring with 4000 equiv. of pyridinium Dowex-100 ion exchange resin for ca. 20 min at room temperature. The resin was filtered and washed with ca. 20ml of water. The filtrate was evaporated to dryness *in vacuo*, then evaporated twice with dry methanol to remove any residual water.

The pyridinium salt was suspended in 2.0ml of dry methanol, and tri-N-octylamine, (90μl, 200μmol), was added. As the mixture was swirled ion exchange occurred and the immiscible tri-N-octylamine dissolved. The solution was evaporated to dryness *in vacuo*, then co-evaporated twice with dry DMF.

The mono-tri-N-octylammonium salt of dAMPS was dissolved in 2.0ml of dry dioxan, and to the solution was added diphenylphosphorochloridate, (50μl, 224μmol) and tri-N-butylamine, (60μl, 248μmol). The reaction was left for ca. 30 min at room temperature, then a ^{31}P n.m.r. spectrum was recorded, which showed the formation of the expected anhydride intermediate as a pair of doublets, δ_P(dioxan) +47 ppm,d, $^2J_{pp}$=29 Hz, (S=P-O-P) and δ_P(dioxan) -24.5 ppm,d, $^2J_{pp}$=29 Hz, [P-O-P-$(OPh)_2$].

The reaction was quenched by the addition of 3.0ml of a 1:1:1 mixture of dry pyridine, dry DMF, and dry methanol, and stirred at room temperature for ca. 2h. A ^{31}P n.m.r. spectrum was

recorded of the reaction mixture, and indicated that all the anhydride intermediate had been consumed, the desired product appearing as a peak at δ_p(pyridine:DMF:MeOH, 1:1:1) +60.3 ppm,s, in the reaction mixture.

The product, dAMPS-OMe, was purified by ion-exchange chromatography on a column of DEAE Sephadex A-25 ion-exchange resin, using a linear gradient of 25-250mM TEAB, pH 8, (750ml of each). dAMPS-OMe was eluted at a buffer concentration of ca. 120mM, but the slower fractions were found to be contaminated with diphenyl phosphate. Pure dAMPS-OMe, (triethylammonium salt), was isolated from the earlier product-containing fractions, (50μmol, 25% yield).

The high-field ^{31}P n.m.r. spectrum, recorded at 121.5 MHz, showed equal intensity resonances at δ_p(DMF:D_4-MeOH 3:1) +57.24 ppm,s, (Sp isomer), and δ_p(DMF:D_4-MeOH 3:1) +57.37 ppm,s, (Rp isomer). The diastereoisomers were assigned to their respective resonances by partial selective digestion using snake venom phosphodiesterase, as described in Chapter 2. H.p.l.c., (reverse phase on Hypersil 5μ ODS column, isocratic elution with 15% methanol in 20mM K_2HPO_4, pH 6.0, flow rate 2.0ml/min), showed retention times of 3.5 min (Sp isomer), and 4.0 min (Rp isomer). The UV spectrum, recorded in H_2O, was found to be characteristic of the adenine chromophore, with λmax of 259nm.

Adenosine 5'-O-methyl phosphorothioate ester, AMPS-OMe.

Dry adenosine, (0.5g, 1.9mMol), was suspended in 6.0ml of dry triethyl phosphate, and dissolved by careful swirling of the flask over a Bunsen flame, the flask being fitted with a calcium chloride drying tube. The clear solution was cooled to 0°C by

immersion in an ice-water bath, then thiophosphoryl chloride, (225μl, 2.2 mMol), was carefully added, and the reaction sealed and stirred at room temperature for ca. 2h, after which time 5.0ml of a 3:1 v/v mixture of methanol:pyridine was added.

The reaction was then allowed to stand at room temperature for a further 30 min, before removal of the methanol:pyridine *in vacuo*. The bulk of the triethyl phosphate was removed by addition of 40-60°C petroleum spirit, and decanting of the supernatant from the precipitated products, three times. The resulting residue was analysed by ^{31}P n.m.r. spectroscopy, which showed the presence of adenosine 5'-O,O-dimethyl phosphorothioate, AMPS$(OMe)_2$, as a singlet at δ_P(MeOH:pyridine 3:1) +71 ppm,s.

The crude mixture was dissolved in saturated sodium hydroxide solution, and stirred at 50°C for 1.5h, after which time ^{31}P n.m.r. spectroscopy indicated complete conversion of AMPS-$(OMe)_2$ into AMPS-OMe, resonating at δ_P(aq. NaOH) +57.4 ppm,s.

The pH of the solution was reduced to ca. 8 by the addition of solid carbon dioxide, and the bulk of the crude product purified by ion-exchange chromatography on DEAE Sephadex ion-exchange resin, using a linear gradient of 25-250mM TEAB, pH 8, (900ml of each). AMPS-OMe was eluted at a buffer concentration of ca. 125mM TEAB. The fractions containing product were combined and evaporated to dryness *in vacuo*, and excess TEAB removed by three co-evaporations with methanol to yield AMPS-OMe, triethylammonium salt, as a glass, (760μmol, ca. 40%). The high-field ^{31}P n.m.r. spectrum, recorded at 121.5 MHz, showed two equal intensity resonances δ_P(DMF:D_4-MeOH 3:1) +57.22 ppm,s, (Sp isomer), and δ_P(DMF:D_4-MeOH 3:1) +57.38 ppm,s, (Rp isomer). Reverse phase

h.p.l.c. (isocratic elution with 6% acetonitrile in 50mM TEAB, pH 6.8, flow rate 1.3ml/min), showed retention times of 9.1 min (Sp isomer), and 11.8 min (Rp isomer). The UV spectrum, recorded in H_2O, was found to be characteristic of the adenine chromophore, with λmax 259nm.

2'-Deoxyguanosine 5'-O-methyl Phosphorothioate ester, dGMPS-OMe.

Dry 2'-deoxyguanosine, (0.5g, 2.0mMol), was suspended in 10.0ml of dry trimethyl phosphate and stirred at room temperature for ca. 1h, during which time most of the 2'-deoxyguanosine dissolved. Thiophosphoryl chloride, (400μl, 4mMol), was added, and the reaction mixture stirred at room temperature for ca. 3h, after which time it was quenched by the addition of 10.0ml of 3:1 v/v methanol:pyridine, and left to stand at room temperature for a further 30 min.

The methanol and pyridine were removed *in vacuo*, and the bulk of the solvent, trimethyl phosphate, removed by addition of toluene, and decanting the supernatant from the precipitated products, three times. ^{31}P n.m.r. spectroscopy of the resulting residue indicated the presence of the desired 2'-deoxyguanosine 5'-O,O-dimethyl phosphorothioate, $dGMPS(OMe)_2$, δ_P(DMF:D_4-MeOH 3:1) +71 ppm,s.

The crude mixture was dissolved in saturated sodium hydroxide solution, and stirred at 50°C for 1.5h, after which time ^{31}P n.m.r. spectroscopy indicated complete conversion of $dGMPS(OMe)_2$ into dGMPS-OMe, which appeared as a signal at δ_P(aq.

NaOH) +57.3 ppm,s, in the spectrum of the reaction mixture.

The pH of the solution was reduced to ca. 8 by the addition of solid carbon dioxide, and the crude product purified by ion-exchange chromatography, as previously described, the product eluting at a buffer concentration of ca. 125mM TEAB. dGMPS-OMe was isolated as the triethylammonium salt, in the form of a glass, (65μmol, 3% yield).

The high-field ^{31}P n.m.r. spectrum, recorded at 121 MHz, showed two equal intensity resonances at δ_P(DMF:D_4-MeOH 3:1) +57.27 ppm,s, (Sp isomer), and δ_P(DMF:D_4-MeOH 3:1) +57.34 ppm,s,(Rp isomer). Reverse phase h.p.l.c. (isocratic elution with 1% acetonitrile in 20mM TEAB, pH 7.0, flow rate of 1.5ml/min), showed retention times of 7.6 min (Sp isomer), and 8.5 min (Rp isomer). The UV spectrum, recorded in H_2O, was found to be characteristic of the guanine chromophore, with a λmax 253nm.

2'-Deoxycytidine 5'-O-methyl Phosphorothioate ester, dCMPS-OMe.

Dry 2'-deoxycytidine, (0.5g, 2.0mMol), was suspended in 9.0ml of dry trimethyl phosphate and dissolved by cautious swirling over a Bunsen flame, the flask being protected by a calcium chloride drying tube. The resulting clear solution was cooled to 0°C in an ice-water bath, then thiophosphoryl chloride, (700ml, 6.9mMol), was added. The reaction was stirred for 24h at room temperature, then quenched by the addition of 9.0ml of 3:1 v/v methanol:pyridine, and allowed to stand for a further 30min.

Pyridine and methanol were removed *in vacuo*, and the bulk of

the trimethyl phosphate extracted into toluene, as previously described. The ^{31}P n.m.r. spectrum of the resulting mixture indicated the presence of 2'-deoxycytidine 5'-O,O-dimethyl phosphorothioate, dCMPS$(OMe)_2$, δ_P(DMF:MeOH 3:1) +71 ppm,s, which was subsequently demethylated and purified, as previously described, to yield dCMPS-OMe as the triethylammonium salt, (350μmol, 16% yield).

The high-field ^{31}P n.m.r. spectrum, recorded at 121 MHz, showed two equal intensity resonances δ_P(DMF:D_4-MeOH 3:1) +57.24 ppm,s, (Sp isomer), and δ_P(DMF:D_4-MeOH 3:1) +57.36 ppm,s, (Rp isomer). Reverse phase h.p.l.c. (isocratic elution with 20mM TEAB, pH 7.0, flow rate of 1.5ml/min), showed retention times of 9.2 min (Sp isomer), and 11.2 min (Rp isomer). The UV spectrum, recorded in H_2O, was found to be characteristic of the cytosine chromophore, with a λmax 271nm.

Thymidine 5'-O-methyl Phosphorothioate ester, TMPS-OMe

Dry thymidine, (0.48g, 2.0mMol), was suspended in 8.0ml of dry triethyl phosphate, and dissolved by swirling in a Bunsen flame, as previously described. After cooling the clear solution, as before, thiophosphoryl chloride, (400μl, 4mMol), was added, and the reaction stirred at room temperature for ca. 48h, after which time it was quenched by addition of 8.0ml 3:1 v/v methanol:pyridine. After standing for a further 30 min the methanol and pyridine were removed *in vacuo*, and the bulk of the triethyl phosphate removed with petroleum spirit, as previously described. A ^{31}P n.m.r. spectrum of the crude material indicated the presence of some thymidine 5'-O,O-dimethyl phosphorothioate,

TMPS$(OMe)_2$, δ_P(DMF:D_4-MeOH 3:1) +71ppm,s, which was subsequently demethylated and purified as previously described. TMPS-OMe was isolated as the triethylammonium salt, (50μmol, 3%).

The high-field ^{31}P n.m.r. spectrum, recorded at 121.5 MHz, showed two equal intensity resonances at δ_P(DMF:D_4-MeOH 3:1) +57.24ppm,s, (Sp isomer), and δ_P(DMF:D_4-MeOH 3:1) +57.37ppm,s, (Rp isomer). Reverse phase h.p.l.c. (isocratic elution with 1% acetonitrile in 20mM TEAB, pH 7.0, flow rate of 1.5ml/min), showed retention times of 9.0 min (Sp isomer), and 10.4 min (Rp isomer). The UV spectrum, recorded in H_2O, was found to be characteristic of the thymine chromophore, with a λmax 267nm.

PREPARATION AND PURIFICATION OF ADENOSINE 5′-PHOSPHOROTHIOATE, AMPS.

The method is based on that used in the preparation of nucleoside 5′-O,O-dimethyl phosphorothioates, except that the reaction mixture was quenched with aqueous base in place of methanol.

Dry adenosine, (0.56g, 2.1mMol), was suspended in 6.0ml of dry triethyl phosphate, and dissolved by swirling in a Bunsen flame, the flask being fitted with a calcium chloride drying tube. The solution was cooled to 0°C in an ice-water bath, then thiophosphoryl chloride, (230μl, 2.3mMol), was added. The reaction was stirred at room temperature for ca. 2h, before quenching with 6.0ml of 3:1 v/v pyridine:water.

After standing for a further 30 min the pyridine and water were removed *in vacuo*, and the triethyl phosphate extracted into 40-60°C petroleum spirit. A ^{31}P n.m.r. spectrum of the resulting product was recorded in TEAB, (100mM, pH 8), and indicated the presence of the desired product, AMPS, as a singlet at δ_P(TEAB) +45ppm,s. The crude product was separated from contaminating inorganic thiophosphate and pyridinium chloride, by ion-exchange chromatography on DEAE Sephadex ion-exchange resin, using 50-300mM TEAB, pH 8.0, (750ml of each). The product was eluted at a buffer concentration of ca. 210 mM, evaporated to dryness, and desalted by three co-evaporations with dry methanol. The AMPS isolated, (1.24mMol, 60%), was compared with authentic material by ^{31}P n.m.r. spectroscopy, h.p.l.c., and UV spectroscopy.

PARTIAL SELECTIVE ENZYMATIC HYDROLYSIS OF NUCLEOSIDE 5'-O-METHYL PHOSPHOROTHIOATE ESTERS USING SNAKE VENOM PHOSPHODIESTERASE

1.Partial Hydrolysis of the Sp diastereoisomer of 2'-deoxyadenosine 5'-O-methyl phosphorothioate ester, dAMPS-OMe

A total of 7.6μmol of dAMPS-OMe, (1:1 mixture of Sp:Rp diastereoisomers), was subjected to partial selective digestion by snake venom phosphodiesterase in three portions. Conditions described below were applied to the partial selective digestion of a sample of 1.6μmol of substrate; volumes of reagents were scaled up as appropriate for digestions involving 2μmol, and 4μmol of substrate.

The triethylammonium salt of dAMPS-OMe, (1.6μmol), was dissolved in 180μl H_2O, and combined with 1M Tris-HCl, pH 9.6, (80μl), 100mM $MgCl_2$ solution, (40μl), and snake venom phosphodiesterase as a suspension in 50% glycerol, pH 6, (100μl, 0.5 units). The reaction was conducted in a glass reacta-vial contained in a heating module set to 36°C. The pH of the incubation mixture was checked using a 1mm strip of narrow-range pH paper, and found to be ca. 9.6. The progress of the reaction was monitored by reverse phase h.p.l.c.

After 24h at 36°C, h.p.l.c. indicated that ca. 28% of the Sp diastereoisomer of dAMPS-OMe had been hydrolysed by the enzyme, as judged by integration of h.p.l.c. peaks, so that the ratio of Sp:Rp in the sample was expected to be ca. 1:1.4. The reaction mixture was diluted with water, (ca. 10ml) and stored frozen

before being combined with the other incubations, and applied to a column of DEAE Sephadex for ion-exchange separation of the partially-digested dAMPS-OMe from the products of digestion. A linear gradient was obtained by gravity mixing of 750ml each of 25mM TEAB, pH 8, and 250mM TEAB, pH 8, and dAMPS-OMe was eluted at a buffer concentration of ca 130mM TEAB. 3.9μmol of Sp-deficient dAMPS-OMe was isolated, as judged by measurement of the UV absorbance of a known volume of aqueous solution at 259nm, and 0.65μmol dAMPS, which eluted at a buffer concentration of ca. 200mM TEAB.

2.Partial Selective Hydrolysis of Sp Diastereoisomer of Adenosine 5'-O-methyl phosphorothioate Ester, AMPS-OMe

The triethylammonium salt of AMPS-OMe, (ca. 9.6μmol of a 1:1 mixture of Rp:Sp diastereoisomers), was dissolved in 400μl H_2O, and combined with 100mM $MgCl_2$ solution, (80μl), 1M Tris-HCl, pH 10, (160μl), and snake venom phosphodiesterase as a suspension in 50% glycerol, pH 6, (200μl, 1.0 units), to a total volume of 840μl. The incubation was conducted in a glass reacta-vial maintained at a temperature of 37°C in a heating module, and 10μl aliquots were removed at intervals for examination of reaction progress by reverse phase h.p.l.c.

After ca. 20h incubation, integration of the h.p.l.c. peaks for the diastereoisomers in the reaction mixture showed a change in the ratio Sp:Rp from 1:1.06 to 1:1.13. After a total of 42h the ratio had changed to 1:1.20, and after 7 days the ratio was 1:1.34, indicating significant digestion of the Sp

diastereoisomer, (ca. 20%), accompanied by the appearance of product peaks at shorter retention time, attributed to a mixture of AMPS, AMP, and adenosine, the products of digestion. The reaction was diluted with ca 10ml H_2O, and applied to a column of DEAE sephadex for ion-exchange separation of the Sp-deficient AMPS-OMe from the digestion products, as described for dAMPS-OMe. 4.1μmol of Sp-deficient AMPS-OMe were recovered, and 1.7μmol of free adenosine. No AMPS or AMP was evident in the UV-detector trace from the ion-exchange purification, indicating that desulphurisation of AMPS, the initial product of hydrolysis, to AMP, and subsequent action of phosphatases to generate free adenosine, had occurred.

3.Partial Selective Hydrolysis of 2'-Deoxycytidine 5'-O-methyl Phosphorothioate Ester, dCMPS-OMe

The triethylammonium salt of dCMPS-OMe, (7.0μmol of 1:1 mixture of Rp:Sp diastereoisomers) was dissolved in 200μl H_2O and combined with 1M Tris-HCl, pH 10, (150μl), 100mM $MgCl_2$ (80μl), and snake venom phosphodiesterase suspension, (200μl, 1 unit). The reaction was conducted in a glass reacta-vial, maintained at 37°C, as previously described.

After ca. 4 days a 10μl aliquot was withdrawn for examination by reverse-phase h.p.l.c. Integration of the h.p.l.c. peaks due to the diastereoisomers of dCMPS-OMe showed a ratio of 1:1.31 Sp:Rp, compared with a ratio of 1:1.06 in the stock solution, indicating that ca. 18% of the Sp diastereoisomer had been

digested. Additional peaks at shorter retention times, assignable to hydrolysis products, were also apparent in the h.p.l.c. trace of the incubation.

The reaction was diluted with ca. 10ml water, combined with a further small scale digestion of 2.0μmol of substrate, and the products separated by ion-exchange purification, as previously described. 6.5μmol dCMPS-OMe was eluted at a buffer concentration of ca 80mM, as judged by UV measurements at 271nm. Ca. 1.0μmol of 2'-deoxycytidine was recovered at the beginning of the concentration gradient, and a mixture of dCMPS and dCMP was eluted at a buffer concentration of ca. 120mM TEAB, thus accounting for the original total of 8.0μmol employed in the reaction.

4)Partial Selective Hydrolysis of Thymidine 5'-O-methyl Phosphorothioate Ester, TMPS-OMe

The triethylammonium salt of TMPS-OMe, (7.0μmol of a 1:1 mixture of Rp:Sp diastereoisomers) was dissolved in 150μl H_2O, and combined with 100mM $MgCl_2$ solution, (75μl), 1M Tris-HCl, pH 10, (350μl), and snake venom phosphodiesterase as a suspension in 50% glycerol, pH 6, (200μl, 1.0 units), to a total volume of 840μl. The incubation was maintained at a temperature of 37°C in a heating module, as previously described, for 4 days, after which time a 10μl aliquot was withdrawn for examination by reverse phase h.p.l.c.

The resultant h.p.l.c. trace indicated significant reduction

in the amount of Sp diastereoisomer relative to the Rp, along with the appearance of peaks at shorter retention times, attributable to the products of hydrolysis. The ratio of Sp:Rp observed was 1:1.8, as judged by h.p.l.c. integration, compared with the ratio of 1:1.0 observed prior to incubation.

The reaction was diluted, and the products separated by ion-exchange chromatography, as previously described. TMPS-OMe was eluted at a buffer concentration of ca. 80mM TEAB, ca. 4.9μmol being recovered, as judged by measurement of the absorbance at 267nm of a solution of the isolated product. There was no evidence in the ion exchange detector trace of TMPS or TMP, but ca. 1.2 μmol thymidine were recovered at the beginning of the gradient.

5)Partial Selective Hydrolysis of 2'-deoxyguanosine 5'-O-methyl Phosphorothioate Ester, dGMPS-OMe

The triethylammonium salt of dGMPS-OMe, (6.0μmol of a 1:1 mixture of Rp:Sp diastereoisomers) was dissolved in 140μl H_2O and combined with 100mM $MgCl_2$ solution, (80μl), 1M Tris-HCl, pH 10, (200μl), and snake venom phosphodiesterase as a suspension in 50% glycerol, pH 6, (200μl, 1.0 units), to a total volume of 620μl. The incubation was maintained at a temperature of 27°C in a heating module, as previously described, for 3 days, after which time a 10μl aliquot was withdrawn for examination by reverse phase h.p.l.c.

The resultant h.p.l.c. trace indicated significant reduction

in the amount of Sp diastereoisomer relative to the Rp, along with the appearance of peaks at shorter retention times, attributable to the products of hydrolysis. The ratio of Sp:Rp observed in the incubation mixture was 1:1.9, as judged by h.p.l.c. integration, compared with the ratio of 1:1.4 observed prior to incubation.

The reaction was diluted, and the products separated by ion-exchange chromatography, as previously described. 4.3μmol of dGMPS-OMe were recovered, as judged by measurement of the absorbance at 253nm of a solution of the isolated product, and ca. 0.77μmol of digestion products.

METHYLATION OF NUCLEOSIDE 5'-O-METHYL PHOSPHOROTHIOATE ESTERS WITH METHYL IODIDE TO GIVE NUCLEOSIDE 5'-S-METHYL-O-METHYL PHOSPHOROTHIOATE TRIESTERS.

Nucleoside 5'-O-methyl phosphorothioate esters can be cleanly and quantitatively methylated to the corresponding S-methyl-O-methyl triesters using excess methyl iodide in methanol at room temperature;-

Methylation of 2'-Deoxyadenosine 5'-O-methyl Phosphorothioate, dAMPS-OMe.

dAMPS-OMe, (10μmol) was dried by repeated co-evaporations of dry methanol, then dissolved in dry methanol, (0.5ml), and stirred at room temperature for 2h with methyl iodide, (75μl, 1.2mMol). Excess methyl iodide was removed in a stream of nitrogen gas, and a ^{31}P n.m.r. spectrum of the reaction mixture recorded, indicating quantitative formation of the S-methyl-O-methyl triesters of dAMPS, resonating at δ_P(MeOH) +32.07ppm,s.

High-field ^{31}P n.m.r. spectroscopy at 121.5MHz permitted resolution of two equal intensity diastereoisomeric resonances, δ_P(3:1 DMF:D_4-MeOH) +30.41ppm,s,(Rp), +30.49ppm,s,(Sp), (separation 8.7 Hz, 0.071 ppm).

The UV spectrum of the methylated material, recorded in H_2O, showed little alteration on methylation, λmax remaining at 259nm, characteristic for the adenine chromophore.

Methylation of Adenosine 5'-O-methyl Phosphorothioate Ester, AMPS-OMe.

AMPS-OMe, (25μmol) was dried by repeated co-evaporations of dry methanol, then dissolved in dry methanol, (0.5ml), and stirred at room temperature for 2.5h with methyl iodide, (200μl, 3.2mMol). Excess methyl iodide was removed in a stream of nitrogen gas, and a ^{31}P n.m.r. spectrum of the reaction recorded, indicating quantitative formation of the S-methyl-O-methyl triesters of AMPS resonating at δ_p(MeOH) +32.07ppm,s.

High-field ^{31}P n.m.r. spectroscopy at 121.5MHz permitted resolution of two equal intensity resonances due to the diastereoisomers of the S-methyl-O-methyl triester, δ_p(3:1 DMF:D_4-MeOH) +30.41ppm,s,(Rp), + 30.49ppm,s,(Sp), (separation 9.9 Hz, 0.082 ppm).

The UV spectrum, recorded in H_2O, showed little alteration on methylation, λmax remaining at 259 nm.

Methylation of 2'-Deoxyguanosine 5'-O-methyl Phosphorothioate Ester, dGMPS-OMe.

dGMPS-OMe, (13μmol) was dried by repeated co-evaporations of dry methanol, then dissolved in dry methanol, (0.5ml), and stirred at room temperature for 3h with methyl iodide, (200μl, 3.2mMol). Excess methyl iodide was removed in a stream of nitrogen gas, and a ^{31}P n.m.r. spectrum of reaction recorded, indicating quantitative formation of the S-methyl-O-methyl triesters of dGMPS resonating at δ_p(MeOH) +32.1 ppm,s.

High-field ^{31}P n.m.r. spectroscopy at 121.5MHz in 3:1 v/v DMF:D_4-MeOH permitted resolution of two equal intensity resonances due to the diastereoisomers of the S-methyl-O-methyl

triester, δ_P(3:1 DMF:D_4-MeOH) +30.42ppm,s,(Rp), +30.48ppm,s,(Sp), separation 7.0 Hz, 0.058 ppm.

The UV spectrum, recorded in in H_2O, showed a shift in λmax on methylation from 253nm, characteristic for the guanine chromophore, to 249nm.

Methylation of 2'-Deoxycytidine 5'-O-methyl Phosphorothioate Ester, dCMPS-OMe.

dCMPS-OMe, (40μmol) was dried by repeated co-evaporations of dry methanol, then dissolved in dry methanol, (0.5ml), and stirred at room temperature for 2.5h with methyl iodide, (300μl, 4.8mMol). Excess methyl iodide was removed on a stream of nitrogen gas, and a ^{31}P n.m.r. spectrum of the reaction recorded, indicating quantitative formation of the S-methyl-O-methyl triesters of dCMPS resonating at δ_P(MeOH) +32.06ppm,s.

High-field ^{31}P n.m.r. spectroscopy at 121.5MHz permitted resolution of the diastereoisomeric S-methyl-O-methyl triester to two equal intensity resonances, δ_P(3:1 DMF:D_4-MeOH) +30.41ppm,s, (Rp), + 30.50ppm,s,(Sp), (separation 11.3 Hz, 0.090 ppm).

The UV spectrum, recorded in H_2O, showed little alteration on methylation, λmax remaining at 271nm, characteristic of the cytosine chromophore.

Methylation of 2'-Deoxythymidine 5'-O-Methyl Phosphorothioate ester, TMPS-OMe.

TMPS-OMe, (15μmol) was dried by repeated co-evaporations of dry methanol, then dissolved in dry methanol, (0.5ml), and stirred at room temperature for 3h with methyl iodide, (100μl, 1.6mMol). Excess methyl iodide was removed in a stream of

nitrogen gas, and a ^{31}P n.m.r. spectrum of the reaction mixture recorded, indicating quantitative formation of the S-methyl-O-methyl triesters of TMPS resonating at δ_P(MeOH) +32.1ppm,s.

High-field ^{31}P n.m.r. spectroscopy at 121.5MHz permitted resolution of the two equal intensity resonances due to the S-methyl-O-methyl triester, δ_P(3:1 DMF:D_4-MeOH) +30.41ppm,s, (Rp), +30.50ppm,s,(Sp), (separation 10.6 Hz, 0.087 ppm).

The UV spectrum, recorded in H_2O, showed little alteration on methylation, λmax remaining at 267nm, characteristic for the thymine chromophore.

METHYLATION OF NUCLEOSIDE 5'-PHOSPHOROTHIOATES WITH DIAZOMETHANE

i)Using methanol as a solvent;

Samples of mono-triethylammonium salts of nucleoside 5'-phosphorothioates, (ca. 25μmol-30μmol), were dried to a gum by repeated co-evaporations with dry methanol, and dissolved in dry methanol, (ca. 0.5-1.0ml). Ethereal diazomethane was added, (ca. 2-5ml), the flask sealed, and the reaction stirred at room temperature for between 1.5 and 8.5h. Excess diazomethane was removed in a stream of nitrogen gas, and products examined by ^{31}P n.m.r. spectroscopy using methanol as solvent. Yields of S-methyl-O-methyl triester, as judged by integration or ^{31}P n.m.r. signals, were variable, generally in the region of 40-80%. Varying amounts of desulphurisation were observed, the extent of which increased as reaction time was increased.

Methylation of 2'-Deoxyadenosine 5'- Phosphorothioate, dAMPS, with Diazomethane in Methanol.

dAMPS, (50μmol), was dissolved in dry methanol, (1.0ml), and stirred with ethereal diazomethane, (3ml, ca. 6 equivs.), for 2.5h at room temperature. After removal of excess diazomethane, the ^{31}P n.m.r. spectrum showed the diastereoisomers of the S-methyl-O-methyl triester of dAMPS at δ_p(MeOH) +32.07ppm,s, (ca. 76% yield), in the presence of corresponding S-methyl diester δ_p(MeOH) +20.6ppm,s, (ca. 21%), and desulphurised material δ_p(MeOH) +0.8ppm,s, (ca.

3%). Increasing the reaction time to 3.5h did not improve the overall yield of triester, and resulted in an increase in the proportion of desulphurised material, (ca. 5%).

The high-field ^{31}P n.m.r. spectrum, recorded at 162MHz, showed the diastereoisomeric S-methyl-O-methyl triesters as a pair of equal-intensity resonances, δ_P(DMF) +29.61ppm,s,(Rp), +29.69ppm,s,(Sp),(separation 12.3 Hz, 0.076ppm).

Increasing the reaction time to 8.25h reduced the yield of triester, as judged by integration of ^{31}P n.m.r. resonances, (ca. 46%), and significantly increased the extent of desulphurisation, (ca. 19%).

Methylation of Adenosine 5'-Phosphorothioate, AMPS, with Diazomethane in Methanol.

AMPS, (50μmol), was dissolved in dry methanol, (1.0ml), and stirred with ethereal diazomethane, (3ml, ca. 6 equivs.), for 2.5h at room temperature. ^{31}P n.m.r. spectroscopy of the reaction mix, after removal of ethereal diazomethane by evaporation, indicated the presence of the S-methyl-O-methyl triester of AMPS δ_P(MeOH) +32.07ppm,s, (81% yield), in the presence or the corresponding S-methyl diester at δ_P(MeOH) +20.6ppm,s, (ca. 16%), and desulphurised material, δ_P(MeOH) +0.8ppm,s, (ca. 3%).

The high-field ^{31}P n.m.r. spectrum, recorded at 121.5 MHz, showed the signal due to the diastereoisomeric triesters as a pair of resonances, δ_P(3:1 DMF:D_4-MeOH) +30.40ppm,s,(Rp), +30.50ppm,s,(Sp), (separation 11.3 Hz, 0.092 ppm), broadened at the base by lower intensity resonances which are attributed to base-methylated triester. The diester signal

was also split in an intensity ratio 1:2, consistent with partial base-methylation. The UV spectrum, recorded in H_2O, showed a shift in λmax from 259nm to 261.5nm, also suggesting that a significant amount of base-methylation had occurred.

Methylation of 2'-Deoxycytidine 5'-Phosphorothioate, dCMPS, with Diazomethane in Methanol.

dCMPS, (80μmol), was stirred with excess diazomethane in methanol for 2h. The ^{31}P n.m.r. spectrum of the resulting mixture showed it to comprise of S-methyl-O-methyl triester, δ_p(MeOH) +32.3ppm,s, (ca. 56%), S-methyl diester, δ_p(MeOH) +20.6ppm,s, (ca. 21%), and desulphurised material, δ_p(MeOH) +0.8ppm,s, (ca. 19%).

Examination of the high-field ^{31}P n.m.r. spectrum, recorded at 162MHz, showed the diastereoisomers of the triester as a pair of resonances, δ_p(DMF) +29.61ppm,s,(Rp), +29.69ppm,s,(Sp), separation 12.3 Hz, 0.076ppm.

ii)Using dimethylformamide as a solvent;

Samples of mono-triethylammonium salts of nucleoside phosphorothioates, (ca. 25μmol-30μmol), were dried to a gum by repeated co-evaporations with dry methanol, and dissolved in dry DMF. Diazomethane was added as a solution in diethyl ether, (ca. 50 equivs. in ca. 5ml), the flask sealed, and the reaction stirred at room temperature for 24-48h. At the end of the reaction time excess diazomethane was removed in a stream of nitrogen gas, and the reaction mixture evaporated to dryness *in vacuo*. Products were analysed by ^{31}P n.m.r.

spectroscopy, using methanol and DMF as solvents. Yields of triester varied from ca. 75% to virtually quantitative as judged by integration in the ^{31}P n.m.r. spectrum of the reaction mixtures. Desulphurisation was not a problem under these conditions, in contrast to the results obtained when methanol was used as the solvent for the methylation reaction.

Methylation of 2'-Deoxyadenosine 5'-Phosphorothioate, dAMPS, with Diazomethane in DMF.

The mono-triethylammonium salt of dAMPS, (30μmol), was dissolved in dry DMF, (1.5ml), and stirred with ethereal diazomethane for ca. 22h at room temperature. After removal of excess diazomethane, the ^{31}P n.m.r. spectrum of the reaction was recorded and showed the S-methyl-O-methyl triesters of dAMPS at δ_P(MeOH) +32.07ppm,s, (ca. 80% yield), in the presence of the corresponding S-methyl diester, δ_P(MeOH) +20.1ppm,s, (ca. 20%). High-field ^{31}P n.m.r. analysis of the reaction mixture at 121.5MHz permitted resolution of the diastereoisomers into a pair of equal intensity resonances, δ_P(3:1 DMF:D_4-MeOH) +30.41ppm,s,(Rp), +30.49ppm,s,(Sp), (separation 9.4 Hz, 0.077 ppm,). Other small peaks in this region were attributed to base-modified triester, and amounted to ca. 10% of the total triester signal, as judged by relative peak intensities. The overall yield of triester was not significantly increased by a further 24h exposure to methylating agent. The UV spectrum, recorded in H_2O, showed no significant alteration on methylation, λmax remaining at 259nm, characteristic for the adenine chromophore.

Methylation of Adenosine 5'-Phosphorothioate, AMPS, with Diazomethane in DMF.

The mono-triethylammonium salt of AMPS, (50μmol), was dissolved in dry methanol, (1.0ml), and stirred with ethereal diazomethane for 24h at room temperature. The ^{31}P n.m.r. spectrum of the resulting mixture showed the S-methyl-O-methyl triesters of AMPS as a signal at δ_P(MeOH) +32.1ppm,s, (ca. 87% yield), and the S-methyl diester as a signal δ_P(MeOH) +20.6ppm,s, (ca. 13%). The high-field ^{31}P n.m.r. spectrum, recorded at 121.5MHz, permitted the diastereoisomeric triester signal to be resolved into a pair of resonances of equal intensity, δ_P(3:1 DMF:D_4-MeOH) +30.41ppm,s,(Rp), +30.49ppm,s,(Sp), separation 10.3 Hz, 0.084ppm. The UV spectrum, recorded in H_2O, showed no significant change on methylation.

Methylation of 2'-Deoxyguanosine 5'-Phosphorothioate, dGMPS, with Diazomethane in DMF.

The mono-triethylammonium salt of dGMPS, (20μmol), was dried, as previously described, and dissolved in 1.0ml dry DMF. Ethereal diazomethane was added and the reaction stirred at room temperature for ca. 24h. The ^{31}P n.m.r. spectrum of the reaction mixture at this stage indicated the presence of S-methyl-O-methyl triesters of dGMPS resonating at δ_P(MeOH) +31.3 ppm,s, (ca. 68% yield), and S-methyl diester, (ca. 32%), at δ_P +19.4ppm,s. However, on examination by high-field ^{31}P n.m.r. spectroscopy at 121.5MHz the triester signal was resolved into a complex set of resonances, attributable to various forms of base-methylated

product. Efforts to adapt the reaction conditions to give a simpler triester signal were unsuccessful, and methylation with diazomethane as a simple route to the S-methyl-O-methyl triester of 2'-deoxyguanosine 5'-phosphorothioate was abandoned. The UV spectrum was found to have altered on methylation, and showed a λmax of 271nm, compared with a value of 259nm prior to methylation.

Methylation of 2'-Deoxycytidine 5'-Phosphorothioate, dCMPS, with Diazomethane in DMF.

The mono-triethylammonium salt of dCMPS, (25μmol), was dissolved in dry DMF, (1.5ml), and stirred with ethereal diazomethane at room temperature for ca. 24h. After removal of excess diazomethane, the ^{31}P n.m.r. spectrum indicated an approximately quantitative yield of S-methyl-O-methyl triesters of dCMPS δ_P(MeOH) +32.07ppm,s. However, on examination of the high-field ^{31}P n.m.r. at 121.5MHz the triester signal was resolved into two pairs of overlapping resonances in an intensity ratio of 1.2:1, δ_P(3:1 DMF:D_4-MeOH) +30.39ppm,s,(N^3-methylated Rp), +30.41ppm,s, (Rp), +30.45ppm,s,(N^3-methylated Sp), +30.49ppm,s,(Sp), separations of 9.4 Hz, 0.077ppm, and 8.2 Hz, 0.067ppm, respectively, shifted on base-methylation by 0.032ppm, upfield. These overlapping pairs of resonances were assigned to the diastereoisomes of unmodified S-methyl-O-methyl triester and N^3-methyl base-methylated triester respectively. The UV spectrum was correspondingly altered, showing a shift in λmax from 271nm to 274nm.

In order to obtain a reasonable predominance of one pair of diastereoisomers, suitable for easy observation and

comparison of ^{18}O-isotope shifts in samples of labelled material, it was necessary to increase the reaction time to ca. 100h. This gave ca. 80% yield of $\underline{N}^3$-methylated triester in the presence of ca. 20% unmodified triester. The corresponding UV spectrum showed a λmax of 278nm, consistent with the literature reports for $\underline{N}^3$-methylated 2'-deoxycytidine.

Methylation of Thymidine 5'-Phosphorothioate, TMPS, with Diazomethane in DMF.

The mono-triethylammonium salt of TMPS, (26μmol), was dissolved in dry DMF, (1.0ml), and stirred at room temperature for 23h with ethereal diazomethane. After removal of excess diazomethane the ^{31}P n.m.r. spectrum was recorded and showed the $\underline{N}^3$-methyl -S-methyl-O-methyl triesters of thymidine at δ_p(MeOH) +32.00ppm, (ca. 86% yield), in the presence of the corresponding $\underline{N}^3$-methyl-S-methyl diester, at δ_p(MeOH) +20.2ppm, (ca. 14%). By increasing the reaction time to 48h a quantitative yield of base-methylated triester was obtainable.

High-field ^{31}P n.m.r. spectroscopy at 121.5MHz permitted resolution of the diastereoisomers to a pair of equal intensity resonances, δ_p(3:1 DMF:D_4-MeOH) +30.41ppm,s,(Rp), +30.50ppm,s,(Sp), separation 10.4 Hz, 0.09 ppm. The UV spectrum, recorded in H_2O, showed a slight shift in λmax on methylation, from 267nm to 265nm.

METHYLATION OF NUCLEOSIDE 5'-PHOSPHOROTHIOATES WITH DIMETHYL SULPHATE.

Methylation of 2'-Deoxyadenosine 5'-Phosphorothioate with Dimethyl Sulphate.

i)With a large excess of methylating agent;

The mono-triethylammonium salt of dAMPS, (26μmol), was dried by repeated co-evaporations of dry methanol *in vacuo*, and dissolved in dry DMF, (0.5ml). Triethylamine, (100μl), and dimethyl sulphate, (250μl, 2.63mMol), were added, and the reaction allowed to stand for 2.25h at room temperature, before removal of the bulk of the solvent *in vacuo*. No attempt was made to remove excess dimethyl sulphate, which was not sufficiently volatile to remove at the pump. The sample was prepared for ^{31}P n.m.r. analysis by addition of DMF, (200μl), D_4-MeOH, (125μl), and triethylamine, (25μl), to a final volume of ca. 600μl, and the resulting ^{31}P n.m.r. spectrum showed a single peak δ_P(3:1 DMF:D_4-MeOH) +30.25ppm,s, attributed to the S-methyl-O-methyl triester of $N^{1,3}$ dimethyl dAMPS.

A high-field ^{31}P n.m.r. spectrum was recorded at 121.5MHz within three hours of sample preparation, and showed the triester signal as a pair of equal-intensity diastereoisomeric resonances, δ_P(3:1 DMF:D_4-MeOH) +30.21ppm,s,(Rp), +30.29ppm,s,(Sp), separation 9.8 Hz, 0.081ppm, (ca. 66% yield), in the presence of an overlapping pair of resonances, (ca. 7%), showing a similar separation.

Also apparent in the spectrum at this stage was a set of four equal intensity resonances upfield of the major triester signal by 0.27 ppm, attributed to de-purinated S-methyl-O-methyl triester, (ca. 27%).

On re-recording the ^{31}P n.m.r. spectrum after the sample had been standing for 12h at room temperature, the peaks due to de-purinated material were found to have increased in intensity at the expense of the original triester signal, (ca. 60%), and after 7 days the ^{31}P n.m.r. spectrum showed depurination to be complete.

ii)With a smaller excess of methylating agent;

The mono-triethylammonium salt of dAMPS, (25μmol), was dried, as before, and dissolved in dry DMF, (300μl), transfered to a 5mm n.m.r. tube, and triethylamine, (25μl), and dimethyl sulphate, (250μl,2.63mMol), added. The progress of the reaction was monitored by ^{31}P n.m.r. spectroscopy. After 15 min the ^{31}P n.m.r. spectrum of the reaction mixture showed S-methyl-O-methyl triester at $\delta_P(DMF:Me_2SO_4)$ +30.05ppm,s, (ca. 10% yield), in the presence of the corresponding S-methyl diester at $\delta_P(DMF:Me_2SO_4)$ +15.5ppm,s, (ca. 90%). After 1h a larger proportion of triester was observed, (ca. 40%), and after 16h the reaction was found to be complete.

The sample was prepared for high-field ^{31}P n.m.r. analysis by addition of DMF, (100μl), and D_4-MeOH, (125μl), to give a final volume of 700μl. The resulting spectrum, recorded at 121.5MHz, showed a major pair of diastereoisomeric resonances, $\delta_P(DMF:D_4\text{-}MeOH:Me_2SO_4)$ +30.21ppm,s,(Rp), +30.29ppm,s,(Sp), (separation 9.8 Hz, 0.080 ppm), in the

presence of the previously observed minor peaks, and a small amount of de-purinated material, (ca.25%). On re-recording the spectrum, after the sample had been standing at room temperature for 7 days, it was found to be unaltered; further depurination of the triester had not occured.

Methylation of Adenosine 5'-Phosphorothioate, dAMPS, with Dimethyl Sulphate.

The mono-triethylammonium salt of AMPS, (30μmol), was dried, as before, and dissolved in dry DMF, (0.5ml), to which was added triethylamine, (100μl), and dimethyl sulphate, (250μl, 2.63mMol). The reaction was allowed to run at room temperature for 1.5h, after which time a ^{31}P n.m.r. spectrum of the reaction mixture, recorded at 60MHz, showed a single peak at δ_P(DMF:Me_2SO_4) +30.05ppm,s. The sample was pumped to remove the bulk of the solvent, then prepared for high-field ^{31}P n.m.r. analysis by addition of DMF, (200μl), and D_4-MeOH, (125μl), to give a final volume of 700μl. The resulting spectrum,recorded at 121.5MHz, showed the triester signal as a pair of equal-intensity diastereoisomeric resonances, δ_P(DMF:D_4-MeOH:Me_2SO_4) +30.19ppm,s,(Rp), +30.31ppm,s,(Sp), (separation 14.1 Hz, 0.116ppm). No change was observed on re-recording the spectrum after the sample had been standing at room temperature for several weeks.

Methylation of 2'-Deoxyguanosine 5'-Phosphorothioate, dGMPS, with Dimethyl Sulphate.

The mono-triethylammonium salt of dGMPS, (20μmol), was dried, as previously described, and dissolved in dry DMF, (0.5ml), to which was added triethylamine, (50μl), and

dimethyl sulphate, (200μl, 2.1mMol). The reaction was stirred overnight at room temperature, then examined by ^{31}P n.m.r. spectroscopy in a 10mm n.m.r. tube, the sample volume being increased by the addition of methanol, (300μl). The resulting spectrum showed a single peak δ_P(DMF:MeOH:Me_2SO_4) +30.25ppm,s, due to the S-methyl--O-methyl triesters of N^7-methyl dGMPS.

The sample was prepared for high-field ^{31}P n.m.r. analysis by pumping to remove the bulk of the solvent, addition of D_4-MeOH, (125μl), and DMF to give a total sample volume of 700μl. The resulting ^{31}P n.m.r. spectrum showed the diastereoisomeric triester resonances, (70% yield), δ_P(DMF:D_4-MeOH:Me_2SO_4) +30.20ppm,s,(Rp), +30.30ppm,s,(Sp), (separation 11.9Hz, 0.098ppm), in the presence of the signals characteristic of depurinated material, (ca. 30%).
On re-recording the high-field ^{31}P n.m.r. spectrum after 6 days at room temperature, the triester was found to have undergone complete de-purination.

This experiment was repeated on a small scale, and the high-field ^{31}P n.m.r. spectrum was accumulated overnight. The 'average' spectrum obtained from overnight accumulation showed ca. 60% depurination.

Methylation of 2'-Deoxycytidine 5'-Phosphorothioate, dCMPS, with Dimethyl Sulphate.

The Mono-triethylammonium salt of dCMPS, (28μmol), was dried, dissolved in dry DMF, (0.8ml), and transferred to a 10mm n.m.r. tube. Triethylamine, (100μl), and dimethyl sulphate, (250μl, 2.63mMol), were added and the progress of the reaction monitored by ^{31}P n.m.r. spectroscopy. After 10

min the spectrum, recorded at 60MHz, showed a peak due to S-methyl-O-methyl triester, δ_P(DMF:Me_2SO_4) +30.05ppm,s, (ca. 70% yield), in the presence of the S-methyl diester at δ_P+16.5ppm. After 1.5h the re-recorded ^{31}P n.m.r. spectrum indicated that the reaction was ca. 90% complete.

The sample volume was reduced, by removal of DMF *in vacuo*, and the sample prepared for high-field ^{31}P n.m.r. analysis by addition of D_4-MeOH, (125μl), and DMF to give a total volume of 600μl. The resulting spectrum showed the triester signal to be composed of two overlapping sets of resonances of ca. equal intensity, as observed for methylations using diazomethane in DMF. The corresponding UV spectrum, recorded in H_2O, showed a λmax of 274nm, intermediate between the values expected for un-modified base, (271nm), and N^3-methylated base, (278nm).

In order to obtain complete N^3 methylation, and hence a clean pair or triester resonances, suitable for the measurement of isotope shifts, it was necessary to methylate first in the absence of triethylamine, as follows: dCMPS, (19μmol), was dissolved in dry DMF, (0.5ml), to which was added dimethyl sulphate, (200μl, 2.1mMol), and the reaction was allowed to stand at room temperature for 30 min. An aliquot was withdrawn and examined by UV spectroscopy, and showed a λmax of 278nm, indicating complete base-methylation at N^3. The corresponding ^{31}P n.m.r. spectrum, recorded at 60MHz, showed a single peak at δ_P(DMF:Me_2SO_4) +21.3ppm,s, due to N^3-methyl -S-methyl diester. Triethylamine, (75μl) was then added to the n.m.r. tube and after 15 min a signal due to the S-methyl-O-methyl triesters of N^3-methyl dCMPS was

observed δ_P(DMF:Me_2SO_4) +30.25ppm,s, (76% yield), the reaction being complete after 1h, as judged by ^{31}P n.m.r. spectroscopy.

High-field ^{31}P n.m.r. spectroscopy at 121.5MHz showed the triester signal as a clean pair of equal intensity resonances, δ_P(DMF:D_4-MeOH:Me_2SO_4) +30.20ppm,s,(Rp), +30.30ppm,s,(Sp), separation 12.4Hz, 0.10ppm.

Methylation of Thymidine 5'-Phosphorothioate, TMPS, with Dimethyl Sulphate.

The mono-triethylammonium salt of TMPS, (9μmol), was dried, as before, and dissolved in dry DMF, (250μl), to which was added triethylamine, (50μl), and dimethyl sulphate, (100μl, 1.05mMol), and the reaction was allowed to run at room temperature for 3h, after which time methylation was judged by ^{31}P n.m.r. spectroscopy to be complete. The sample was prepared for high-field ^{31}P n.m.r. analysis by the addition of DMF, (50μl), and D_4-methanol, (125μl).

The resulting high-field ^{31}P n.m.r. spectrum showed a clean pair of equal intensity diastereoisomeric resonances, δ_P(DMF:D_4-MeOH,Me_2SO_4) +30.20ppm,s,(Rp), +30.30ppm,s,(Sp), separation 12.5 Hz, 0.10 ppm due to the S-methyl-O-methyl triester of TMPS.

METHYLATION OF PARTIALLY HYDROLYSED NUCLEOSIDE 5'-O-METHYL PHOSPHOROTHIOATES TO GENERATE SAMPLES OF NUCLEOSIDE 5'-S-METHYL-O-METHYL PHOSPHOROTHIOATE TRIESTERS FOR CONFIGURATIONAL ASSIGNMENT:

1.1 Generation of Sp deficient sample of diastereoisomers of dAMPSMeOMe and configurational assignment by ^{31}P n.m.r. spectroscopy:

The Sp deficient sample of dAMPS-OMe, (3.9μmol), isolated from the incubation with snake venom phosphodiesterase, was freed from excess TEAB and dried to a gum by repeated co-evaporations of dry methanol. The residue was dissolved in 3:1 v/v DMF: D_4-MeOH (500μl), and examined by high field ^{31}P n.m.r. spectroscopy. The resulting resonances were broad, in spite of attempts to maximise resolution; δ_P(DMF:D_4-MeOH) +57.24ppm,s,(Sp), +57.37ppm,s,(Rp), the ratio of Sp:Rp 1:1.36, as judged by ^{31}P n.m.r. integration.

The n.m.r. sample was evaporated to dryness at the pump and dissolved in dry methanol (500μl). Excess methyl iodide was added, (35μl, 560μmol), and the reaction was sealed and stirred magnetically at room temperature for ca. 1.75h. After this time the reaction was evaporated to dryness *in vacuo*, and the sample prepared for ^{31}P n.m.r. analysis by dissolving in 3:1 v/v DMF:D_4-MeOH, (500μl). The resulting high-field ^{31}P n.m.r spectrum, which was accumulated overnight, showed signals due to the diastereoisomers of the S-methyl-O-methyl triester, δ_P(DMF:D_4-MeOH) +29.61ppm,s,(Rp), +29.69ppm,s,(Sp), in the presence of unreacted O-methyl diester. The signals due to the unreacted diester were broad and poorly resolved whilst those due to the triester were sharp and well resolved, both pairs of signals showing a ratio of Sp:Rp of ca. 1:1.36. It was estimated

from ^{31}P n.m.r. integration that ca. 40% of the diester remained.

1.2. Configurational assignment of base-methylated dAMPSMeOMe;

Part of the sample of Sp-deficient dAMPS-OMe, (1.6μmol), was evaporated to dryness *in vacuo* and dissolved in dry DMF, (200μl), to which was added dimethyl sulphate, (20μl, 210μmol). The homogenous reaction was allowed to stand at room temperature for ca. 4h before preparation for ^{31}P n.m.r. analysis by addition of further DMF, (155μl), and D_4-MeOH, (125μl) to give a final volume of ca. 500μl, ca. 3:1 v/v DMF:D_4-MeOH.

The resulting ^{31}P n.m.r spectrum, recorded at 121.5 MHz and accumulated over several hours, showed a pair of resonances of similar shift, separation, and relative intensity as the original sample of dAMPSMeOMe triester, indicating that methylation at the purine base does not affect configurational assignments. Also observed in the spectrum were signals characteristic of depurinated S-methyl-O-methyl phosphorothioate triester; these increased in intensity at the expense of the original signal until, after ca. 12h, depurination was complete.

2.1 Generation of Sp deficient sample of diastereoisomers of AMPSMeOMe and configurational assignment by ^{31}P n.m.r. spectroscopy:

The Sp deficient sample of AMPS-OMe, (4.0μmol), isolated from the incubation with snake venom phosphodiesterase, was freed from excess TEAB and dried to a gum by repeated co-evaporations of dry methanol. The residue was dissolved in dry methanol (500μl), and excess methyl iodide added, (35μl, 560μmol). The reaction was sealed and stirred magnetically at room temperature for ca. 2h after which time it was evaporated to dryness *in vacuo*, and the

sample prepared for ^{31}P n.m.r. analysis by dissolving in 3:1 v/v DMF:D_4-MeOH, (500μl). The resulting high-field ^{31}P n.m.r spectrum, which was accumulated overnight, showed signals due to the diastereoisomers of the S-methyl-O-methyl triester, δ_P(DMF:D_4-MeOH) +30.41ppm,s,(Rp),+30.49ppm,s,(Sp), as the only resonances, indicating that methylation of the diester to the corresponding triester had proceeded cleanly and quantitatively. The intensity ratio of Sp:Rp was ca. 1:1.6, as judged by integration.

2.2. Configurational assignment of base-methylated AMPSMeOMe;

Part of the sample of Sp-deficient AMPS-OMe, (1.6μmol), was evaporated to dryness in vacuo and dissolved in dry DMF, (100μl), to which was added triethylamine, (25μl), and dimethyl sulphate, (75μl, 780μmol). The homogenous reaction was allowed to stand at room temperature for ca. 5h before preparation for ^{31}P n.m.r. analysis by addition of further DMF, (200μl), and D_4-MeOH, (125μl) to give a final volume of ca. 500μl, ca. 3:1 v/v DMF:D_4-MeOH.

The resulting ^{31}P n.m.r spectrum, recorded at 121.5 MHz and accumulated over several hours, showed a pair of resonances of similar shift, separation, and relative intensities as the original sample of AMPSMeOMe triester, indicating that methylation at the purine base does not affect configurational assignments.

3.1 Generation of Sp deficient sample of diastereoisomers of dCMPSMeOMe and configurational assignment by ^{31}P n.m.r. spectroscopy:

The Sp deficient sample of dCMPS-OMe, (6.5μmol), isolated from

the incubation with snake venom phosphodiesterase, was freed from excess TEAB and dried to a gum by repeated co-evaporations of dry methanol. The residue was dissolved in dry methanol (500μl), and excess methyl iodide added, (50μl, 800μmol). The reaction was sealed and stirred magnetically at room temperature for ca. 3.5h after which time it was evaporated to dryness *in vacuo*, and the sample prepared for ^{31}P n.m.r. analysis by dissolving in 3:1 v/v DMF:D_4-MeOH, (500μl). The resulting high-field ^{31}P n.m.r spectrum, which was accumulated overnight, showed signals due to the diastereoisomers of the S-methyl-O-methyl triester, δ_P(DMF:D_4-MeOH) +30.41ppm,s,(Rp), +30.49ppm,s,(Sp), as the only resonances, ie. methylation of the diester had proceded cleanly and quantitatively. The intensity ratio of Sp:Rp was ca. 1:1.65, as judged by integration.

3.2. Configurational assignment of base-methylated dCMPSMeOMe;

Part of the sample of Sp-deficient dCMPS-OMe, (2.6μmol), was evaporated to dryness *in vacuo* and dissolved in dry DMF, (200μl), to which was added ethereal diazomethane (ca. 1.5ml, 500μmol). The reaction was stirred magnetically at room temperature for ca. 12h, after which time excess diazomethane was removed on a stream of nitrogen gas and the reaction mixture evaporated to dryness *in vacuo*. The sample was prepared for ^{31}P n.m.r. analysis by dissolving in 500μl of 3:1 v/v DMF:D_4-MeOH.

The resulting ^{31}P n.m.r spectrum, recorded at 121.5 MHz and accumulated overnight showed two overlapping pairs of resonances each pair having an intensity ratio of ca. 1:1.4 Sp:Rp. The ratio of the pairs of resonances to each other was ca. 1:1.4; in both pairs the lower intensity Sp diastereoisomeric resonance was downfield of the corresponding Rp resonance, so that the

configurational assignments were shown to be unaffected by base-methylation. Observed shifts were δ_P(DMF:d_4-MeOH) +30.39ppm,s,($\underline{N}^3$-Me Rp), +30.41ppm,s,(Rp), +30.45ppm,s,($\underline{N}^3$-Me Sp), +30.49ppm,s, (Sp). The separation of the pairs of resonances were 0.075ppm, 9.12Hz, and 0.067ppm, 8.16Hz, the resonances being shifted upfield by ca. 0.03ppm as a result of base-methylation.

4.1 Generation of Sp deficient sample of diastereoisomers of TMPSMeOMe and configurational assignment by ^{31}P n.m.r. spectroscopy:

The Sp deficient sample of TMPS-OMe, (4.9μmol), isolated from the partial hydrolysis with snake venom phosphodiesterase, was freed from excess TEAB and dried to a gum by repeated co-evaporations of dry methanol. The residue was dissolved in dry methanol (500μl), and excess methyl iodide added, (40μl, 640μmol). The reaction was sealed and stirred magnetically at room temperature for ca. 3.5h after which time it was evaporated to dryness *in vacuo*, and the sample prepared for ^{31}P n.m.r. analysis by dissolving in 3:1 v/v DMF:D_4-MeOH, (500μl). The resulting high-field ^{31}P n.m.r spectrum, which was accumulated overnight, showed signals due to the diastereoisomers of the $\underline{S}$-methyl-$\underline{O}$-methyl triester of TMPS, δ_P(DMF:D_4-MeOH) +30.41ppm,s,(Rp), +30.50ppm,s,(Sp), as the only resonances, ie. methylation of the diester was complete. The intensity ratio of Sp:Rp was ca. 1:1.65, as judged by integration.

4.2. Configurational assignment of base-methylated TMPSMeOMe;

Part of the sample of Sp-deficient TMPS-OMe, (2.0μmol), was evaporated to dryness *in vacuo* and dissolved in dry DMF, (200μl), to which was added ethereal diazomethane (ca. 2.0ml, ca.

500μmol). The reaction was sealed and stirred magnetically at room temperature for ca. 12h, after which time excess diazomethane was removed on a stream of nitrogen gas and the reaction mixture evaporated to dryness *in vacuo*. The sample was prepared for ^{31}P n.m.r. analysis by dissolving in 500μl of 3:1 v/v DMF:D_4-MeOH.

The resulting ^{31}P n.m.r spectrum, recorded at 121.5 MHz and accumulated overnight showed a clean pair of diastereoisomeric resonances as the only signals, δ_P(DMF:D_4-MeOH) +30.42ppm,s,(Rp), +30.51ppm,s,(Sp), in a ratio Sp:Rp 1:1.6, the lower intensity Sp resonance was again downfield of the corresponding Rp resonance, so that the configurational assignments were shown to be unaffected by base-methylation. The separation of the resonances was 0.086ppm, 10.Hz.

4.3 Further Check on the effect of base-methylation on configurational assignments of TMPSMeOMe:

A further 2.0μmol of the original assymmetric sample of TMPSMeOMe was combined with an approximately equivalent sample of TMPSMeOMe with 1:1 ratio of Sp:Rp, prepared by methyl iodide methylation of TMPS-OMe, as previously described. The resulting sample was expected to show a reduced ratio of Sp:Rp but an improved signal:noise ratio in the ^{31}P n.m.r. spectrum. The sample was divided in half, and one half was subjected to treatment with excess diazomethane using conditions described above, in order to affect base methylation. The samples were then recombined and examined by ^{31}P n.m.r spectroscopy at 121.5MHz. The resulting spectrum showed two pairs of overlapping resonances due to the diastereoisomers of the base-methylated and

non base-methylated material in approximately equal amounts. The separation on the upfield pair of resonances was 0.087ppm, 10.6Hz, and that on the downfield pair was 0.088ppm, 10.7Hz, base-methylation being accompanied by a shift of 0.016ppm. Within each pair of resonances the ratio of Sp:Rp was ca. 1:1.2, and in both cases the resonance assigned to the Sp diastereoisomer resonated downfield of that due to the Rp diastereoisomer.

5.1 Generation of Sp deficient sample of diastereoisomers of dGMPSMeOMe and configurational assignment by ^{31}P n.m.r. spectroscopy:

The Sp deficient sample of dGMPS-OMe, isolated from the partial hydrolysis with snake venom phosphodiesterase, was freed from excess TEAB and dried to a gum by repeated co-evaporations of dry methanol. Part of the resulting sample, (2.1μmol), was dissolved in dry DMF (100μl), to which was added triethylamine, (20μl), and dimethyl sulphate, (45μl, 470μmol). The homogenous reaction mixture was allowed to stand overnight at room temperature, then the sample prepared for ^{31}P n.m.r. analysis by addition of DMF, (210μl), and D_4-MeOH, (125μl), to give a total volume of ca. 500μl of ca. 3:1 v/v DMF:D_4-MeOH. The resulting high-field ^{31}P n.m.r spectrum, accumulated overnight, showed signals due to the diastereoisomers of the base-methylated S-methyl-O-methyl triester, δ_P(DMF:D_4-MeOH,Me_2SO_4) +30.20ppm,s,(Rp), +30.30ppm,s,(Sp), separation 0.098ppm, 11.9Hz, in the presence of signals characteristic of depurinated S-methyl-O-methyl triester, these latter signals representing ca. 60% of the total resonance intensity.

6.1 Configurational assignment of the depurinated S-methyl-O-methyl triester:

Samples of methyl sulphate methylated dAMPSMeOMe and dGMPSMeOMe were allowed to undergo complete depurination and the ^{31}P n.m.r spectra re-recorded. The resulting spectra showed depurination to be accompanied by an upfield shift of ca 0.5ppm, the signals due to the diastereoisomers of each epimer appearing as a pair of overlapping resonances. Within each pair the Sp distereoisomer was assignable as the lower intensity resonance, the α and β epimers being formed in equal amount, as indicated by the two Rp diastereoisomers and the two Sp diastereoisomers showing equal intensity resonances. Shifts were $\delta P(DMF:D_4\text{-}MeOH:Me_2SO_4)$ +29.51ppm,s,(Rp), +29.56ppm,s,(Sp), +29.75ppm,s,(Rp), +29.77ppm,s,(Sp). The epimers were not assigned.

METHYLATION OF A SAMPLE OF Sp [^{16}O,^{18}O]dAMPS AS A VALIDATION OF THE ANALYSIS METHOD:

A sample of Sp [^{16}O,^{18}O]dAMPS isolated from the stereospecific cleavage of Sp 5'-O-(2'-deoxyadenosyl)-3'-O-thymidyl phorophorothioate in ^{18}O-labelled water by Nuclease P1 was combined with unlabelled dAMPS to give a 5μmol sample of phosphorothioate with 40% isotope enrichment. The sample was dried to a gum by three co-evaporations of dry methanol and dissolved in dry methanol, (500μl), to which was added ethereal diazomethane, (ca.3ml, 200-300μmol). The reaction was sealed and stirred at room temperature for 2.5h, after which time excess diazomethane was removed in a stream of nitrogen gas. The reaction mixture was evaporated to dryness *in vacuo*, and dissolved in DMF:D_4MeOH (3:1 v/v), to which was added a few crystals of 8-hydroxyquinoline as a chelating agent, for high-field ^{31}P n.m.r. analysis.

The resulting high-field ^{31}P n.m.r. spectrum, recorded at 121.5 MHz, showed the signal due to the S-methyl-O-methyl phosphorothioate triester δ_P(DMF:D_4-MeOH) +30.41ppm,s,(Rp), +30.49ppm,s,(Sp), in the presence of S-methyl phosphorothioate diester, δ_P(DMF:D_4-MeOH) +16.5 ppm,s. The separation between the diastereoisomers of the triester was 0.071ppm, 8.7 Hz, and the $^{31}P(^{18}O)$ shift on the downfield Sp resonance was 2.3Hz, 0.019ppm, whilst that on the upfield Rp resonance was 5.7Hz, 0.047ppm.

STEREOCHEMICAL ANALYSIS OF THE HYDROLYSIS REACTION CATALYSED BY BOVINE INTESTINAL MUCOSA PHOSPHODIESTERASE

Initial Digestion Experiment Showing Sp AMPS-OMe to be a Substrate.

AMPS-OMe, (6.0μmol of a 1:1 mixture of Sp:Rp isomers), was dried to a gum in a reacta-vial, and dissolved in H_2O, (200μl), to which was added Tris-HCl, (45μl, 1.0M, pH 8.2), and enzyme suspension, (100μl, ca. 1.6 units), to give a digestion mixture pH 8, 100mM in Tris-HCl, 9mM in Sp AMPS-OMe. The reaction was incubated at 30°C in a reacta-vial heating block, and aliquots removed for examination by h.p.l.c. After 17h, integration of h.p.l.c. peaks indicated a reduction of ca. 44% in the Sp diastereoisomer of AMPS-OMe, relative to the Rp diastereoisomer, (the diastereoisomers having been previously assigned under these h.p.l.c. conditions using snake venom phosphodiesterase). After 40h ca. 90% digestion of the Sp diastereoisomer was observed. The initial digestion product was identified by co-injection with authentic material to be AMPS, which desulphurised to AMP. This in turn was a substrate for phosphatase associated with the enzyme, resulting in the release of free adenosine, identified by co-injection with authentic material. N.B. Ratios of Sp:Rp were examined using a mobile phase of 6% acetonitrile in 50mM TEAB, pH 6.8, which gave retention times of 9.1 min (Sp isomer), 11.8 min (Rp isomer), with a flow rate of 1.3 ml/min. Under these conditions AMP and AMPS co-eluted, with retention time of 3.3 min. In order to examine product ratios a mobile phase of 2.4%

acetonitrile was employed, giving retention times of 4.3 min for AMP, 5.3 min for AMPS.

Incubation conditions were varied with respect to temperature, [substrate], [enzyme], and ionic strength, in order to achieve an adequate yield of AMPS for stereochemical analysis. It was found that the rate of desulphurisation was reduced relative to the rate of digestion when lower temperatures, and larger amounts of enzyme were employed.

Digestion of Sp Adenosine-5'-O-methyl Phosphorothioate Ester in Presence of ^{18}O-labelled Water

AMPS-OMe, (16μmol of a 1:1 mixture of Sp:Rp diastereoisomers), was equally divided between four Eppendorf tubes; Tris-HCl buffer was added to each, (20μl, 1.0M, pH 8.2), and water removed on a Speed Vac Concentrator. To the resulting gums in each tube was added ^{18}O-labelled water, (100μl of 98 atom%), and enzyme suspension, (80μl, ca. 1.25 units). Digestions were performed at an ambient temperature of ca. 14°C, aliquots being removed at intervals for h.p.l.c. analysis.

After 2 days the yield of AMPS was judged to be optimal, and the incubations were combined and halted by freezing. Unlabelled AMPS, (0.8μmol), was added, and the resulting mixture of AMPS and ^{18}O-AMPS applied to a column of DEAE-Sephadex ion-exchange resin, the digestion products being separated using a linear gradient of 0-300mM TEAB, pH 8.0, (500ml of each). Ca. 70 fractions were collected, fractions 13-18 containing Sp-deficient AMPS-OMe, (10.2μmol), fractions 35-40 containing the mixture of

[^{16}O,^{18}O]AMPS, and unlabelled AMPS, (2.0μmol), identified by co-injection with authentic material on h.p.l.c.

Methylation of [^{16}O,^{18}O]AMPS of Unknown Configuration With Dimethyl Sulphate.

The [^{16}O,^{18}O]AMPS, (2.0μmol, ca. 34% enriched), isolated from the digestion mixture was dried by repeated co-evaporations of dry methanol, and dissolved in dry DMF, (100μl), to which was added triethylamine, (20μl), and dimethyl sulphate, (50μl). The reaction was allowed to stand at room temperature for 2h, then reduced to a minimum volume by evaporation *in vacuo*, and prepared for ^{31}P n.m.r. analysis by addition of DMF, (200μl), and D_4-methanol, (125μl), to give a sample of composition ca. 3:1 v/v DMF:D_4-MeOH.

The resulting ^{31}P n.m.r. spectrum showed the triester signals δ_P(DMF:Me_2SO_4,D_4-MeOH) +30.39ppm,s,(Rp), δ_P(DMF:Me_2SO_4,D_4-MeOH) +30.51ppm,s,(Sp), to be the only phosphorus-containing material. Two isotope shifts were observable on the diastereoisomeric resonances, the ratio of labelled to unlabelled material being 2.2:1, as measured from intensities of ^{31}P n.m.r. resonances, consistent with an ^{18}O-enrichment of ca. 32%. A small shift, (0.020ppm, 2.4 Hz), was observed on the Sp resonance, and a larger shift, (0.045ppm, 5.5 Hz), the diastereoisomeric separation of the unlabelled resonances being 14.8 Hz, 0.12 ppm.

Determination of an Approximate Km Value for Sp AMPS-OMe.

The rate of digestion of Sp AMPS-OMe by the enzyme in the presence of the Rp isomer, (a 1:1 mixture of diastereoisomers was used), was determined over a substrate concentration range of 0.065-0.500 mM. Reactions were conducted in Eppendorf tubes maintained at 23°C in a reacta-vial heating block, in a volume of 100μl buffered at pH 8.2 by 100mM Tris-HCl, and containing 0.03mg, 0.02 units of enzyme suspension. Reactions were monitored by withdrawal of samples and examination by reverse-phase h.p.l.c. The extent of hydrolysis was measured by the reduction in the amount of the Sp diastereoisomer relative to the Rp diastereoisomer, as judged by h.p.l.c. integration. Initial rates of hydrolysis at different substrate concentrations were calculated by monitoring the first 10% of the reaction. Approximate values for Km of 67μM, and for λmax of 1.3μmol min^{-1} $(mg\ protein)^{-1}$ were obtained by Least Squares Analysis of a Lineweaver-Burk plot.

CONFIGURATIONAL ANALYSIS OF A SAMPLE OF [^{16}O,^{18}O]TMPS OF UNKNOWN CONFIGURATION AT PHOSPHORUS

The sample of [^{16}O,^{18}O]TMPS, (ca.1.5μmol), isolated from the hydrolysis of d[Tp(S)A] in ^{18}O-labelled water by mung bean nuclease, was combined with an approximately equivalent amount of unlabelled TMPS to give a total sample of ca. 3.4μmol with ca.45% isotope enrichment. This sample was dried to a gum by repeated co-evaporations of dry methanol, and dissolved in dry DMF, (200μl). Ethereal diazomethane was added, (1-1.5ml, ca. 200-300 μmol), and the reaction was sealed and stirred at room temperature for 36h, after which time excess diazomethane was removed on a stream of nitrogen gas. The methanol solution was evaporated to dryness, and the resulting sample dissolved in DMF:D_4MeOH (500μl, 3:1 v/v), for analysis by high-field ^{31}P n.m.r. spectroscopy.

The resulting ^{31}P n.m.r. recorded at 121.5 MHz, showed a quantitative yield of diastereoisomers of the S-methyl-O-methyl phosphorothioate triesters of TMPS as a clean pair of resonances, δ_P(DMF:D_4-MeOH) +30.41ppm,s,(Rp), +30.50ppm,s,(Sp), each showing a $^{31}P(^{18}O)$ isotope-shifted resonance due to the corresponding diastereoisomer of the [^{16}O,^{18}O]-S-methyl-O-methyl phosphorothioate triesters. The shift on the upfield resonance due to the Sp diastereoisomer was the smaller at 2.5 Hz, 0.021ppm, whilst that on the downfield Rp diastereoisomer was 5.7 Hz, 0.047ppm.

STEREOCHEMICAL ANALYSIS OF IODINE MEDIATED DESULPHURISATION

Desulphurisation of Sp 5'-O-[2'-deoxyadenosyl]-3'-O-thymidyl Phosphorothioate, Sp d[Tp(S)A]

Sp d[Tp(S)A], (16μmol), was dissolved in H_2O, (100μl), and a solution of iodine, (60mg, 160μmol, in pyridine, 250μl), added. The reaction mixture was transferred to a 5mm n.m.r. tube, and its progress monitored by ^{31}P n.m.r. spectroscopy. When desulphurisation was judged to be complete, as indicated by the disappearance of the ^{31}P n.m.r. resonance due to d[Tp(S)A], $\delta P(H_2O$:pyridine) +55.5 ppm,s, and concomitant increase in that due to d[TpA], $\delta_P(H_2O$,pyridine) +0.8 ppm,s. The reaction was diluted with water, and the bulk of the iodine extracted by washing with diethyl ether. The aqueous layers were combined, and evaporated to dryness, *in vacuo*.

Preparation of the Methyl Esters of d[TpA], and Sp ^{18}O-d[TpA]

d[TpA], (8.0μmol), obtained as described above, was dissolved in H_2O, (2.0ml), to which was added Sp ^{18}O-d[TpA], (5.2μmol), obtained from the stereospecific desulphurisation of Rp d[Tp(S)A] using N-bromosuccinimide in ^{18}O-labelled H_2O. The solution was stirred with K^+ Dowex ion-exchange resin, (1.0ml), for 20 min at room temperature, then the resin filtered off at the pump, and washed with ca. 30ml of H_2O.

The filtrate was reduced to a volume of ca. 3 ml by evaporation *in vacuo*, and 18-crown-6, (8.8 mg, 33μmol), added.

The solution was then evaporated to dryness, and co-evaporated twice with dry DMF, before being left on the pump for ca. 1h. The sample was then dissolved in dry DMSO, (400μl), and stirred overnight with methyl iodide, (100μl, 1.6 mMol).

Excess methyl iodide was removed on a stream of nitrogen gas, and the sample prepared for high-field ^{31}P n.m.r. analysis by addition of 8-hydroxyquinolene, (few mgs), and D_6-DMSO, (200μl), and filtering. The resulting spectrum, figure 4.1, shows the two resonances due to the diastereoisomers of the unlabelled 5'-O-(2'-deoxy N^1-methyladenosyl)-3'-O-thymidyl-O-methyl phosphate triesters, $\delta_P(D_6\text{-DMSO})$ +0.88 ppm,s,(Sp), and $\delta_P(D_6\text{-DMSO})$ +0.80 ppm,s,(Rp), with two isotope shifts, of 1.95Hz, (0.016 ppm), and 4.97Hz, (0.041 ppm), respectively. Hence the diastereoisomers of the methyl esters were assigned to corresponding resonances from the knowledge of the configurations of the ^{18}O-labelled material.

Desulphurisation of Sp d[Tp(S)A] in the Presence of ^{18}O-Labelled Water, by Iodine in Pyridine.

Sp-d[Tp(S)A], (8.0μmol), was dried by repeated co-evaporations of dry DMF, and left on the pump for ca. 1h, before dissolving in ^{18}O-labelled H_2O, (100μl), to which was added a solution of iodine, (20 mg, 80μmol), in dry pyridine, (300μl). The reaction mixture was transferred to a 5mm n.m.r. tube, and its progress monitored by ^{31}P n.m.r. spectroscopy.

After ca. 1.5h, when desulphurisation was judged to be

complete, the reaction was diluted with ca. 5ml of H_2O, and the excess iodine extracted into diethyl ether. The aqueous washings were combined with an approximately equivalent sample of d[TpA], (8.0μmol), and the mixture converted to the K^+ salt, as before, by stirring with ca. 1ml of Dowex ion-exchange resin. After filtering, 18-crown-6 (6.8 mg, 23μmol), was added, and the solution evaporated to dryness *in vacuo*, then co-evaporated twice with dry DMF, before being left on the pump for ca. 1h.

The sample of K^+ 18-crown-6 salt was dissolved in dry DMSO, (375μl), and stirred overnight with methyl iodide, (125μl, 2.0 mMol). Excess methyl iodide was removed, and the sample prepared for high-field ^{31}P n.m.r. analysis, as before. The resulting spectrum, (Figure 4.2), shows the resonances due to the diastereoisomeric methyl esters, $\delta_P(D_6\text{-DMSO})$ +0.88ppm,s,(Sp), and $\delta_P(D_6\text{-DMSO})$ +0.80ppm,s,(Rp), each signal showing two equal intensity isotope shifted resonances, indicative of epimerisation at phosphorus,the magnitude of the isotope shifts being 1.95Hz, 0.016ppm, and 4.97Hz. 0.041ppm, on each resonance.

Desulphurisation of Sp d[Tp(S)A] in the Presence of ^{18}O-labelled Water by Iodine in Lutidine.

Sp d[Tp(S)A], (5.2μmol), was dissolved in ^{18}O-labelled H_2O, (100μl, 66 atom%), to which was added iodine, (13mg, 50μmol), dissolved in lutidine, (300μl), and allowed to stand overnight at room temperature. The reaction was then diluted with water, and the iodine extracted, as before. The sample

was then converted to the K^+ salt by stirring with K^+ Dowex ion-exchange resin, and the K^+ 18-crown-6 complex prepared by addition of 18-crown-6, (23 mg, 87μmol), as before.

The dry K^+ 18-crown-6, complex was dissolved in dry DMSO, (400μl), and stirred with methyl iodide, (150μl, 2.4mMol), overnight, the sample then being prepared for high-field ^{31}P n.m.r. analysis in 2:1 v/v DMSO:D_6DMSO, as before. The resulting spectrum, Figure 4.3, shows the methyl triesters, δ_P(D_6-DMSO) +0.88 ppm,s,(Sp) δ_P(D_6-DMSO) +0.80ppm,s,(Rp), again with two isotope shifted resonances on each diastereoisomer. This time one pair of isotope-shifted resonances is significantly more intense, the Sp resonance showing a more intense resonance shifted by 4.95Hz, 0.041ppm, whilst the more intense resonance on the signal due to the Rp diastereoisomer is shifted by 1.95Hz, 0.016ppm. The relative intensities of the isotope-shifted signals indicate that the reaction has proceeded with predominant INVERSION of configuration at phosphorus, (75%).

Desulphurisation of Dimethyl Phosphorothioate, $(MeO)_2PSO^-Na^+$

1.Using Aqueous Pyridine;

Iodine, (100μmol, 0.026g), was dissolved in 350μl of a ca. 3:1 v/v mixture of pyridine/H_2O. $(MeO)_2PSO^-Na^+$, (3.4mg, 21μmol), was added, and the mixture stirred at room temperature for ca. 1h. The crude reaction mixture was then transferred to an n.m.r. tube and the ^{31}P n.m.r. spectrum recorded, which showed $(MeO)_2PO_2^-Na^+$ as a signal δ_P(pyridine:H_2O) +3.23ppm,s, in the presence of unreacted $(MeO)_2PSO^-Na^+$, resonating at δ_P(pyridine:H_2O) +58.49ppm,s. Integration of the ^{31}P n.m.r. resonances indicated that desulphurisation was ca. 75% complete.

2.Using Aqueous Lutidine;

Iodine, (250μmol, 0.065g), was dissolved in 425μl of a ca. 3:1 v/v mixture of lutidine/H_2O. $(MeO)_2PSO^-Na^+$, (4.0mg, 25μmol), was added, and the mixture stirred at room temperature. After ca. 20min the reaction mixture was transferred to an n.m.r. tube and the ^{31}P n.m.r. spectrum recorded, which showed $(MeO)_2PO_2^-Na^+$ as a signal δ_P(pyridine:H_2O) +2.42ppm,s, in the presence of unreacted $(MeO)_2PSO^-Na^+$, δ_P(pyridine:H_2O) +52.00ppm,s. Integration of the ^{31}P n.m.r. resonances indicated that desulphurisation was ca. 70% complete.

3.Using Aqueous Dioxan with Tri-N-Butylamine;

Iodine, (250μmol, 0.065g), was dissolved in 350μl of a ca. 2:1 v/v mixture of dioxan/H_2O which contained a few drops of tri-N-butylamine. $(MeO)_2PSO^-Na^+$, (3.4mg, 21μmol), was added, and the mixture stirred at room temperature. After ca. 20min the reaction mixture was transferred to an n.m.r. tube and the ^{31}P n.m.r. spectrum recorded, which showed no signal due to $(MeO)_2PO_2^-Na^+$. The reaction was allowed to stir at room temperature for a further 1.5h, after which time the ^{31}P n.m.r. spectrum was re-recorded. No desulphurised material was observed, but unidentified peaks, probably due to disulphides, were observed, $\delta_P(H_2O$:dioxan) +33.7ppm,s, and $\delta_P(H_2O$:dioxan) +29.6ppm,s, were observed, in addition to the signal due to unreacted $(MeO)_2PSO^-Na^+$, resonating at $\delta_P(H_2O$:dioxan) +54.85ppm,s. On addition of a few drops of pyridine to the n.m.r. sample, and re-recording the spectrum after 10min, a signal due to $(MeO)_2PO_2^-Na^+$ was observed, integration of which indicated ca. 45% desulphurisation, suggesting that pyridine is necessary for the desulphurisation reaction to occur.

SUMMARY

Stereochemical studies of enzyme-catalysed substitution at phosphorus provide useful information about the mechanism of an enzymatic reaction by allowing the distinction to be made between a single-step mechanism, characterised by inversion of configuration, and a double displacement mechanism, involving the formation of a covalent enzyme-bound intermediate, and characterised by overall retention of configuration at the phosphorus centre.

The phosphorothioate approach to such stereochemical studies, although chemically more straightforward than the isotopically chiral phosphate approach, has been limited in its application by the lack of a general method for the configurational analysis of nucleoside 5′-[^{16}O,^{18}O] phosphorothioates. An enzymatic method for the configurational analysis of [^{16}O,^{18}O]AMPS[65] and [^{16}O,^{18}O]dAMPS is available,[33,104] which relies on the specificity of adenylate kinase, but this is not generally applicable.

The analysis method described in this thesis represents a chemically simple, and generally applicable route to the configurational analysis of all four major deoxyribonucleoside 5′-[^{16}O,^{18}O] phosphorothioates and [^{16}O,^{18}O]AMPS. The analysis procedure involves isolation of the chiral phosphorothioate from the enzyme reaction by ion-exchange chromatography, followed by methylation using excess diazomethane or dimethyl sulphate in a "one-pot" procedure at room temperature, to generate the correponding diastereoisomeric S-methyl-O-methyl triester. Since these diastereoisomers have been assigned to their corresponding ^{31}P n.m.r. resonances, the position of the

^{18}O-isotpe can be located by examination of the relative magnitudes of $^{31}P(^{18}O)$-isotope shifts[112] in the ^{31}P n.m.r. spectrum of the S-methyl-O-methyl triesters. The diasteroisomer in which the ^{18}O-isotope is in the P=O position shows a larger isotope shift, ca. 0.05ppm, relative to the unlabelled diastereoisomer, than that in which the ^{18}O-isotope is in the P-OMe position, ca. 0.02ppm.

This analysis method may also be extended to the configurational analysis of other chiral nucleoside 5'-[^{16}O,^{18}O] phosphorothioates, provided that the absolute configurations of the S-methyl-O-methyl triesters are assigned. It is expected that these assignments could be made using the the route described here, i.e. by exploiting the stereoselectivity of snake venom phosphodiesterase for the Sp diastereoisomer of the corresponding nucleoside 5'-O-methyl phosphorothioate diester, to generate Sp-deficient samples of the desired S-methyl-O-methyl triester.

The method described here is applicable to the configurational analysis of samples of ca. 2μmol of chiral nucleoside phosphorothioate, but it seems likely that F.A.B mass spectrometry[128] could be applied to enhance the sensitivity of the method by 50-100 fold, if necessary. The diastereoisomers of the nucleoside 5'-S-methyl-O-methyl phosphorothioates have been shown to be resolved by h.p.l.c., (Dr. M. Hamblin, unpublished work), allowing small scale separations of the diasteroisomers to be performed by h.p.l.c. By first assigning the diastereoisomers to their h.p.l.c. peaks by examination of Sp-deficient samples, as described for ^{31}P n.m.r. assignments, and assuming that a suitable fragmentation pattern can be achieved in the mass spectra of the separated diastereoisomers, this analysis method could be

extended to the configurational analysis of much smaller samples of chiral nucleoside 5′-[^{16}O,^{18}O] phosphorothioates.

A stereochemical study of the hydrolysis reaction catalysed by bovine intestinal mucosa 5′-nucleotide phosphodiesterase shows the Sp diastereoisomer of adenosine 5′-O-methyl phosphorothioate to be hydrolysed in ^{18}O-labelled water to give [^{16}O,^{18}O]AMPS and methanol. Configurational analysis of the sample of [^{16}O,^{18}O]AMPS by methylation with dimethyl sulphate and examination by ^{31}P n.m.r. spectroscopy showed it to have the Sp configuration at phosphorus, indicating that the hydrolysis reaction proceeds with overall retention of configuration. This result is in agreement with earlier kinetic studies on this enzyme[133] which also implicate a covalent enzyme intermediate in the reaction pathway, and with the more recent isolation and sequencing of a phosphorylated active site peptide leading to the identification of threonine as the phosphorylated active site residue.[136]

A stereochemical study of the enzyme mung bean nuclease has also been undertaken. Mung bean nuclease selectively hydrolyses the Sp diastereoisomer of d[Ap(S)T] in ^{18}O-labelled water to give [^{16}O,^{18}O]TMPS and 2′-deoxyadenosine. Isolation and configurational analysis of the latter, by treatment with excess diazomethane and subsequent ^{31}P n.m.r. analysis, shows it to have the Sp configuration and hence that the hydrolysis reaction has proceeded with inversion of configuratiion at phosphorus. This result is in agreement with stereochemical results for other single-strand specific nucleases, eg. nuclease S1[65] and nuclease P1,[68] which also recognise the Sp diastereoisomer of a dinucleoside phosphorothioate and catalyse its hydrolysis with inversion of configuration.

In some cases phosphorothioate substrate analogues are not handled by an enzyme, so that the oxygen chiral phosphate approach must be used in any stereochemical study. A convenient route to an oxygen chiral phosphate analogue is provided by the stereospecific exchange of sulphur by oxygen in a single diastereoisomer of the corresponding phosphorothioate, several methods for achieving such an exchange are available.[160,161,168-173] An important requirement of such an exchange is the absence of side reactions with the nucleoside bases. The iodine-mediated desulphurisation of an oligonucleotide has been shown[156] to proceed cleanly and quantitatively, but the stereochemical course of the reaction had not previously been investigated. When a stereochemical study of this exchange was undertaken using a dinucleoside phosphorothioate, Rp d[Tp(S)A], in ^{18}O-labelled aqueous pyridine, configurational analysis of the resulting oxygen chiral dinucleoside phosphate showed that the desulphurisation had occurred with epimerisation at the phosphorus centre, consistent with the involvment of pyridine as a nucleophilic catalyst. When less nucleophilic lutidine was used in place of pyridine the substitution occurred with ca. 75% inversion of configuration, an adequate stereoselectivity to make this method of desulphurisation a useful one.

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